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MONTH	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	130 131	132/1 132/2 132/3	133/1 133/2	134/1 134/2	135/1 135/2	136/1 136/2 136/3	137/1 137/2	138/1 138/2	139/1 139/2	140/1 140/2 140/3	142 144/1	144/2 144/3
Biomedical Applications	143/1		143/2		143/3		143/4		143/5		143/6	
Chromatographic Reviews				141/1				141/2				141/3

Scope. The *Journal of Chromatography* publishes papers on all aspects of chromatography, electrophoresis and related methods. Contributions consist mainly of research papers dealing with chromatographic theory, instrumental development and their applications. The section *Biomedical Applications*, which is under separate editorship, deals with the following aspects: developments in and applications of chromatographic and electrophoretic techniques related to clinical diagnosis (including the publication of normal values); screening and profiling procedures with special reference to metabolic disorders; results from basic medical research with direct consequences in clinical practice; combinations of chromatographic and electrophoretic methods with other physicochemical techniques such as mass spectrometry. In *Chromatographic Reviews*, reviews on all aspects of chromatography, electrophoresis and related methods are published.

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CONTENTS

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Volume 4

GEORGE R. PETTIT, *Professor of Chemistry and Director, Cancer Research Institute, Arizona State University, U.S.A.*

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This volume will be of value particularly to those working in the fields of amino acids, peptides and proteins.

CONTENTS: Headings to Methods. Preface. 1. Racemization. 2. Amino Acid Protecting Groups. 3. Peptide Bond-Forming Methods. 4. Segment Condensation Synthesis. 5. Solution Phase Repetitive Synthesis. 6. Solid Phase Repetitive Synthesis. 7. Glossary and Tabular Survey. Appendix. Laboratory Methods. Bibliography.

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SIMPLE AND EFFICIENT METHOD OF ZONE SPREADING CORRECTION IN GEL PERMEATION CHROMATOGRAPHY

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(First received December 14th, 1976; revised manuscript received April 1st, 1977)

SUMMARY

A method of zone spreading correction in gel permeation chromatography is described that is applicable in the case of a Gaussian-type spreading function. Instead of the integral Tung equation, an equivalent partial differential equation is solved numerically and in this way the necessary calculations are considerably simplified. The method is applied to artificial and real chromatograms and the results are compared with those of some known correction procedures.

INTRODUCTION

The imperfect resolving power of real chromatographic columns, due to zone spreading, distorts the results of gel permeation chromatographic (GPC) analyses of polymers. In order to obtain accurate data on molecular weight distributions and on molecular weight averages, it is therefore imperative to use special correction procedures that partly or completely eliminate the influence of the dispersion in the column.

From the mathematical point of view, the zone spreading correction requires an integral (Tung) equation to be solved by a suitable numerical method. In the literature one can find many different procedures[1–9], but most of them require, with only minor exceptions[10,11], fast computers with large storage space. In this paper, a zone spreading correction procedure is proposed that is very simple and fast, such that the necessary calculations can be carried out, if need be, even with a simple desk calculator.

The procedure is based on the assumption that the spreading function in the Tung equation is Gaussian, a condition which is usually well fulfilled with the highly efficient columns used in modern GPC instruments.

THEORETICAL

The relationship between the original, normalized chromatogram, $f(x)$, and the chromatogram corrected for zone spreading, $w(x)$, is described by the Tung integral equation

$$f(x) = \int_{-\infty}^{\infty} K(x,y)\, w(y)\, \mathrm{d}y \qquad (1)$$

where x is the elution volume. Its kernel, $K(x,y)$, is usually called the spreading function and represents the elution curve of a monodisperse polymer with a molecular weight corresponding to the elution volume y. In the following discussion we limit ourselves to the case of a Gaussian spreading function, which we shall write as

$$K(x,y) = \sqrt{(h/\pi)} \exp[-h\,(x-y)^2] \tag{2}$$

The spreading factor, h, is inversely proportional to the extent of sample spreading in a given column.

Firstly, we shall show that under these conditions the integral eqn. 1 represents a solution of a boundary value problem analogous to a certain problem of free diffusion. This equivalent boundary value problem can be stated as follows: Solve

$$D \cdot \frac{\partial^2 f(x,t)}{\partial x^2} = \frac{\partial f(x,t)}{\partial t} \tag{3}$$

subject to the boundary conditions

$$f(x, 0) = w(x) \tag{4}$$

$$f(\infty, t) = 0 \tag{5}$$

$$f(-\infty, t) = 0 \tag{6}$$

[This problem can be given the following physical interpretation: if the concentration of a compound in a one-dimensional space (space coordinate x) has initially the shape $w(x)$, find how the shape has changed by free diffusion proceeding for a time t_d, if the diffusion coefficient, assumed to be constant, is D.]

The solution of this boundary value problem[12] is of the form

$$f(x,t_d) = \frac{1}{2\sqrt{\pi\, D t_d}} \int_{-\infty}^{\infty} \exp\left[-\frac{(x-y)^2}{4D\,t_d}\right] w(y)\,\mathrm{d}y \tag{7}$$

which is the same as that of the Tung integral eqn. 1 with the kernel given by eqn. 2, provided that we put

$$D\,t_d = 1/4h \tag{8}$$

It follows that the calculation of the corrected chromatogram can be reduced to the determination of the boundary condition $w(x)$ in the problem given by eqns. 3–6, where the function $f(x,t_d)$ is known [it is identical with the actual, uncorrected chromatogram $f(x)$] and the quantity h can be obtained in advance by calibrating the column with a series of narrow fractions, *e.g.*, by the reverse-flow method[13].

In order to obtain the final correction equations in the simplest form, we shall solve this problem by a simple method of finite differences. Its essence rests on using the Taylor expansion to estimate the values of the sought function in points with abscissae that differ by a small increment from those where the values of the func-

tion are known. For the derivatives in the Taylor series we substitute their numerical analogues obtained by approximating the given function by an interpolation polynomial.

This procedure can be repeated in an optional pre-selected number of steps (k), where the function values calculated in step i serve as a basis for a computation of derivatives in step $i + 1$. Starting from the known values of $f(x,t_a)$ (uncorrected chromatogram), we reach the sought function $w(x)$ after k steps. The differential eqn. 3 is instrumental in transforming the derivatives with respect to the coordinate x (only these are experimentally available) into derivatives with respect to t, to be substituted into the Taylor series.

The above principle can be modified in many different ways in order to obtain a more precise solution (e.g., see ref. 14), but all of these procedures require more sophisticated algorithms. In this work we employed only the Taylor series method and a higher precision was obtained by substituting higher-order derivatives.

Taylor expansion of the function $f(x,t)$ in t can be written as

$$f(x,t-\Delta t) = f(x,t) - \Delta t \cdot \frac{\partial f(x,t)}{\partial t} + \frac{\Delta t^2}{2} \cdot \frac{\partial^2 f(x,t)}{\partial t^2} - \frac{\Delta t^3}{6} \cdot \frac{\partial^3 f(x,t)}{\partial t^3} + \ldots \quad (9)$$

assuming that all partial derivatives exist.

Using a notation more suitable for discrete quantities, *i.e.*,

$$f(x_i, t_n) = f_{i,n}, \left.\frac{\partial f(x,t)}{\partial t}\right|_{\substack{x = x_i \\ t = t_n}} = \frac{\partial f_{i,n}}{\partial t} \quad (10)$$

and similarly for higher derivatives, eqn. 9 can be rewritten as

$$f_{i,n-1} = f_{i,n} - \Delta t \cdot \frac{\partial f_{i,n}}{\partial t} + \frac{\Delta t^2}{2} \cdot \frac{\partial^2 f_{i,n}}{\partial t^2} - \frac{\Delta t^3}{6} \cdot \frac{\partial^3 f_{i,n}}{\partial t^3} \ldots \quad (11)$$

It is evident from eqn. 3 that $\partial f_{i,n}/\partial t$ can be directly substituted; further, it holds that

$$D \cdot \frac{\partial^4 f_{i,n}}{\partial x^4} = \frac{\partial^3 f_{i,n}}{\partial x^2 \partial t} \quad (12)$$

and also

$$D \cdot \frac{\partial^3 f_{i,n}}{\partial x^2 \partial t} = \frac{\partial^2 f_{i,n}}{\partial t^2} \quad (13)$$

so that for the second derivative in t we obtain the relationship

$$D^2 \cdot \frac{\partial^4 f_{i,n}}{\partial x^4} = \frac{\partial^2 f_{i,n}}{\partial t^2} \quad (14)$$

Similarly, it holds for the third derivative in t that

$$D^3 \cdot \frac{\partial^6 f_{i,n}}{\partial x^6} = \frac{\partial^3 f_{i,n}}{\partial t^3} \quad (15)$$

etc. Substituting now into eqn. 11, we obtain the relationship

$$f_{i,n-1} = f_{i,n} - D\Delta t \cdot \frac{\partial^2 f_{i,n}}{\partial x^2} + \frac{D^2 \Delta t^2}{2} \cdot \frac{\partial^4 f_{i,n}}{\partial x^4} - \frac{D^3 \Delta t^3}{6} \cdot \frac{\partial^6 f_{i,n}}{\partial x^6} + \ldots \quad (16)$$

in which we find only derivatives in x and different powers of the product $D\Delta t$. If the calculation is performed in k steps, we can express Δt in terms of t_d:

$$\Delta t = t_d/k \quad (17)$$

and according to eqn. 8 we obtain for $D\Delta t$

$$D\Delta t = 1/(4kh) \quad (18)$$

Substituting from eqn. 18 into eqn. 16, we obtain a final equation for calculation of the quantity $f_{i,n-1}$, where all coefficients are numerically accessible. If we now calculate the partial derivatives in x using relationships obtained by approximating the function $f_{i,n}$ by an interpolation polynomial and if we put $r = (4kh\Delta x^2)^{-1}$, we obtain the following equations directly applicable to the calculation of quantities $f_{i,n-1}$:

$$f_{i,n-1} = f_{i,n}(1 + 2r) - r(f_{i-1,n} + f_{i+1,n}) \quad (19)$$

if the Taylor series is truncated after the term with the first derivative;

$$f_{i,n-1} = f_{i,n}\left(1 + \frac{5}{2}r + 3r^2\right) - \left(f_{i-1,n} + f_{i+1,n}\right)\left(\frac{4}{3}r + 2r^2\right) + \\ + \left(f_{i-2,n} + f_{i+2,n}\right)\left(\frac{1}{12}r + \frac{1}{2}r^2\right) \quad (20)$$

from the Taylor series with the second derivative; and finally

$$f_{i,n-1} = f_{i,n}\left(1 + \frac{49}{18}r + \frac{14}{3}r^2 + \frac{10}{3}r^3\right) - \left(f_{i-1,n} + f_{i+1,n}\right)\left(\frac{3}{2}r + \frac{13}{4}r^2 + \frac{5}{2}r^3\right) + \\ + \left(f_{i-2,n} + f_{i+2,n}\right)\left(\frac{3}{20}r + r^2 + r^3\right) - \left(f_{i-3,n} + f_{i+3,n}\right)\left(\frac{1}{90}r + \frac{1}{12}r^2 + \frac{1}{6}r^3\right) \quad (21)$$

for the expansion including the third derivative. In these equations the subscript i corresponds to the values of the elution volume, *i.e.*, to the abscissae of the chromatogram, the subscript n corresponds to the quantity t in such a way that $t = t_d$ for $n = k$ (where k is the chosen number of steps) and $t = 0$ for $n = 0$.

Eqns. 19–21 were derived on the assumption that D in eqn. 3 is constant; this is equivalent (*cf.*, eqn. 8) o the assumption that the spreading factor, h, is independent of the elution volume. It would be possible to solve an equivalent boundary value problem also for the case $D = D(x)$, but the main advantage of the proposed procedure ,*viz.*, the simplicity of the final correction equations (eqns. 19–21), would be lost. In correcting the chromatograms where the spreading factor was a function of the

elution volume, we used a procedure common to other known correction methods[4,6,10] varying values of r, calculated from the known relationship $h = h(x)$, were substituted into eqns. 19–21.

EXPERIMENTAL

Chromatograms of poly(methyl methacrylate) and polystyrene were obtained with a Waters Model ALC 100 instrument with four Styragel columns ($5 \cdot 10^6$, $1.5 \cdot 10^5$, $1.5 \cdot 10^4$ and $5 \cdot 10^2$ Å) in series. The flow-rate of solvent (tetrahydrofuran) was 1 ml/min and the detector was a differential refractometer. The calibration graph for polystyrene samples was linear and followed the relationship

$$\log_{10} M = \frac{95.1859 - v}{7.4349} \tag{22}$$

where v is the elution volume, which throughout this whole paper is expressed in counts (1 count = 2.40 ml), and M is the molecular weight. The spreading factor, h, of the Gaussian kernel in the Tung integral equation depended on the elution volume through the second-order polynomial

$$h = -0.93102 + 0.02541\, v - 0.00007\, v^2 \tag{23}$$

RESULTS AND DISCUSSION

In order to verify the efficiency of the proposed correction method, we applied it to artificial chromatograms and also to real chromatograms of poly(methyl methacrylate) and polystyrene. In constructing two artificial chromatograms we used a superposition of two Schulz–Zimm molecular weight distribution functions (chromatogram A) and one Schulz–Zimm distribution function (chromatogram B), in both instances with known molecular weight averages (M_n and M_w). In chromatogram B a very narrow distribution with $M_n = 1.00 \cdot 10^5$ and $M_w = 1.10 \cdot 10^5$ was used; in chromatogram A two narrow Schulz–Zimm molecular weight distributions with parameters $M_n^{(1)} = 4.5 \cdot 10^5$, $M_w^{(1)} = 5.0 \cdot 10^5$, weight fraction $\alpha_1 = 0.6$ and $M_n^{(2)} = 1.35 \cdot 10^6$, $M_w^{(2)} = 1.5 \cdot 10^6$, weight fraction $\alpha_2 = 0.4$ were superimposed, to yield a composite distribution function with

$$M_n = \left[\frac{\alpha_1}{M_n^{(1)}} + \frac{\alpha_2}{M_n^{(2)}}\right]^{-1} = 6.137 \cdot 10^5$$

and

$$M_w = \alpha_2 M_w^{(2)} + \alpha_1 M_w^{(1)} = 9.0 \cdot 10^5$$

From the known course of the initial molecular weight distribution, g(M), a corrected normalized chromatogram $w(v)$ was calculated with the relationship

$$w(v) = -\mathrm{g}(M) \cdot M \cdot 2.303\, (\mathrm{d} \log M / \mathrm{d}v)$$

The value −0.1345 calculated from the experimental calibration (eqn. 22) was substituted for the derivative in this equation. The uncorrected chromatograms $f(v)$ were then calculated by numerical integration of eqn. 1: in case A a constant spreading factor $h = 0.2$ was used, whereas in case B an elution-volume-dependent h according to eqn. 23 was employed. To adjust the precision of the input data to the precision expected in real chromatograms, the heights of calculated normalized "uncorrected" chromatograms were multiplied by 1000 and rounded off to an even first decimal (which corresponds to a precision of ±0.2 mm for a chromatogram with a maximum height of about 14 cm).

Preliminary calculations showed that the correction based on the simplest equation (eqn. 19) is inadequate and cannot be used in practice. On the other hand, eqns. 20 and 21 require the numerical calculation of higher derivatives and a low precision in reading the heights of chromatograms (especially at their extreme ends, where the relative error is the highest) can lead to artificial oscillations in the corrected curve. The occurrence of this undesirable effect is minimized if a judiciously selected integral multiple of the true volume count increment, $\Delta v_{calc} = l\Delta v$, with $l = 1,2,3...$, is used in eqns. 20 and 21 instead of Δv itself. The modified equations are then

$$f_{i,n-1} = f_{i,n}\left(1 + \frac{5}{2}r + 3r^2\right) - \left(f_{i-l,n} + f_{i+l,n}\right)\left(\frac{4}{3}r + 2r^2\right) + \\ + \left(f_{i-2l,n} + f_{i+2l,n}\right)\left(\frac{1}{12}r + \frac{1}{2}r^2\right) \qquad (20a)$$

and

$$f_{i,n-1} = f_{i,n}\left(1 + \frac{49}{18}r + \frac{14}{3}r^2 + \frac{10}{3}r^3\right) - \left(f_{i-l,n} + f_{i+l,n}\right)\left(\frac{3}{2}r + \frac{13}{4}r^2 + \frac{5}{2}r^3\right)$$

$$+ \left(f_{i-2l,n} + f_{i+2l,n}\right)\left(\frac{3}{20}r + r^2 + r^3\right) - \left(f_{i-3l,n} + f_{i+3l,n}\right)\left(\frac{1}{90}r + \frac{1}{12}r^2 + \frac{1}{6}r^3\right) \qquad (21a)$$

In view of the lower relative precision in reading the heights at the extreme ends of chromatograms, equations of lower order were used in the actual calculations for abcissae $i = 1$ to $i = 3l$ and $i = N - 3l + 1$ to $i = N$, where N is the total number of experimental points (*e.g.*, in a computer program based on eqn. 21a, eqns. 19 and 20 were used at the extreme ends).

The artificial chromatogram A was used to study the influence of the number of calculating steps (k) and the influence of the quantity Δv_{calc} on the correction efficiency of eqn. 21a. Fig. 1 clearly shows that the correction efficiency increases with the number of iterations (k), although the small improvement in resolution brought about by increasing the number of steps from $k = 5$ to $k = 15$ hardly justifies tripling the computation time.

Even for $k = 1$ (*i.e.*, with a direct, non-iterative application of eqn. 21a) one attains a distinct although by no means perfect resolution of both superimposed peaks.

Fig. 2 shows a significant influence of the diminishing Δv_{calc} on the correction efficiency of eqn. 21a for a constant number of iterations $k = 3$. (However, a further decrease to $\Delta v_{calc} = \Delta v = 0.5$ leads to considerable oscillations of the computed

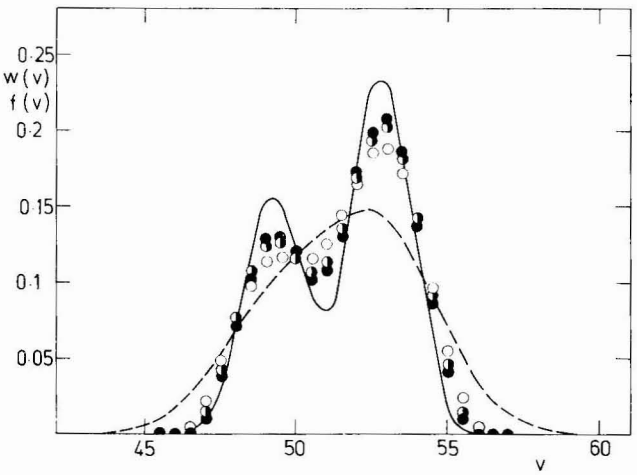

Fig. 1. Influence of the number of iteration steps (k) on the correction efficiency of eqn. 21a applied to artificial chromatogram A. Calculated with $\Delta v_{calc} = 1.5$. Broken line, uncorrected chromatogram $f(v)$; solid line, known course of the corrected chromatogram $w(v)$; ○, $k = 1$; ◑, $k = 5$; ●, $k = 15$.

function w.) It is also evident that even with a moderate number of iterations ($k = 3$), a very good correction efficiency can be achieved. This fact emerges even more clearly from Fig. 3, where the results obtained by applying eqns. 20a and 21a in three iteration steps are compared with a very efficient correction procedure, *viz.*, Method 2 of Ishige *et al.*[8]. (We have had very useful experience with this algorithm* for some years and accordingly we use it as a standard for comparison purposes.) Fig. 3 also shows a chromatogram corrected according to the method of Pierce and Armonas[10]; this method is comparable with the procedure proposed here in terms of simplicity and rapidity of calculation.

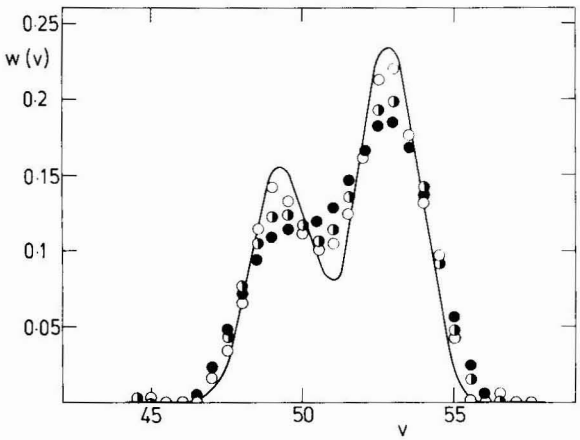

Fig. 2. Influence of the value of Δv_{calc} on the correction efficiency by eqn. 21a applied to the artificial chromatogram A. Calculated with $k = 3$. Curve, original course of $w(v)$; ○, $\Delta v_{calc} = 1$; ◑, $\Delta v_{calc} = 1.5$; ●, $\Delta v_{calc} = 2$.

* The program written in Fortran IV was made available by courtesy of Prof. Hamielec.

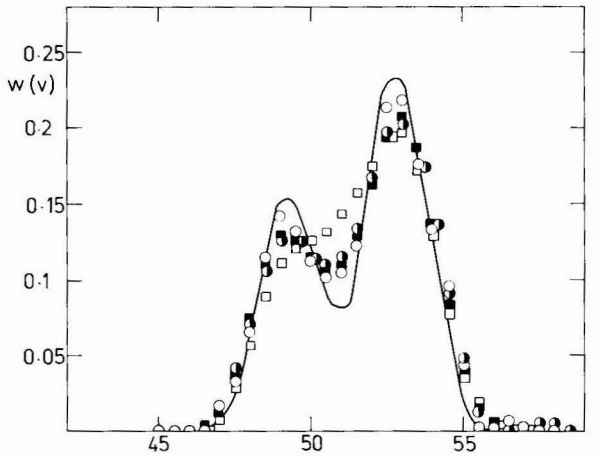

Fig. 3. Comparison of the correction efficiency of different methods applied to the artificial chromatogram A. ○, This work, eqn. 21a, $k = 3$, $\Delta v_{calc} = 1$; ◑, this work, eqn. 20a, $k = 3$, $\Delta v_{calc} = 1$; ■, Ishige *et al.*[8] (Method 2); □, Pierce and Armonas[10]; curve, original course of $w(v)$.

It follows from the comparison of the curves in Fig. 3 that the efficiency of the simple correction based on the sevenpoint eqn. 21a is comparable with, if not better than, the sophisticated algorithm used by Ishige *et al.* The correction eqn. 20a also affords good results, whereas the Pierce and Armonas method gives only a slight in-

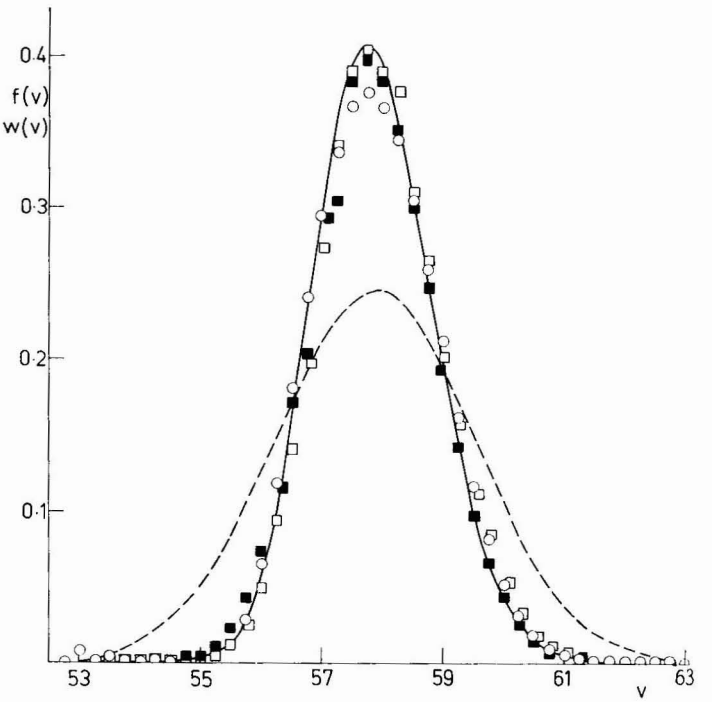

Fig. 4. Corresponding comparison to that in Fig. 3 for the artificial chromatogram B. Notation as in Fig. 3. Broken line, uncorrected chromatogram $f(v)$.

dication of a resolution of both peaks. [In this connection we should mention the interesting finding that our method based on eqns. 20a and 21a works very well with $\Delta v_{calc} = 1$, satisfactorily with $\Delta v_{calc} = 1.5$ and unsatisfactorily with $\Delta v_{calc} = \Delta v = 0.5$, whereas the computer program used by Ishige *et al.* yields good results only in the last instance. With $\Delta v_{calc} = 1$ it iterated very slowly, did not manage to satisfy the prescribed tolerance (0.01) and the final corrected curve closely resembled that obtained by the method of Pierce and Armonas.]

Computer programs for all of the methods were written in Basic and implemented on a Wang 2200 desk-top programmable calculator with 12 kB storage capacity. Net computation times for the correction of chromatogram A in Fig. 3 were: $2\frac{1}{2}$ min. with eqn. 21a, 1 min for the method of Pierce and Armonas, and 83 min for the method of Ishige *et al.*

A corresponding comparison of our method (eqn. 21a) with the procedures of Ishige *et al.* and Pierce and Armonas for the artificial chromatogram B is shown in Fig. 4. In this instance of a unimodal, very narrow molecular weight distribution ($M_n/M_w = 1.1$) all of the methods gave very good results and the calculated curves fitted the original function $w(v)$.

Finally, in Figs. 4 and 5 the proposed correction procedure (eqn. 21a, $\Delta v_{calc} = 1.5$) is compared with the methods of Ishige *et al.* and Pierce and Armonas on real chromatograms obtained with polystyrene and poly(methyl methacrylate); a spread-

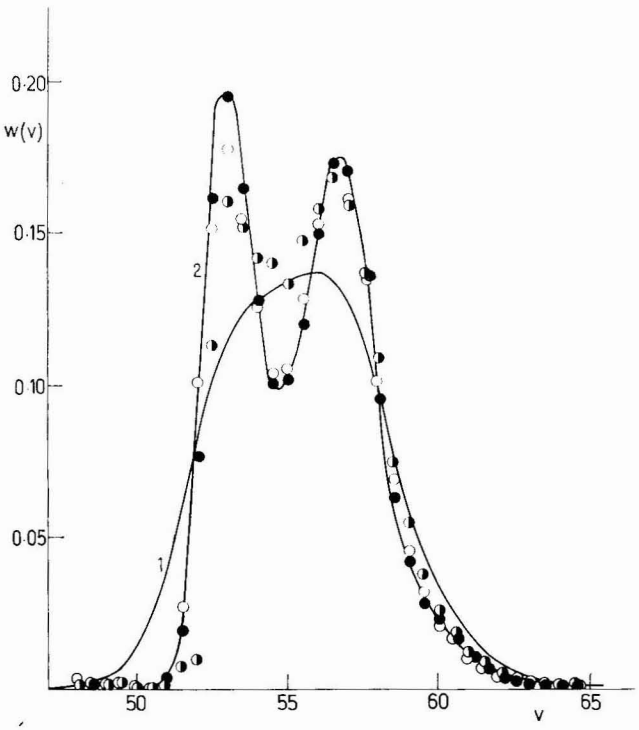

Fig. 5. Comparison of different methods of correction applied to a real chromatogram of polystyrene. 1 = Normalized, uncorrected chromatogram; 2 = Ishige *et al.*[8]; ○, this work, eqn. 21a, $k = 5$, $\Delta v_{calc} = 1.5$; ◑, Pierce and Armonas[10].

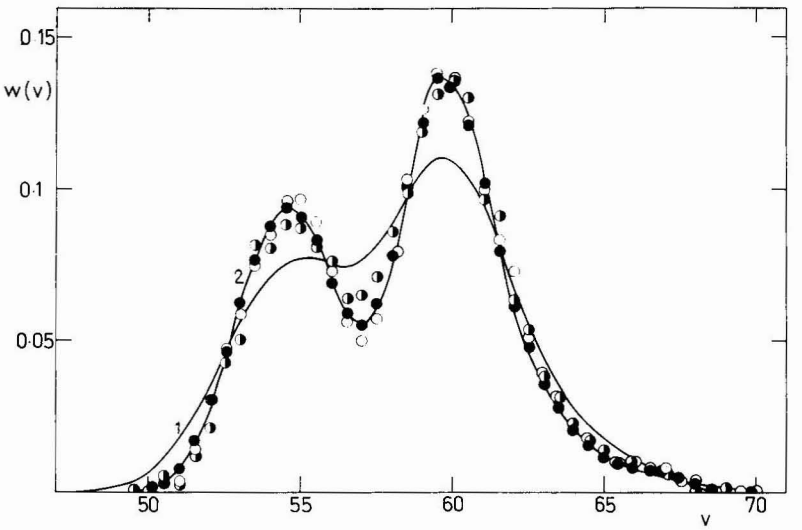

Fig. 6. Corresponding comparison to that in Fig. 5 for a real chromatogram of poly(methyl methacrylate). Notation as in Fig. 5.

ing factor, $h(v)$, given by eqn. 23 was used throughout. The chromatogram of the polystyrene sample in Fig. 5 indicates the presence of two strongly superimposed peaks; all three correction methods were able to resolve them. Eqn. 21 and the method of Ishige *et al.* gave again very similar corrected chromatograms; the resolution of the method of Pierce and Armonas was much worse. A very similar situation arises in the correction of a chromatogram of poly(methyl methacrylate) shown in Fig. 6, although in this instance the separation of both peaks is somewhat greater; as a result, even the method of Pierce and Armonas gives a satisfactory result. The net computation times with the Wang 2200 calculator were as follows: (a) for the chromatogram in Fig. 5, *ca.* $1\frac{3}{4}$ min (Pierce and Armonas), *ca.* $2\frac{1}{2}$ min (eqn. 21a) and 72 min (Ishige *et al.*); (b) for the chromatogram in Fig. 6, *ca.* 2 min (Pierce and Armonas),

TABLE I

MOLECULAR WEIGHT AVERAGES CALCULATED BY DIFFERENT CORRECTION METHODS FROM CHROMATOGRAMS OF POLYSTYRENE AND POLY(METHYL METHACRYLATE)

Method	*Sample*	$M_n \cdot 10^4$	$M_w \cdot 10^4$	$M_z \cdot 10^4$
Uncorrected	Polystyrene	7.615	14.969	25.115
Pierce and Armonas[10]		7.862	12.594	17.610
Eqn. 21a, $\Delta v_{calc} = 1.5$, $k = 5$		8.164	13.933	19.850
Ishige *et al.*[8]		8.236	13.867	19.249
Uncorrected	Poly(methyl methacrylate)*	3.536	12.148	30.000
Pierce and Armonas[10]		3.813	10.802	22.872
Eqn. 21a, $\Delta v_{calc} = 1.5$, $k = 5$		3.742	10.826	21.814
Ishige *et al.*[8]		3.790	11.263	23.565

* Calculated from a calibration graph for polystyrene assuming the validity of the universal calibration according to Grubisic *et al.*[15].

ca. 3 min (eqn. 21a) and 32 min (Ishige *et al.*). Corrected values of number-, weight- and *z*-averages obtained by the three methods for both real samples are given in Table I.

From the point of view of correction efficiency, eqn. 21a is to be preferred to the simpler eqn. 20a. It is recommended that one should work with $\Delta v_{calc} = 1$, or $\Delta v_{calc} = 1.5$, with a number of iteration steps not greater than $k = 5$. In all of the examples studied, the proposed method of zone spreading correction based on eqn. 21a proved to be equally efficient but much simpler and faster than the method of Ishige *et al.* It is particularly suitable for laboratories with only a small computer with restricted storage capacity; with lower demands on accuracy, eqn. 21a can be used in a single step and the calculation can then be carried out with a simple desk-top calculator. The proposed procedure is limited to the case of a Gaussian kernel in the Tung integral equation, but it is, however, capable of accommodating a variable spreading factor, $h(v)$. The method does not require a fine sub-division of the abscissa in reading the chromatogram heights, which speeds up considerably the processing of a large number of experimental results.

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CLUSTER EXPANSION FOR RETENTION VOLUME IN GAS ADSORPTION CHROMATOGRAPHY

ADSORPTION OF HYDROCARBONS ON GRAPHITE

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SUMMARY

A cluster expansion for retention volume in gas adsorption chromatography is developed. Theoretical evaluations are made of the temperature dependence of the integrals W_1, which are directly proportional to Henry's constant, and of the dependence of retention volumes of benzene, hexane and pentane on their density in the free gas phase. The results are compared with experimental gas chromatographic results and with an application of a graphitized lamp-black as an absorbent. Good agreement is found for isosteric heats of adsorption and reciprocal temperature dependence of integrals W_1, but the two evaluations of the dependence of retention volume on adsorbate density are not in agreement. Reasons for these divergences are suggested.

INTRODUCTION

For many years graphitized blacks have found experimental use for theoretical and practical reasons[1–5]. The well-defined topography of the adsorptive centres enables the calculation of many thermodynamic characteristics of the surface adsorption of hydrocarbons and noble gases and the chromatographic separation of many complicated mixtures of hydrocarbons (*e.g.* geometrical isomers, butanes, xylenes)[2].

Molecular statistical adsorption theory has been used for the calculation of the adsorption equilibrium coefficient, the retention volume, and the differential energy and entropy of adsorption for various hydrocarbons of very different molecular

shape, size, and polarizability[1–5]. The aim of this paper is to introduce a cluster expansion for retention volume in gas adsorption chromatography[6,7] which could be used to calculate, from chromatographic measurements, microscopic characteristics of adsorption systems, to predict retention values and to optimize chromatographic separation processes.

CLUSTER EXPANSION FOR RETENTION VOLUME IN GAS ADSORPTION CHROMATOGRAPHY

Consider an assembly of molecules of fixed activity α and volume V, near an inert solid surface (inert in the sense that its own thermodynamic properties are unaffected by the presence of adsorbed gas and merely seem to provide an external potential field). The grand canonical partition function is given by[8]

$$\Xi = \sum_{N>0} \frac{\alpha^N}{N!} Z_N \tag{1}$$

where Z_N is the configurational integral. Expansion of $\ln \Xi$ into activity power leads to

$$\ln \Xi = \sum_{l=1} B_l \alpha^l \tag{2}$$

where B_l's are the generalized cluster integrals, relative to l molecules

$$l!\, B_l = \int_V \cdots \int \mathrm{d}r^l \prod_{i=1}^{l} g_i \sum_{i,j} (\Pi f_{ij})_c \tag{3}$$

In the above $g_i = \exp(-v_i/RT)$, $f_{ij} = \exp(-u_{ij}/RT) - 1$, $v_i = v(r_i)$ is the gas–solid potential energy, and $u_{ij} = u(r_i, r_j)$ is the mutual interaction energy of two molecules, localized at r_i and r_j. We neglect the third and higher order effects and assume that $u_{ij} = u(|r_i - r_j|)$. We will also assume that the total interaction energy of l molecules can be represented as a sum of pair-wise energies. The index c in eqn. 3 denotes that to each particular product $(\Pi f_{ij})_c$ there corresponds a connected graph, such that each molecule of the set is represented by a g_i-vertex and each factor f_{ij} by a line joining two vertices. Defining v_i such that

$$g_i = \begin{cases} g_i \text{ for } r_i \in V \\ 0 \text{ for } r_i \notin V \end{cases}$$

integration of eqn. 3 may be extended over all configurational space.

Next, using Bellemans[9,10] procedure of reduction of B_l, we can expand $\ln \Xi$ into density n in the homogeneous ($v_i = 0$) part of system powers.

For this purpose we can define a new type of cluster integral W_l, relative to l molecules[9,10].

$l!\, W_l =$ {the sum of the contributions of all connected graphs of l distinct square vertices, such that the basic part of these graphs consists of white squares, and all terminal subparts of black squares}.

The contribution to W_l from a given graph of l squares has been calculated as follows:

(i) to each white vertex corresponds a factor g_i

(ii) with each terminal subpart of λ black squares is associated a factor $\{-1 + \prod_{i=1}^{\lambda} g_i\}$

(iii) with each link joining two vertices is associated a factor f_{ij}

(iv) integrate over the coordinates in ∞ (in the case of white squares it is is reduced to integration over V).

For example

$$1!\, W_1 = \int_V (g_1 - 1)\, \mathrm{d}r_1 = \blacksquare - \square$$

$$2!\, W_2 = \int_V g_1 \left\{ \int_\infty (g_2 - 1) f_{12}\, \mathrm{d}r_2 \right\} \mathrm{d}r_1 = \blacksquare - \square \tag{4}$$

Thus, using Bellemans' general proof, we have[9,10]

$$l^2 B_l = (V + W_1) \sum_{[nk]} \prod_k \frac{(l\beta_k)^{n_k}}{n_k!} + l \sum_{[m,nk]} m\, W_m \prod_k \frac{(l\beta_k)^{n_k}}{n_k!} \tag{5}$$

$$\Sigma k n_k = l-1 \qquad \Sigma k n_k = l-m \quad (m>1)$$

or in the form of Cauchy integrals

$$l^2 B_l = \frac{V + W_1}{2\pi i} \oint_C \frac{\mathrm{e}^{l\varphi(\zeta)}}{\zeta^l}\, \mathrm{d}\zeta + \frac{l}{2\pi i} \oint_C \frac{\mathrm{e}^{l\varphi(\zeta)}}{\zeta^l} \sum_2^\infty m\, W_m \zeta^{m-1}\, \mathrm{d}\zeta \tag{6}$$

where $\varphi(\zeta) = \Sigma \beta_k \zeta^k$, β_k are irreducible Mayer integrals and C is a closed contour around the origin leaving outside all singularities of the integrand except the pole in zero.

Substituting eqn. 6 into eqn. 2, and making use of the Lagrange theorem on the existence[11] of a unique root $\zeta_0 = n$, which satisfies the equation $\zeta_0 \exp[-\varphi(\zeta_0)] = \alpha$, we obtain

$$\bar{N} = n\, W_1 + \sum_2^\infty m\, W_m\, n^m \left(1 - \sum_1^\infty k\, \beta_k\, n_k\right)^{-1} \tag{7}$$

where $\bar{N}$ is the average number of adsorbed molecules and n is the bulk density. Consequently, the cluster expansion for retention volume V_N in gas adsorption chromatography, obtained via the Conder–Purnell[12] equation may be written as follows:

$$V_N(n) = W_1 + \left\{ \sum_{k,m} k\, m\, (m-k)\, \beta_k\, W_m\, n^{m+k-1} \right\} \left\{ 1 - \sum_k k\, \beta_k\, n^k \right\}^{-2} \tag{8}$$

The integral W_1 is simply related to Henry's constant

$$W_1 = \frac{K_H}{RT} \tag{9}$$

To describe the adsorption of complex molecules on graphite it is necessary to take into account the dependence of v on the distance between the molecule mass centre and the surface, and on the orientation of the molecule. However, hydrocarbon molecules are composed of few different atoms, so there is evidence in favour of representing v as a sum of the potential functions of intermolecular interactions of atom pairs $v^{(ij)}$, where index i denotes i-th carbon atom of graphite, and index j the j-th atom of adsorbate molecule[1–5]

$$v = \sum_{i,j} v^{(ij)} \tag{10}$$

Generally, the potential energy of interactions $v^{(ij)}$ may be approximated by the Lennard-Jones (W) (12–6) function

$$v^{(ij)} = 4\varepsilon_j \left[\left(\frac{\sigma_j}{\varrho_{ij}} \right)^{12} - \left(\frac{\sigma_j}{\varrho_{ij}} \right)^{6} \right] \tag{11}$$

where ϱ_{ij} denotes the distance between the i-th carbon atom of graphite and the j-th atom of adsorbate molecule.

The distance ϱ_{ij} is determined by specifying the origin r_0 and the orientation Θ of a suitable molecular frame with respect to a crystalline reference system. Thus, in the notation of Battezatti *et al.*[3],

$$\varrho_{ij} = | r_i - r_j | \tag{12}$$

$$r_j = \{\hat{R}(\Theta) | r_0\} r_{0j} \tag{13}$$

where $\{\hat{R}(\Theta) | r_0\}$ is a roto-translation operator[3]. The potential energy interactions of the k-th atom of adsorbate with graphite $v^{(k)}$ may be written[3,8]

$$v^{(k)}(r) = v_0^{(k)}(z) + \sum_{1} \omega_{(z)}^{(km)} f_m(s_1, s_2) \tag{14}$$

where

$$v_0^{(k)}(z) = \frac{2\pi \varepsilon_k}{| a_1 \times a_2 |} \sum_{n=0} \left\{ \frac{\sigma_k^{12}}{5(z + n d)^{10}} - \frac{\sigma_k^{6}}{(z + n d)^4} \right\} \tag{15}$$

$$\omega^{(k,m)} = \frac{\pi \varepsilon_k}{| a_1 \times a_2 |} \left\{ \frac{\sigma_k^{12} g_m^5}{1920 z^5} K_5(g_m z) - \frac{\sigma_k^6 g_m^2}{2z^2} K_2(g_m z) \right\} \tag{16}$$

where d is the interlayer distance of graphite, K_n is the modified Bessel function of the second kind and order n, $f_m(s_1,s_2)$ are the functions defined by Steele, the vector $s_1 a_1 + s_2 a_2$ is the projection of r on the graphite plane, a_1 and a_2 being the graphite unit lattice vectors, and g_m is the length of the reciprocal lattice vectors, defined by

Steele[8]. As mentioned by Ricca, the principal advantage of this method lies in the functional form of the potential. It is also much more economical in computer time.

Evaluation of integrals W_1 and W_2

The integral W_1 is given by the following equation[2]

$$W_1 = \int_V \{\exp(-v(r_m, \Theta)/RT) - 1\} J \, dr_m \tag{17}$$

where r_m denotes the coordinates of centre of mass and J is the jacobian. The integration is made over all configurational space available to the adsorbate molecules.

Eqn. 17 is valid for quasirigid molecules and ignores the perturbation from the surface to the internal vibrational degrees of freedom, which is negligible for a non-specific adsorption on homogeneous surface of graphite. On the other hand, perturbations to the internal rotational degrees of freedom are not negligible. In calculations of W_1 we have used the method of Vidal-Madjar *et al.*[1,2], in which the graphite surface is substituted by a continuum. The potential energy of interactions of a complex molecule with this continuum is treated as an average value averaged over positions of r_m on the graphite lattice. For adsorption of benzene on graphite we have adopted model "C" of Vidal-Madjar *et al.*[1,2], whereas in the case of adsorption of pentane and hexane we used model "F".

In model C, reserved for the description of planar (or quasi-planar) molecules, the system of coordinates connected with adsorbate molecule OXYZ is chosen so that OX and OY are the two main angles of the molecular plane, OZ corresponds to the largest moment of inertia (perpendicular to the molecular plane), θ is the angle of the molecular plane to the adsorbent surface and ψ defines the orientation of the molecule within that plane. Let us denote by v_{z_0}'' the value of $\partial^2 v/\partial z^2$ for $z = z_0$, where z_0 is the equilibrium distance at $\theta = 0$ and $v_\theta'' = \partial^2 v/\partial\theta^2$. In this approximation[2]

$$v(z, \theta, \psi) = v_0(z = z_0) + \frac{1}{2} v_{z_0}''(z - z_0)^2 + \frac{1}{2} v_\theta''(\psi) \sin^2\theta \tag{18}$$

and consequently

$$W_1 = A \sqrt{\frac{2\,II\,RT}{v_{z_0}''}}\, 2\,II\,RT \exp(-v_0/RT) \int_0^{2\pi} \frac{1}{v_\theta''(\psi)} \, d\psi \tag{19}$$

where A denotes the surface area.

For the description of adsorption of pentane and hexane we used the model "F". Because of the strong anisotropic field of adsorption these molecules have a preferential orientation with their axis of the lowest moment of inertia roughly parallel to the surface. The angle between OZ and the perpendicular to the adsorbent surface is designated θ.

We assume rotation around OY and oscillation around OZ. The movement around OX can be either a hindered rotation F_a or an oscillation F_b. Thus we have

$$F_a: v(z, \theta, \psi) = v_0 + \frac{1}{2} v_{z_0}''(z - z_0)^2 + \frac{k_\theta}{2} \cos^2\theta + \frac{1}{2} v_\psi'' \sin^2\psi \tag{20}$$

$$F_b\colon v(z,\theta,\psi) = v_0 + \frac{1}{2}v''_{z_0}(z-z_0)^2 + \frac{1}{2}v''_{\theta,\pi/2}\cos^2\theta + \frac{1}{2}v''_{\psi}\sin^2\psi \tag{21}$$

where $(k_\theta, v''_\psi, v''_{\theta,\pi/2})$ are force constants[2]. Consequently[2]

$$F_a\colon W_1 = \frac{A}{2\Pi}(2\Pi RT)^{3/2}(v''_{z_0}\, v''_\psi\, k_\theta)^{-1/2}\exp\left(-\frac{v_0}{RT}\right) \tag{22}$$

$$F_b\colon W_1 = \frac{A}{2\Pi}(2\Pi RT)^{3/2}(v''_{z_0}\, v''_\psi\, v''_{\theta,\pi/2})^{-1/2}\exp\left(-\frac{v_0}{RT}\right) \tag{23}$$

The problem of numerical evaluation of integrals W_2 is more complicated. For this purpose we assume that the adsorbate molecules interact via simple LJ (12–6) potential

$$u(r) = 4\,\varepsilon_{gg}\left[\left(\frac{\sigma_{gg}}{r}\right)^{12} - \left(\frac{\sigma_{gg}}{r}\right)^{6}\right] \tag{24}$$

with the parameters ε_{gg} and σ_{gg}. These parameters can be treated as averages[4]. The integrals W_2 were evaluated according to eqn. 4.

To investigate the practicality of the above theory, we compared the results of the theoretical calculations of the temperature dependence of integrals W_1 for benzene, hexane and pentane, as well as the dependence of their retention volumes on their densities in the free gas phase, with experimental data. In the theoretical calculations adequate literature data were used.

Experiments were carried out by the chromatographic method using as model adsorbent graphitized lamp-black, which was also examined spectroscopically.

Numerical calculations

The adsorption potential energies were calculated for a whole molecule and for different locations of centre of mass r_m with respect to the graphite plane. The value of v_0 and z_0 were taken as average values, averaged over all considered locations of r_m. These are summed in Table I. Next, the values of W_1 were calculated according to eqns. 19, 22 and 23. They are compared with experimental results in Figs. 1–3. Table II presents the values of isosteric heats of adsorption in the zero coverage limit, calculated according to the equation

$$q^0_{st} = RT + R\frac{\partial \ln W_1}{\partial(1/T)} = RT + u^0_{st} \tag{25}$$

TABLE I

THE VALUES OF v_0 AND z_0 FOR BENZENE, HEXANE AND PENTANE ON GRAPHITE

	Benzene	*Hexane*	*Pentane*
z_0 (Å)	3.32	3.78	3.72
v_0 (cal/mole)	−10,260	−11,100	−9740

TABLE II

THEORETICAL AND EXPERIMENTAL VALUES OF q_{st}^0 (cal/mole) AT 360 °K

Adsorbate	*Theort.*	*Exptl.*
Benzene	10,030	9960
Hexane	10,420	10,240
Pentane	9920	10,030

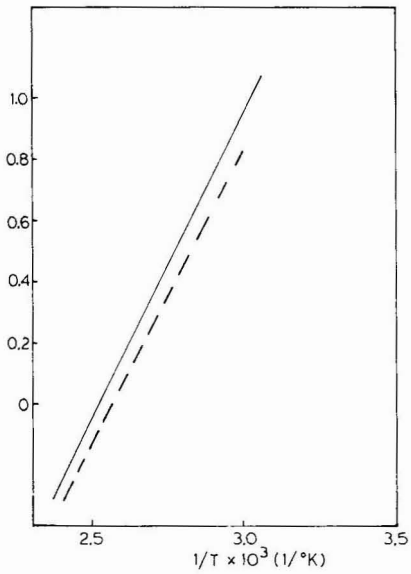

Fig. 1. Variation of the integrals W_1 as a function of temperature for benzene on graphitized lampblack. The solid line ($u_{st} = 9.31$) was calculated according to eqn. 19, and the broken line ($u_{st}^0 = 8.934$) was obtained experimentally.

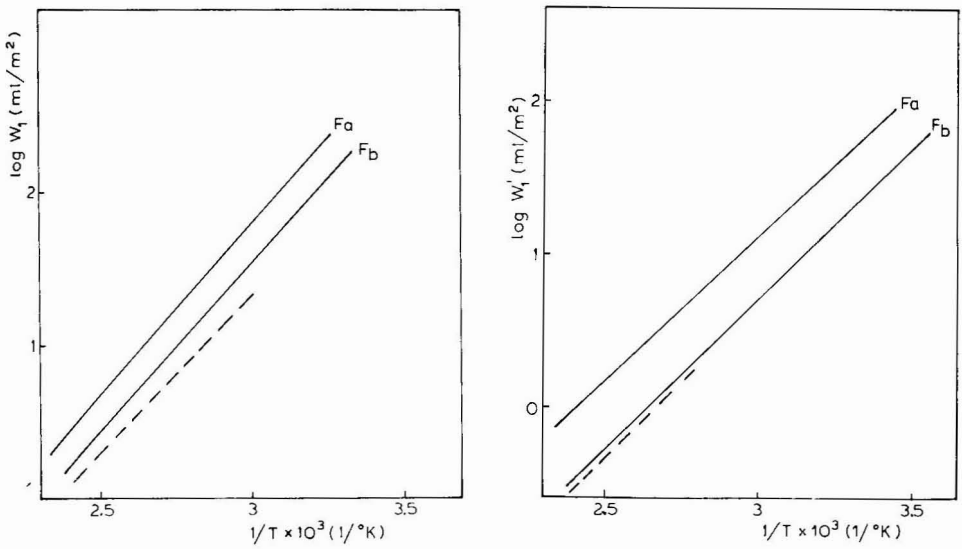

Fig. 2. Variation of the integrals W_1 as a function of temperature for *n*-hexane on graphitized lamp black. The solid lines denote the results of theoretical calculations ($u_{st}^0 = 10.2$ kcal/mole) and the broken line ($u_s^0 = 9.52$ kcal/mole) was obtained experimentally.

Fig. 3. As for Fig. 2, but for *n*-pentane. Solid lines: $u_{st}^0 = 9.2$ kcal/mole); broken line: $u_{st}^0 = 9.31$ kcal/mole.

The second part of our calculations are concerned with the evaluation of the initial plot of $V_N = V_N(n)$ for pentane at 387.7 °K and 395.6 °K, calculated according to eqn. 8, truncated at n^2. The effective values of the parameters ε_{gg} and σ_{gg} were taken from the paper by Lal and Spencer[12]. They were $\varepsilon_{gg} = 568$ cal/mole, and $\sigma_{gg} = 4.13$ Å. The results of our calculations are shown in Fig. 4.

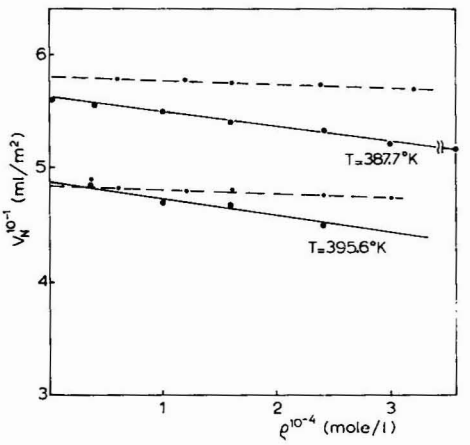

Fig. 4. The initial plot of V_N against n. The points denote the experimental data, whereas the dashed lines were calculated according to eqn. 8, truncated to n^2.

EXPERIMENTAL

Commercial lamp-black, made in the USSR, was graphitized in an Acheson resistance furnace. The specific surface areas of carbon blacks were measured by the method of thermal desorption of nitrogen. Elemental analysis of the adsorbents was carried out using the Hewlett-Packard analyser CHN Model F & M 185. The microcrystalline structure was examined by the Dron-1 diffractometer with a cupric lamp and nickel filter. The degree of graphitization was established measuring the ratio of the intensities of total line 112 and total line 110 in relation to the natural madagascaric graphite. Electron paramagnetic resonance (EPR) measurements were carried out at room temperature using EPR spectrometer type RE-1301, with diphenylpicryl hydrazide as reference. Photomicrographs were obtained with a Tesla B-613 electron microscope with resolving power 4.5 Å. The characterisics of carbon blacks before and after graphitization are summarized in Table III. Photomicrographs of lamp-black before and after graphitization are presented in Fig. 5 and 6.

The chromatographic measurements were carried out on an ICSO Chromatograph with a detector of thermal conductivity. Hydrogen passed over molecular sieves 4A and 5A was used as carrier gas, flow-rate 40 ml/min. The chromatographic column was 1 m long and 4 mm I.D., and contained 6.87 g of adsorbent. The adsorbent particle sizes 0.30–0.49 mm were selected for use. Adsorbate samples (*n*-pentane, *n*-hexane and benzene, 0.1 to 1.0 μl) were introduced on the column with the Hamilton syringe. The absolute retention volume and adsorbate density were measured in the

TABLE III

CHARACTERIZATION OF PHYSICOCHEMICAL PROPERTIES OF GRAPHITIZED AND NON-GRAPHITIZED CARBON BLACKS

Carbon black	*Surface area* (m^2/g)	*Elementary composition* (%)		*Size of the crystal* (Å)		*Concentration of spine* ($1/g$)	*Degree of graphitization* (%)
		C	*H*	*Lc*	*La*		
Lamp-black, non-graphitized	17.0	95.1	0.6	17.3	27.3	$7.4 \cdot 10^{21}$	—
Lamp-black, graphitized	15.2	99.0	0.3	126	197	$1 \cdot 10^{21}$	53

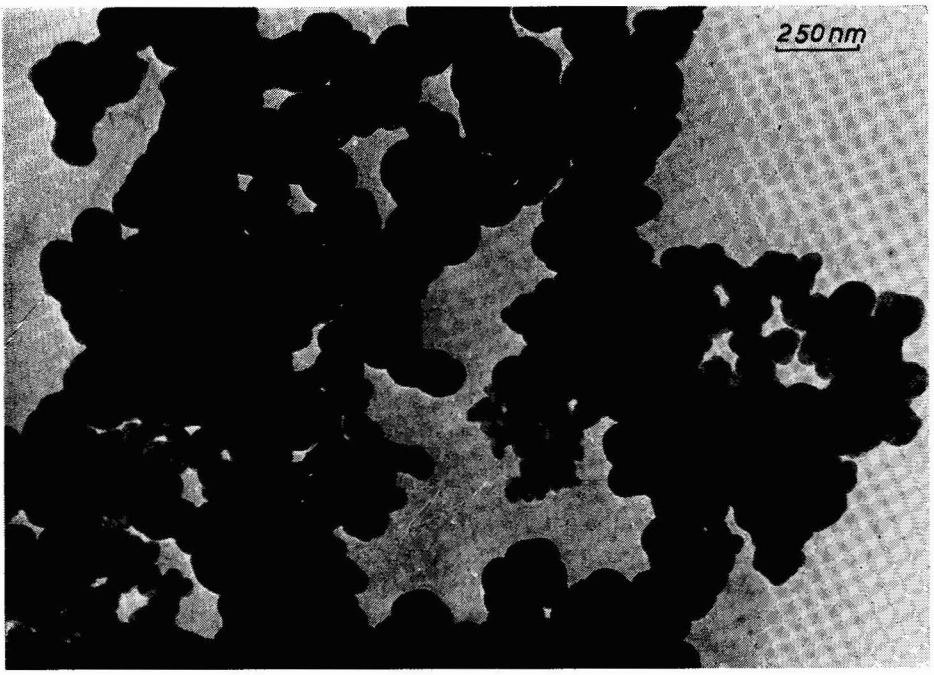

Fig. 5. Electron micrograph of the lamp-black before graphitization.

range 360–420 °K. Conditions were chosen to eliminate dynamic effects as far as possible.

RESULTS AND DISCUSSION

The Henry's constants (integrals W_1) of hydrocarbons adsorbed on graphitized blacks have been evaluated by many authors, using various models. We used the models of adsorption potential proposed by Vidal-Madjar *et al.*[2], which we felt were applicable because they permitted the authors to obtain excellent agreement between values of retention volume, differential energy of adsorption and entropy of adsorp-

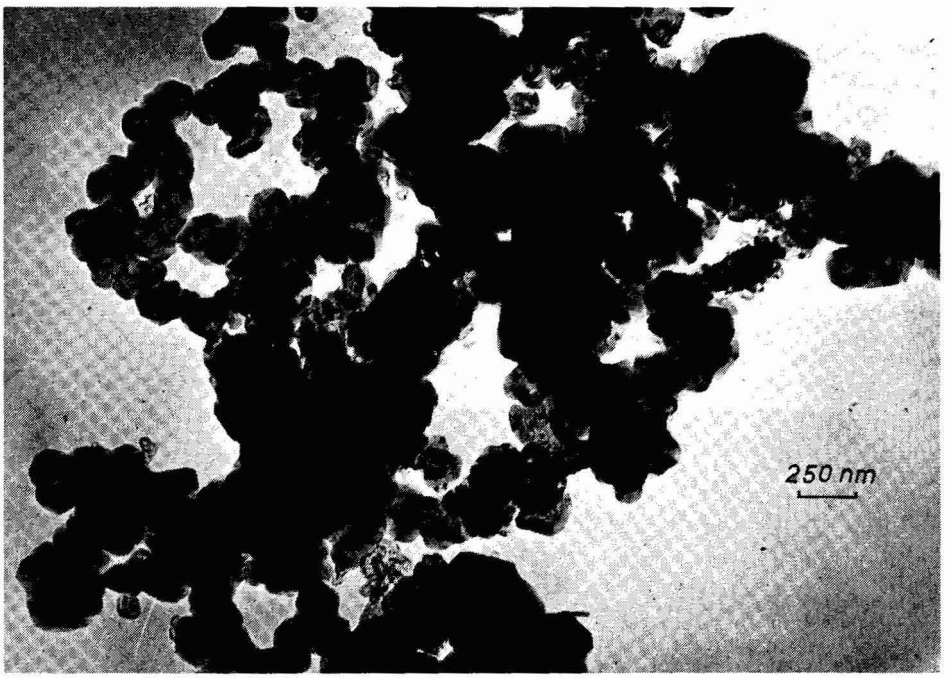

Fig. 6. Electron micrograph of the lamp-black after graphitization.

tion calculated theoretically and obtained experimentally. We also obtained good agreement between the results of theoretical thermodynamic evaluations and experimental data, as can be seen from Figs. 1–3, in which solid lines indicate calculated results and dashed lines experimental results. In theoretical evaluations of integrals W_1 we assumed an adsorbent surface area of 15 m/g, *i.e.* corresponding to the surface area by tested graphitized lampblack is characterized. Heats of adsorption calculated from eqn. 25 ($T = 360$ °K) were compared with heats measured chromatographically, and were in reasonable agreement (Table II). The main aim of our work was to find a way of evaluating retention volume as a function of adsorbate density in the free gas phase based on the proper model of adsorption and interaction potentials. The results of this type of calculation for pentane adsorbed on tested graphitized black are presented in Fig. 4. In these calculations the surface area was treated as a best-fit parameter. The experimental value for A was 15 m^2/g, and the calculated value 11 m^2/g. Fig. 4 shows that the agreement between the theoretical dependence $V_N = V_N(n)$ and the experimental data is not good. There are many reasons for this, the two chief being that the experiments were conducted in dynamic conditions, and that the crystalline structure of tested black deviated from the ideal.

The chromatographic process is dynamic by nature, and thus our assumption of perfect equilibrium, which would hold in static conditions, is not entirely valid here. Therefore, although the chromatographic conditions we used secured the minimal influence of diffusion and kinetic effects, the effect of these factors on the retention data could not be eliminated. The imperfect structure of graphitized lamp-black is illustrated by the degree of its graphitization, amounting to 53 °C in

relation to pure madagascaric graphite. Also the blacks adsorb gas molecules on a surface which consists of randomly orientated microcrystallites. These deviations from the perfect structure cause errors in the calculation of W_1.

Another reason for the discrepancies between the results is connected with the assumed model of two molecules of adsorbate in the superficial phase. The energy of interaction between gas and solid state was assumed in accordance with Guiochon's F_b model[1,2]. The assumption of effective values of the LJ (12—6) potential in our calculations has considerable faults, because that approach treats adsorbate molecules as spheres. In addition, we assumed that the energy of interaction does not depend on molecular orientation in relation to the adsorbent surface.

It is obvious that these assumptions are not adequate in the case of adsorbed molecules, as the adsorbent forces molecular orientation of adsorbate (Battezatti *et al.*[3]). However, these assumptions make it possible to carry out numerical calculations of the adsorption potential. More realistic assumptions would lead to closer agreement between the results.

It would appear that this method of theoretical evaluation of retention volumes can be used to predict retention volumes of chromatographed substances. Graphitized blacks are characterized by a special topography of adsorption centres which makes possible the separation of complex mixtures of organic compounds.

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DYNAMISCHER STRÖMUNGSTEILER FÜR PRÄPARATIVE GAS-CHROMATOGRAPHEN

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SUMMARY

Dynamical flow splitter for use in preparative-scale gas chromatography

A dynamical carrier gas splitting system for use in preparative-scale gas chromatography was developed. The splitter consists of a combination of a capillary system heated by the thermostat of the chromatographic apparatus and an unheated needle valve fitted outside the thermostat. In this way troubles resulting from condensation of high-boiling substances in the splitter and leaks in the needle valve were avoided. Results of theoretical and experimental investigations concerning the pneumatic basic principle and the dynamical characteristics of this splitting system are described. This splitter allows a stepless change in gas flow ratio from 1:50 to 1:600. Practical use shows that the splitter is characterized by high reliability in automatic recycling runs as well as in case of large sample size and high boiling substances.

EINLEITUNG

Flammenionisationsdetektoren (FID) werden vorteilhaft in präparativen Gas-Chromatographen eingesetzt, weil preiswerter Stickstoff als Trägergas verwendet werden kann, geringe Empfindlichkeit gegenüber Temperatur-, Druck- und Gasmengenstromschwankungen besteht, eine stabile Nullinie vorliegt und auf Grund hoher Detektorempfindlichkeit die Sammlung von Spurenverunreinigungen möglich ist.

Die Verwendung eines FID erfordert jedoch die Aufteilung des Eluates auf Sammelvorrichtung und Detektor mit Hilfe eines Strömungsteilers, der im beheizten Teil des Säulenthermostaten angebracht sein muss, um eine vorzeitige Kondensation des getrennten Produktes auszuschliessen.

Der Strömungsteiler muss für eine optimale und störungsfreie Arbeitsweise des

automatischen präparativen Gas-Chromatographen nachstehend aufgeführte Forderungen erfüllen:

Einstellbarkeit des Teilerverhältnisses über einen weiten Bereich,
Temperaturunempfindlichkeit des Stellgliedes für das Teilerverhältnis,
bequeme Zugänglichkeit zum Strömungsteiler im Thermostatenraum,
hohe zeitliche Konstanz des Teilerverhältnisses.

Die bisher übliche Aufteilung des Eluates mittels Nadelventilen[1] oder unterschiedlich dimensionierter, auswechselbarer Kapillaren[2] führt in der Praxis oft zu Schwierigkeiten, da die Anwendung beheizter Nadelventile häufig zu unüberwindlichen Dichtungsproblemen führt, während auswechselbare Kapillaren keine kontinuierliche Optimierung und Korrektur des Teilungsverhältnisses während des Betriebes gestatten. Einen prinzipiellen Ausweg aus diesen Schwierigkeiten eröffnet die Möglichkeit der pneumatischen Umschaltung von Gaswegen[3,4]. In dem hier beschriebenen dynamischen Strömungsteiler werden durch Kombination von Kapillaren und Nadelventil die Vorteile der Beheizbarkeit mit denen der kontinuierlichen Veränderung des Gasstromes verknüpft, wobei die Kapillaren im Thermostatenraum und das Nadelventil zur Vermeidung von Dichtungsproblemen ausserhalb des Thermostatenraumes angebracht sind. Einige praktische Trennaufgaben demonstrieren Funktionsweise, Flexibilität und Einsatzgrenzen des dynamischen Strömungsteilers.

THEORETISCHE GRUNDLAGEN

Nach Ferner[5] lässt sich für pneumatische Widerstände im Bereich niederer Drücke und laminarer Strömungen die Dimension Ohm analog zu den bekannten Begriffen der Elektrotechnik verwenden. Diese Analogie ermöglicht die quantitative Berechnung der Teilströme J_1 zur Sammelvorrichtung und J_3 zum FID sowie des Teilerverhältnisses ausgehend von dem Ersatzschaltbild (Fig. 1) in Abhängigkeit vom Hilfsgasstrom J_H oder dem Eluatstrom J.

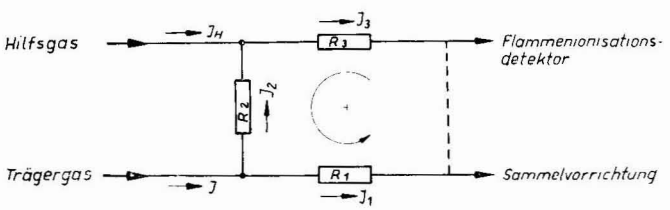

Fig. 1. Ersatzschaltbild dynamischer Strömungsteiler.

Gemäss den Kirchhoffschen Sätzen gilt für die Anordnung nach Fig. 1 unter der Annahme, dass der Druck an der FID-Düse gleich dem an der Sammelvorrichtung ist:

(Maschensatz) $$J_1 R_1 - J_3 R_3 - J_2 R_2 = 0 \quad (1)$$

(Knotenpunktsatz) $$J - J_1 - J_2 = 0 \quad (2)$$

$$J_H + J_2 - J_3 = 0 \quad (3)$$

Mit diesem Ansatz ergibt sich nach einigen Zwischenrechnungen für den Teilstrom zur Sammelvorrichtung

$$J_1 = J \frac{R_2 + R_3}{R_1 + R_2 + R_3} + J_H \frac{R_3}{R_1 + R_2 + R_3} \tag{4}$$

und zum FID

$$J_3 = J_H \frac{R_1 + R_2}{R_1 + R_2 + R_3} + J \frac{R_1}{R_1 + R_2 + R_3} \tag{5}$$

Das Teilerverhältnis lässt sich aus dem durch R_1 strömenden Teil J_1 im Verhältnis zu dem effektiv durch R_2 strömenden Eluatanteil J_2^* zum FID ermitteln.

$$T = \frac{J_1}{J_2^*} \tag{6}$$

Durch Grenzwertbetrachtung der Gleichung 4 und Gleichung 5 für $J = 0$ und $J_H = 0$ erhält man für J_2^*

$$J_2^* = (J_3)_{J_H=0} - (J_1)_{J=0} \tag{7}$$

$$= J \frac{R_1}{R_1 + R_2 + R_3} - J_H \frac{R_3}{R_1 + R_2 + R_3} \tag{8}$$

und damit für das Teilerverhältnis

$$T = \frac{J(R_2 + R_3) + J_H R_3}{J R_1 - J_H R_3} \tag{9}$$

In Fig. 2 ist das Teilerverhältnis in Abhängigkeit vom Gasstrom J_H mit dem Eluatstrom J als Parameter (5, 10, 15 l/h) aufgetragen, wobei das Grundteilerverhältnis $T_0 = 50 : 1$ beträgt ($R_1 : R_2 : R_3 = 1 : 25 : 25$). Die zugehörigen Gasströme J_1 und J_3 zeigt die Fig. 3.

Aus Fig. 2 ist zu erkennen, dass die Einstellbarkeit des Teilerverhältnisses für $T > 200 \ldots 500$ unbefriedigend ist, wobei der niedrigere Wert für einen Trägergasstrom von 5 l/h und der hohe Wert für 15 l/h gilt. Diese Betrachtung setzt eine lineare Kennlinie des Nadelventils voraus.

Das Verhältnis der Widerstände R_2 und R_3 beeinflusst die Einstellcharakteristik des Teilers sowie die zum FID strömende Gasmenge in Abhängigkeit vom Teilerverhältnis. Beispielsweise steigt bei einem Trägergasmengenstrom von 15 l/h die dem FID zugeführte Teilstrommenge in Abhängigkeit vom Teilerverhältnis von $T_0 = 50 : 1$ bis $T = 1000 : 1$ von 0.3 l/h (T_0) auf $\approx$ 0,6 l/h (T_{1000}).

Fig. 4 zeigt die Abhängigkeit des Teilerverhältnisses mit veränderten Widerstandsverhältnissen. Für $R_2 : R_3 = 1 : 8$ liegt eine steile Steuercharakteristik $T = f(J_H)$ vor bei relativ geringer Gasmengenstromänderung zum FID und bei $R_2 : R_3 = 1{,}25 : 1$ eine flache Steuercharakteristik bei mehr als doppelter Gasmengenstromänderung.

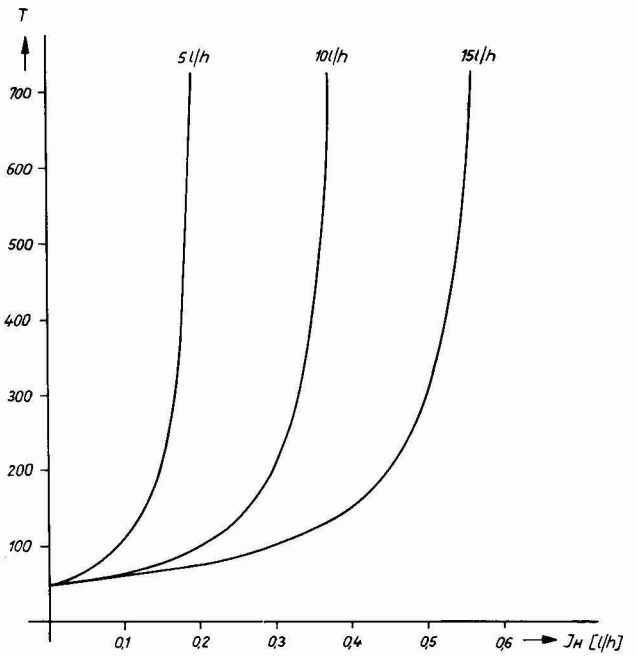

Fig. 2. Teilerverhältnis T in Abhängigkeit vom Hilfsgasstrom J_H, Parameter: Eluatstrom $J = 5$; 10 und 15 l/h, Grundteilerverhältnis $T_0 = 50{:}1$, Widerstandsverhältnis $R_1{:}R_2{:}R_3 = 1{:}25{:}25$.

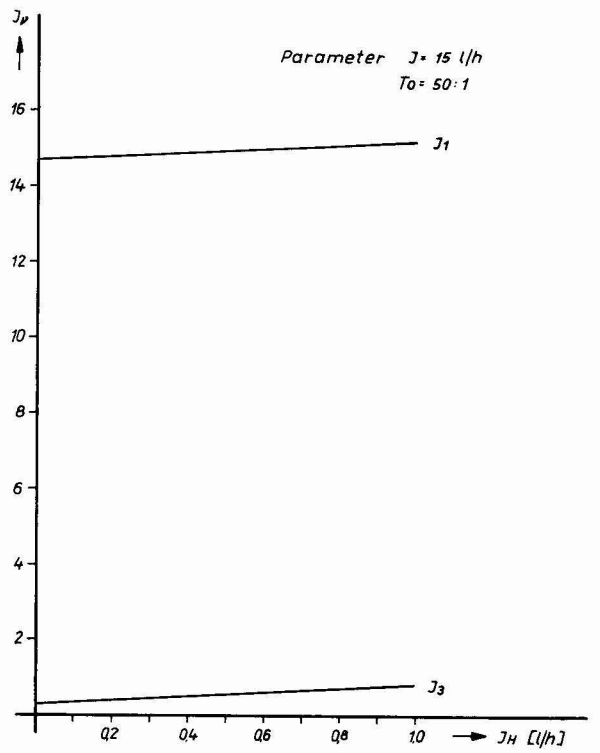

Fig. 3. Gasmengenstrom zur Sammelvorrichtung (J_1) und zum FID (J_3) in Abhängigkeit vom Hilfsgasstrom J_H, bei einem Tragergasstrom $J = 15$ l/h und einem Grundteilerverhältnis $T_0 = 50{:}1$.

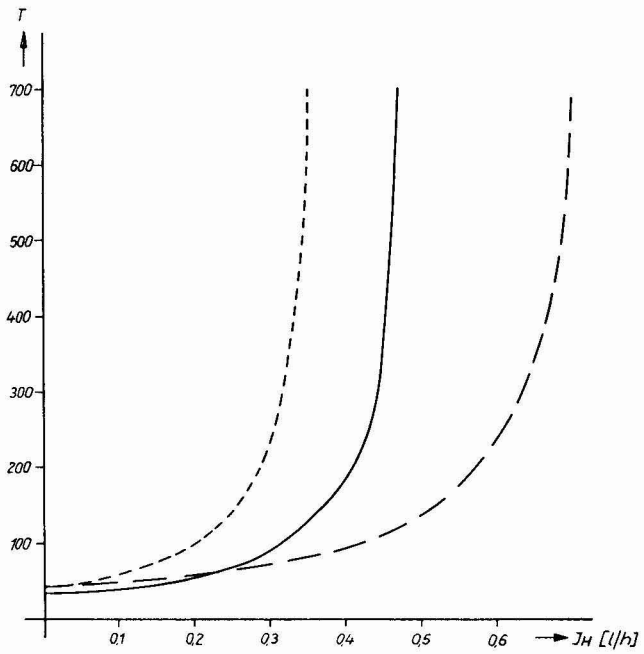

Fig. 4. Teilerverhältnis in Abhängigkeit vom Gasstrom J_H, bei verschiedenen Widerstandsverhältnissen $R_1:R_2:R_3$, Eluatstrom $J = 15$ l/h. ———, Grundteilerverhältnis $T_0 = 35:1$, $R_1:R_2:R_3 = 1:5:30$. – – – –, Grundteilerverhältnis $T_0 = 45:1$, $R_1:R_2:R_3 = 1:5:40$. — — —, Grundteilerverhältnis $T_0 = 45:1$, $R_1:R_2:R_3 = 1:25:20$.

AUFBAU UND FUNKTION DES DYNAMISCHEN STRÖMUNGSTEILERS

Der dynamische Strömungsteiler[6] besteht aus einem festen System von drei Kapillaren und einem Nadelventil (Fig. 5). Die Kapillaren befinden sich im Thermostaten zwischen Säulenausgang und Sammelvorrichtung (R_1) sowie FID (R_2, R_3). Das nichtbeheizte Nadelventil ist ausserhalb des Thermostaten angeordnet. Die Auf-

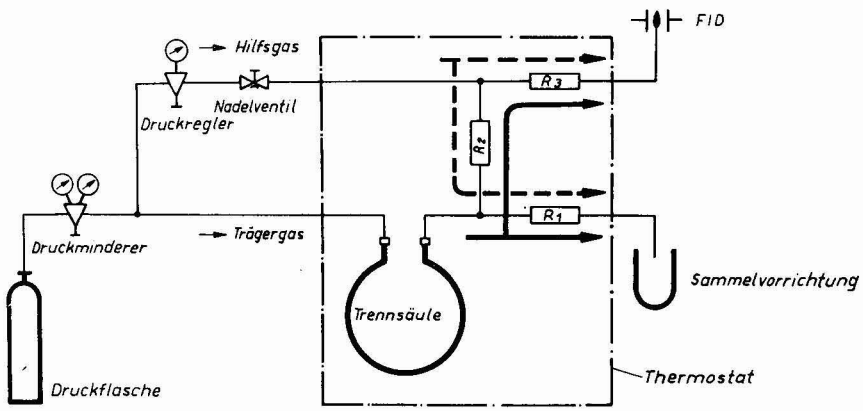

Fig. 5. Schema dynamischer Strömungsteiler.

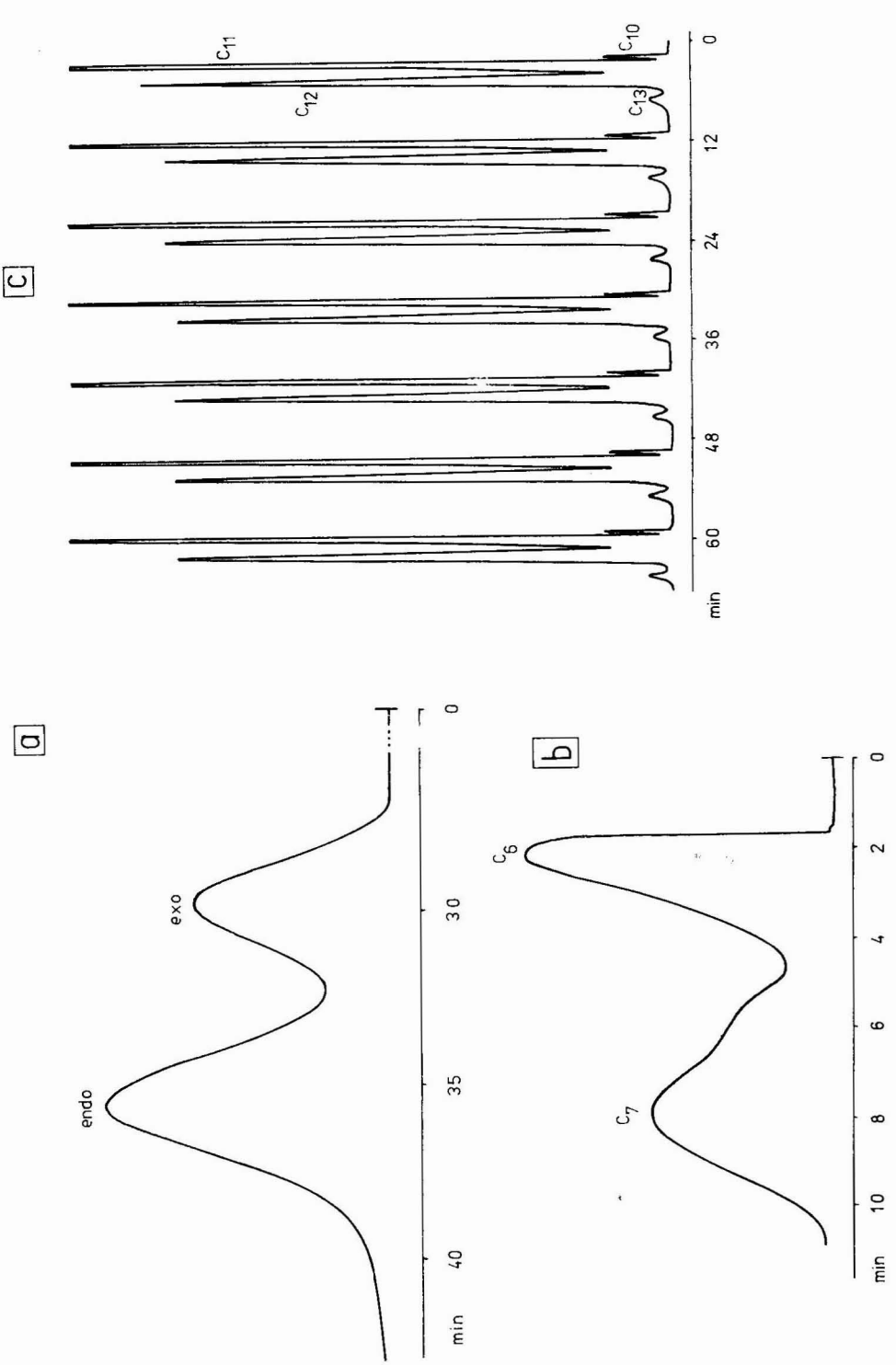

Fig. 6. Präparative Trennungen von Probenmengen zwischen 0.06 und 3 ml. (a) 0.06 ml endo-,exo-Bicyclo(2,2,1)hepten-2-carbonsäure-isobutylester-4; (b) 3 ml eines *n*-Hexan–*n*-Heptan-Gemisches; (c) zyklisch wiederholte Auftrennung von 0.4 ml eines *n*-Kohlenwasserstoffgemisches (Parex I mit den Komponenten n-$C_{10}H_{22}$ . . . n-$C_{13}H_{28}$).

teilung des Eluates wird durch Zuführung eines Gasstromes über das Nadelventil an dem Verbindungspunkt der Kapillaren R_2, R_3 bewirkt. Die Gasmenge kann sowohl durch Druckänderung vor dem Nadelventil oder Betätigung des Nadelventils eingestellt werden. Damit lässt sich das Teilerverhältnis in weiten Grenzen zwischen ca. 1 : 10 bis 1 : 500 in Abhängigkeit vom Grundteilerverhältnis einstellen.

Die Vorteile des dynamischen Strömungsteilers bestehen darin, dass das Nadelventil nur von reinem Trägergas durchströmt wird und bei Raumtemperatur betrieben werden kann. Alle mit dem Eluat in Berührung kommenden Teile können ohne Funktionsbeeinträchtigung auf Thermostattemperatur erwärmt werden, und somit sind Störungen durch Veränderung des Teilerverhältnisses infolge Kondensation weitestgehend ausgeschlossen. Der Schwierigkeitsgrad des Trennproblems und die Empfindlichkeit des FID beeinflussen die Wahl des Grundteilerverhältnisses. Als Grundteilerverhältnis T_0 wird die Aufteilung des Eluates auf Sammelvorrichtung und FID definiert, wenn kein Gasstrom über das Nadelventil eingespeist wird (Gl. 1), wobei $J = J_1 + J_3$ dem aus der Säule austretenden Trägergasstrom entspricht.

$$T_0 = \frac{J_1}{J_3}, J_H = 0 \tag{10}$$

Um den FID im optimalen Empfindlichkeitsbereich betreiben zu können, wird über ein zusätzliches Regelsystem ein konstanter Trägergasstrom von 2 l/h unmittelbar vor dem FID eingespeist.

PRAKTISCHE ANWENDUNG

Der vorstehend beschriebene dynamische Strömungsteiler wurde als Bauelement des kommerziellen Präparativ-Zusatzes zum Gas-Chromatographen GCHF 18.3, VEB Chromatron, Berlin, praktisch erprobt[7,8]. Es wurden Trennsäulen von 10 mm Innendurchmesser verwendet, die je nach Trennproblem 1, 2 oder 3 m lang waren. Die Dosierung erfolgte manuell mittels Spritze oder automatisch mit Hilfe der Druck-Zeit-Dosierung des Präparativ-Zusatzes. Die Teilerverhältnisse variierten zwischen dem Grundteilerverhältnis von 1 : 50 bis zu e nem Teilerverhältnis von 1 : 600.

Messen des Teilerverhältnisses

Zur Bestimmung des Teilerverhältnisses wird zunächst die Berghöhe h_0 einer Eichsubstanz beim Grundteilerverhältnis T_0, im vorliegenden Falle 50 : 1, und anschliessend die Berghöhe h_T bei dem gewählten Teilerverhältnis T bei gleicher Detektorempfindlichkeit gemessen. Das Teilerverhältnis errechnet sich aus den Berghöhen h_0 und h_T bei gegebenem Grundteilerverhältnis nach der Gleichung

$$T = \frac{h_0 (T_0 + 1)}{h_T} - 1 \tag{11}$$

Erfolgt die Dosierung der Eichsubstanz mit einer dem Flüssigkeitsinjektor parallel geschalteten Gasschleife, so kann das Teilerverhältnis bequem und schnell gemessen und ein für die anstehende Trennaufgabe günstiges Teilerverhältnis gewählt werden.

Häufig genügt eine Abschätzung des Teilerverhältnisses mit Hilfe der Signalgrösse, die bei einer gegebenen Detektorempfindlichkeit und Probenmenge erfahrungsgemäss zu erwarten ist. Werden kleinere Substanzmengen im präparativen Masstab getrennt, so ist ein weniger grosses Teilerverhältnis günstiger, um nicht durch hohe Detektorempfindlichkeit bei Grundlinieninstabilitäten eine unerwünschte Kühlfallenansteuerung auszulösen.

Ein über einen längeren Zeitraum reproduzierbares Teilerverhältnis lässt sich entsprechend der in Fig. 2 dargestellten T–J_H-Kurven am besten im Bereich bis $T = 300$ und höheren Strömungsgeschwindigkeiten realisieren.

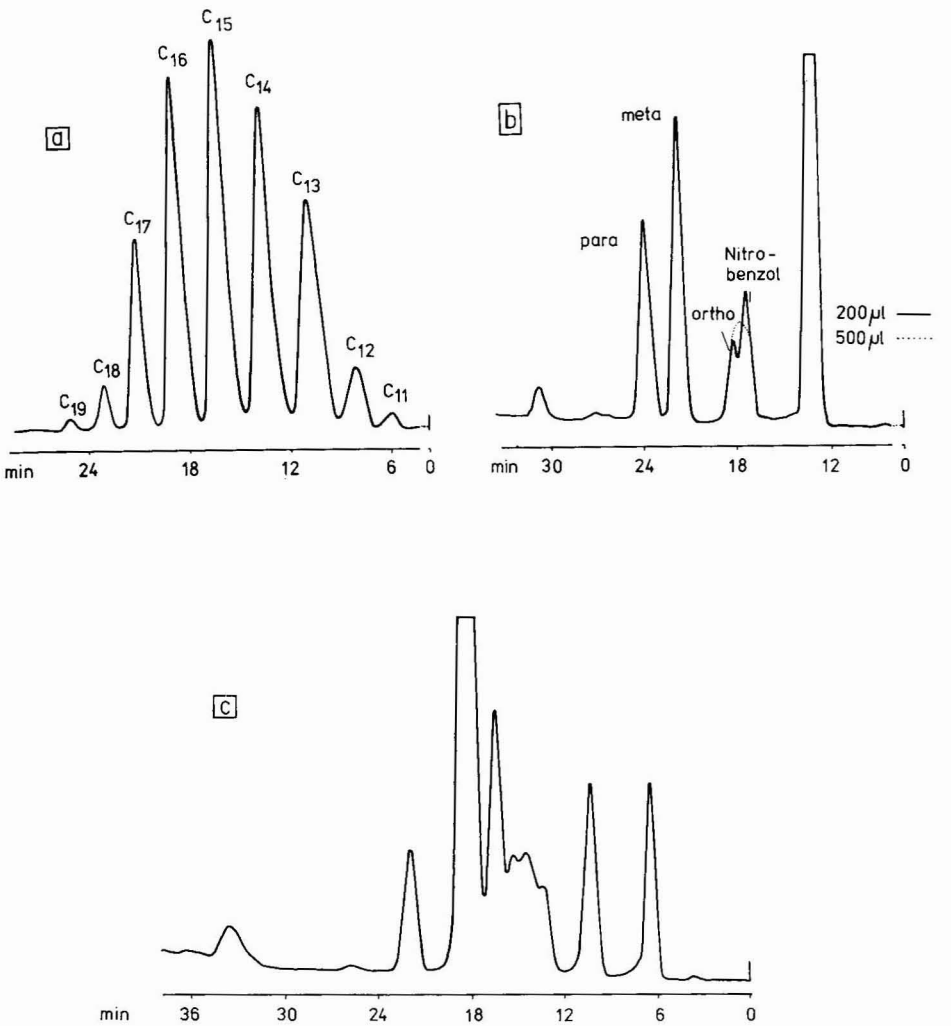

Fig. 7. Präparative Trennungen von hochsiedenden Substanzgemischen. (a) 0.7 ml eines *n*-Kohlenwasserstoffgemisches (Parex II mit den Komponenten *n*-$C_{11}H_{24}$ ... *n*-$C_{19}H_{40}$); (b) 0.5 ml *o*-, *m*-, *p*-Nitrophenyldimethylfluorsilan; (c) 0.5 ml eines hochsiedenden Gemisches substituierter Zyklosiloxane zur spektroskopischen Identifizierung.

Trennergebnisse

Ein Vergleich der in Fig. 6 dargestellten Chromatogramme zeigt, dass die dosierten Probenmengen lediglich durch die notwendigen chromatographischen Arbeitsbedingungen bestimmt werden. Um eine hinreichende Auftrennung zu erhalten, können beispielsweise von den schwierig trennbaren endo-exo-Isomeren nur 60 μl, von Parex I 0.4 ml und von dem leicht trennbaren Testgemisch *n*-Hexan–*n*-Heptan 3 ml pro Zyklus dosiert werden, ohne die Funktionsweise des Teilers nachteilig zu beeinträchtigen. Fig. 6c demonstriert ausserdem die gute Konstanz der Grundlinie und Reproduzierbarkeit der Gas-Chromatogramme im automatischen Dauerbetrieb.

Wie die in Fig. 7 dargestellten präparativen Trennungen von hochsiedenden Substanzgemischen weiten Siedebereiches zeigen, arbeitet der dynamische Strömungsteiler auch bei Säulentemperaturen um 300° für die präparative Gas-Chromatographie im Milliliterbereich störungsfrei. Das eingestellte Teilerverhältnis bleibt hinreichend konstant und reproduzierbar, solange das Druckniveau des gesamten Systems nicht durch Verschmutzung der Kapillaren und des Auffangsystems durch viskose, polymerisierende oder auskristallisierende Verbindungen krass geändert wird.

Die Arbeitsbedingungen gehen aus Tabelle I hervor.

TABELLE I

ARBEITSBEDINGUNGEN FÜR DIE DURCHFÜHRUNG DER PRÄPARATIVEN TRENNUNGEN

Substanz	*Fig.*	*Siedebereich (°C/Torr)*	*Trennflüssigkeit*	*Säulenlänge (m)**	*Säulentemperatur (°C)*	*Trägergas, Stickstoff (ml/min)*
Bicyclo(2,2,1)hepten-2-carbonsäure-isobutylester-4	6	103 –105/13	20% Carbowax 20M	3	150	237
n-Hexan–*n*-Heptan	6	68.6–98.4/760	23% OE 4011	2	100	167
Parex I	6	150.6–234/760	20% Silikongummi BC	2	200	200
Parex II	7	195.8–330/760	20% Silikongummi BC	2	180–280 4°/min	200
Nitrophenyldimethylfluorsilan						
o-	7	107 –112/3	20% Silikongummi BC	2	100–270 6°/min	200
m-		105 –115/10				
p-		Fp. 42				
Cyclosiloxangemisch	7	84 –121/0.6	20% Silikongummi BC	2	150–300 4°/min	200

* Innendurchmesser: 10 mm.

ZUSAMMENFASSUNG

Ausgehend von auf der Grundlage eines elektrischen Ersatzschaltbildes durchgeführten theoretischen und experimentellen Untersuchungen der Strömungsverhält-

nisse in Kapillaren wurde ein dynamischer Strömungsteiler für die präparative Gas-Chromatographie entwickelt. Durch eine Kombination von beheizten Kapillaren und einem unbeheizten Nadelventil kann eine kontinuierliche Veränderung des zum Flammenionisationsdetektor bzw. zur Sammelfalle geführten Gasstromes erreicht werden, ohne dass Störungen durch vorzeitige Kondensation des Produktes oder Undichtigkeiten auftreten. Das Ergebnis ist grosse Zuverlässigkeit im Dauerbetrieb sowohl bei Dosierung grosser Substanzmengen pro Trennzyklus als auch bei der präparativen Trennung hochsiedender Substanzen.

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CHROM. 10,077

HIGH-SPEED GAS CHROMATOGRAPHIC ANALYSIS IN PROCESS CONTROL*

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SUMMARY

Among the different criteria for a process gas chromatograph to be included in a closed loop control, the analytical credibility is one of the most important and can be improved by averaging the results of several analyses carried out during the response time of the process. This criterion resulted in high-speed gas chromatographic analysis being developed.

Modified adsorption chromatography with Spherosil is one means of reducing the time of analysis to 1 min without difficulty. Several examples are presented to show the potential of such a technique in process gas chromatography.

INTRODUCTION

In order to be included in a closed loop system for the control of chemical processes, some types of on-line analysers must have their analytical credibility increased. With a process gas chromatograph the analytical credibility can be improved by averaging the results of several analytical measurements carried out within the response time of the process.

These requirements led to the development of high-speed chromatography.

The problem of reliability has already been solved with a technique called the "deferred standard"[1–4], which consists on on-line checking of the process chromatograph. High-speed chromatographic analysis has been known for many years; there are examples in the literature as early as 1957[5] of separations performed within 1 min on capillary columns. However, for use in process gas chromatography (PGC) a simpler solution with similar performances is required.

SIMPLE AND ECONOMIC HIGH-SPEED GC ANALYSIS

In a previous paper on PGC[6], the properties of modified adsorption chroma-

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tography were summarized, leading from classical analysis times (15–30 min), to accelerated analysis (5–15 min) and then to rapid analysis (1–5 min). The final step, the achievement of ultra-rapid analysis, with analysis times less than 1 min, is the result of studies undertaken during several years on Spherosil in modified gas–solid chromatography (GSC)[7–9].

When modified with thin layers of stationary phases, the main properties of Spherosil are as follows: decreased coefficient of mass transfer owing to a layer thickness of stationary phase between 10 and 20 Å (ref. 10); flatness of the Van Deemter curve, allowing faster carrier gas flow-rates, with a minimum loss in column efficiency; and efficient micro-packed columns, usable with common detectors; the final parameter to be considered in order to achieve very rapid analyses is the specific surface area. By considering these properties consecutively, the time of an analysis can be reduced by a factor of up to 30 without any difficulty.

As an illustration of this improvement, Fig. 1 shows the same mixture of five chlorinated hydrocarbons separated in different ways. In Fig. 1A, classical partition chromatography effects the separation in 25 min. In Fig. 1B, the separation is now achieved in 5 min, simply as a result of using modified adsorption instead of partition chromatography. This step is called "accelerated analysis". In Fig. 1C, the separation time is halved again by increasing the linear velocity of the carrier gas. However, in order to keep the volumetric flow-rate compatible with common industrial detectors (thermal conductivity or flame ionization), the column diameter has to be decreased. This is the "rapid analysis" step. In general, micro-packed columns of I.D. 1 mm are more efficient than 1/4-in. columns packed with the same material. Fig. 1D corresponds to "ultra-rapid analysis", which can be considered the cumulative result of the different properties considered above.

It has been shown[7–9] that the straightforward rules which govern any separation in modified gas–solid chromatography with Spherosil are as follows: at a constant specific surface area, S, and with a variable film thickness, d_f, the selectivity is variable; and with a variable S but constant d_f, the selectivity is constant. The latter rule means that superimposable chromatograms can be obtained on Spherosil of different specific surface areas, provided that the total surface energy is the same for the different columns. In other words, if d_f is constant, the product of the specific surface area of Spherosil and the column length has to be a constant, to first approximation:

$$S \cdot L = \text{constant} \tag{1}$$

By assuming that a homogeneous and reproducible packing is obtainable, the weight, W, of the packing can be replaced with the column length, L. Shortening an analytical sequence will involve increasing the specific surface area and reducing the column length, and consequently the dead volume of the column will be smaller.

Fig. 2 is an illustration of superimposable chromatograms of chlorinated hydrocarbons separated on two different types of Spherosil, with $S = 28$ and 200 m^2/g, but coated with a constant layer thickness of stationary phase. It can easily be seen that the amount of stationary phase is proportional to the specific surface area:

$$\frac{W_1}{S_1} = \frac{W_2}{S_2} \qquad \frac{2}{28} \approx \frac{14}{200} = 0.07$$

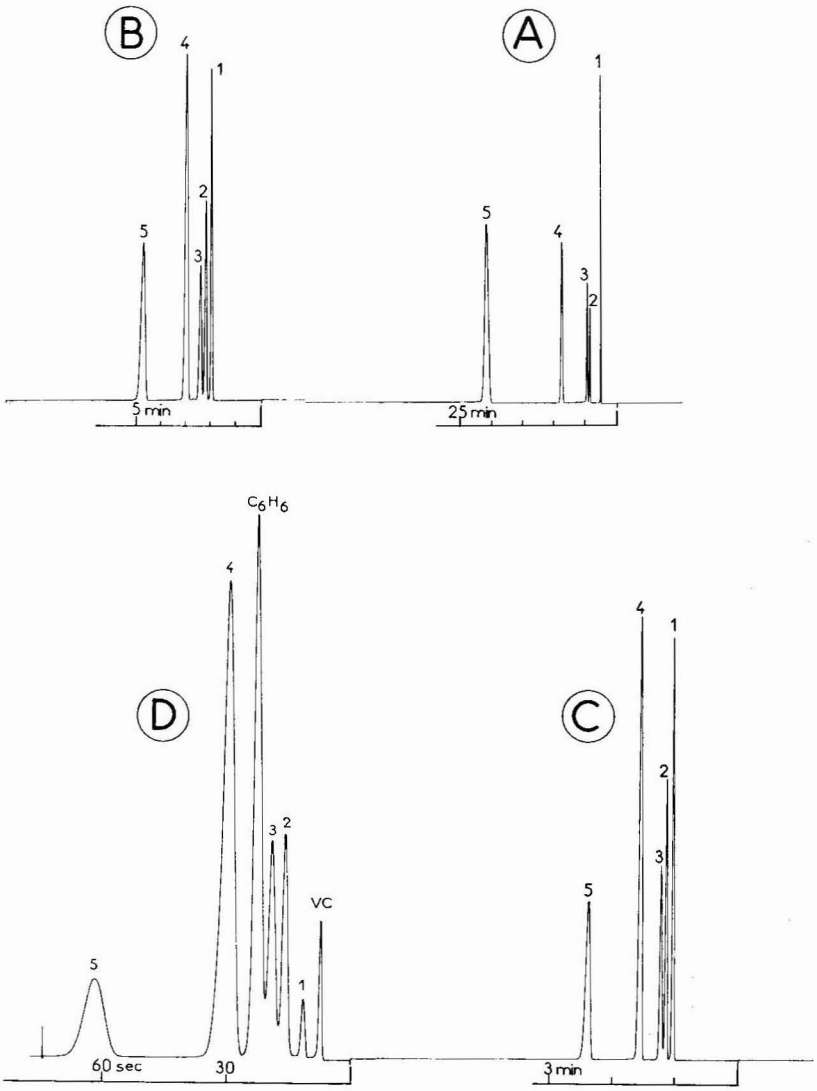

Fig. 1. Separation of a mixture of chlorinated hydrocarbons. Peaks: (1) vinylidene chloride; (2) methylene chloride; (3) carbon tetrachloride; (4) 1,2-dichloroethane; (5) 1,1,2-trichloroethane; VC, vinyl chloride; C_6H_6, benzene. (A) Partition chromatography (classical analysis). Column, 4 m × 4 mm I.D., Chromosorb P (d_p = 145–175 μm) + Carbowax 20M (20 g per 100 g); temperature, 130°; flow-rate (N_2), 3 l/h; Δp, 1.8 bar; injection, 1 μl of liquid. (B) Modified gas–solid chromatography (accelerated analysis). Column, 4 m × 4 mm I.D. Spherosil (28 m^2/g, d_p = 125–200 μm) + Carbowax 20M (2 g per 100 g); temperature, 130°; flow-rate (N_2), 3 l/h; Δp, 2.5 bar; injection, 1 μl of liquid. (C) Modified gas–solid chromatography (rapid analysis). Column, 5 m × 1 mm I.D. Spherosil (28 m^2/g, d_p = 125–200 μm) + Carbowax 20M (2 g per 100 g); temperature, 130°; flow-rate (N_2) 0.9 l/h; Δp, 5 bar, injection, 1 μl of liquid. (D) Modified gas–solid chromatography (ultra-rapid analysis). Column, 0.7 m × 1 mm I.D. Spherosil (200 m^2/g, d_p = 80–90 μm) + Carbowax 20M (14 g per 100 g); temperature, 130°; flow-rate (N_2), 1.25 l/h; Δp, 5 bar; injection, 0.05 μl of liquid.

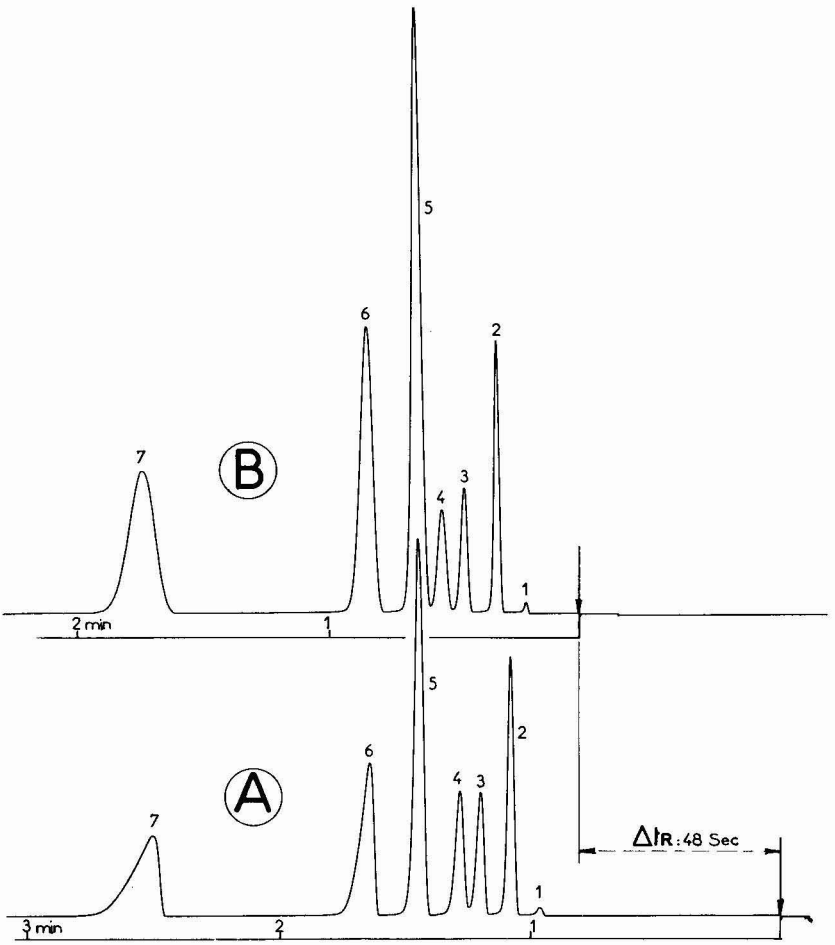

Fig. 2. Superimposable chromatograms of chlorinated hydrocarbons obtained on two different types of Spherosil. (A) Column, 5 m × 1 mm I.D. Spherosil (28 m^2/g, d_p = 125–200 μm) + Carbowax 20M (2 g per 100 g) temperature, 130°; flow-rate (N_2), 3 l/h. (B) Column, 0.7 m × 1 mm I.D. Spherosil (200 m^2/g, d_p = 80–90 μm) + Carbowax 20M (14 g per 100 g); temperature, 130°; flow-rate (N_2), 0.40 l/h. Peaks: (1) vinyl chloride; (2) vinylidene chloride; (3) methylene chloride; (4) carbon tetrachloride; (5) benzene; (6) 1,2-dichloroethane; (7) 1,1,2-trichloroethane.

and that the product $S \cdot L$ is constant:

$$S_1 \cdot L_1 = S_2 \cdot L_2$$

$$28 \cdot 5 = 200 \cdot 0.70 = 140$$

By applying the above properties (using a micro-packed column of coated Spherosil, increasing S, reducing L and increasing the carrier flow-rate), the mixture of chlorinated hydrocarbons containing two additional compounds (vinyl chloride and

benzene) is now separated in 72 sec on a column of I.D. 1 mm and length 70 cm packed with Spherosil (200 m^2/g) coated with Carbowax 20M (14 g per 100 g).

In addition, it must be pointed out that under the conditions used 950 theoretical plates (1350 plates/m) generated by the 70-cm column were sufficient to perform this separation, in spite of the 6-fold higher flow-rate than the optimum. Increasing the flow-rate still further would lead to an analysis time within the 60 sec required, but the resolution between the peaks would be poorer, despite the fact that the Van Deemter curve is rather flat in modified GSC (Fig. 3), and that the flow-rate can be increased.

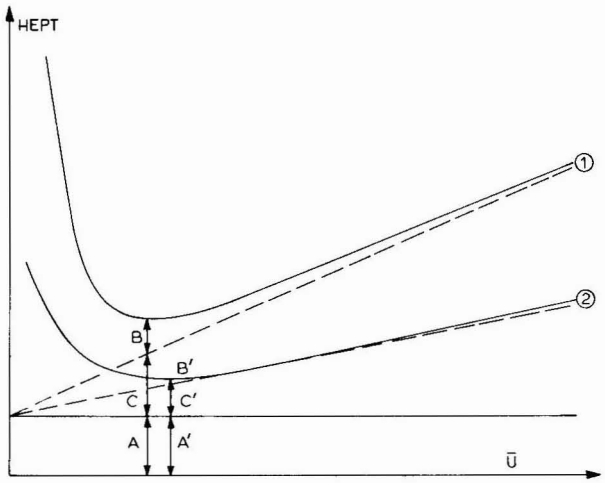

Fig. 3. Schematic curves of Van Deemter equation: HETP = f(u). (1) In partition chromatography; (2) in modified adsorption chromatography.

The resolution (R), which depends on the column efficiency and selectivity, is given by the well known equation

$$R = \frac{1}{4}\left(\frac{\alpha-1}{\alpha}\right)\cdot\left(\frac{k'_2}{1+k'_2}\right)\sqrt{N_2}$$

where

α = relative retention, i.e. the ratio K_2/K_1 of the partition coefficients of components 2 and 1;

k'_2 = capacity factor of component 2;

N = number of theoretical plates, calculated on peak 2.

In modified GSC on Spherosil, a decrease in resolution can be partly balanced on the one hand by using higher specific surface areas, which gives a higher selectivity[8] (see Figs. 5A and 5B), and on the other hand by trying to increase the total number of theoretical plates or, better, the number of effective plates per second as speed of analysis is the main concern.

The solution of the last problem was given by Huber *et al.*[11], who recommended that the particle size should be reduced to 30–50 μm in GC, by analogy with the effect of particle size in high-performance liquid chromatography. According to Huber *et al.*, column efficiencies of 10,000 plates/m are feasible, but there is a pressure drop, which in this instance can reach 30–50 bar. However, when applied to shorter columns

of coated Spherosil, this concept may be convenient and compatible with the present PGC technology, which tolerates no more than 8–10 bar of back-pressure in the pneumatic circuits.

Figs. 4A and 4B illustrate the Huber *et al.* theory combined with the effect of a high specific surface area of Spherosil and the selectivity of the liquid stationary phase. The same mixture of chlorinated hydrocarbons is still separated within 60 sec, but in this instance a column only a 8 cm long and 1 mm I.D. of Spherosil (200 m^2/g) coated with β,β'-oxydipropionitrile (ODPN, 14.5 g per 100 g), with a particle size distribution between 25 and 40 μm, was sufficient to achieve this separation (Fig. 4A). Despite a 4-fold greater flow-rate than the optimum, the column back-pressure was only 1.9 bar and the column generated 250 theoretical plates (3,125 plates/m) or 5 N_{eff}/sec. Fig. 4B shows the limits of the technique: a 4-cm column filled with the same packing still allows a useful separation of the mixture.

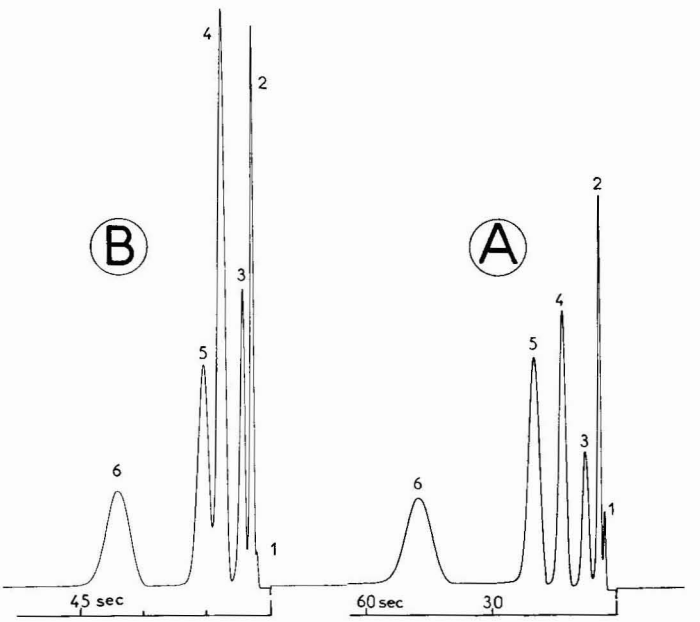

Fig. 4. Separation of chlorinated hydrocarbons on a short GC column. (A) Column, 8 cm × 1 mm I.D. Spherosil (200 m^2/g, d_p = 25–40 μm) + β,β'-oxydipropionitrile (14.5 g per 100 g); temperature, 85°; flow-rate (N_2), 0.66 l/h; Δp, 1.9 bar; injection, 0.05 μl of liquid. (B) Column, 4 cm × 1 mm I.D. Spherosil (200 m^2/g, d_p: 25–40 μm) + β,β'-oxydipropionitrile (14.5 g per 100 g); flow-rate (N_2), 0.22 l/h; Δp, 0.75 bar, injection, 0.02 μl of liquid. Peaks: (1) vinyl chloride; (2) vinylidene chloride; (3) carbon tetrachloride; (4) benzene; (5) 1,2-dichloroethane; (6) 1,1,2-trichloroethane.

This technique of shortening the column in GC when using a support with a higher specific surface area is similar to that already used in high-performance liquid chromatography[12].

Although these column performances can be increased by using suitable chromatographs avoiding dead volumes up- and down-stream of such short columns, or by improving the packing technique for small particles, etc., it must be borne in mind

that the aim is not to attain spectacular performances but only to perform high-speed PGC analyses with a reduced operating cost. A compromise among the different parameters such as column length, specific surface area of Spherosil, particle diameter and amount of sample injected has to be found when the existing equipment is to be used. However, a process gas chromatograph specially designed for short columns would be able to overcome the problem of the installation of the analyser with regard to the location of the sample injection. The compact analytical unit of such a chromatograph could be located very close to the sample probe, so that the influence of the nature and the response time of the sampling system would be minimized.

EXAMPLES OF HIGH-SPEED GC ANALYSIS

All of the experiments were carried out on a Carlo Erba 2400 laboratory chromatograph equipped with flame-ionization detectors. The shortest columns were connected to the injection port and to the detector, with intermediate empty tubes, respectively, of length 15 cm, I.D. 1 mm, and length 15 cm, I.D. 0.25 mm.

Figs. 5A and 5B show a synthetic blend of normal aliphatic C_6–C_{16} hydrocarbons separated at 180° on 1-mm I.D. column, under the following conditions: In Fig. 5A, the separation was achieved in $3\frac{1}{2}$ min by using a 35-cm column of Spherosil (93 m^2/g) coated with Carbowax 20M (20 g per 100 g) and a nitrogen flow-rate of 0.24

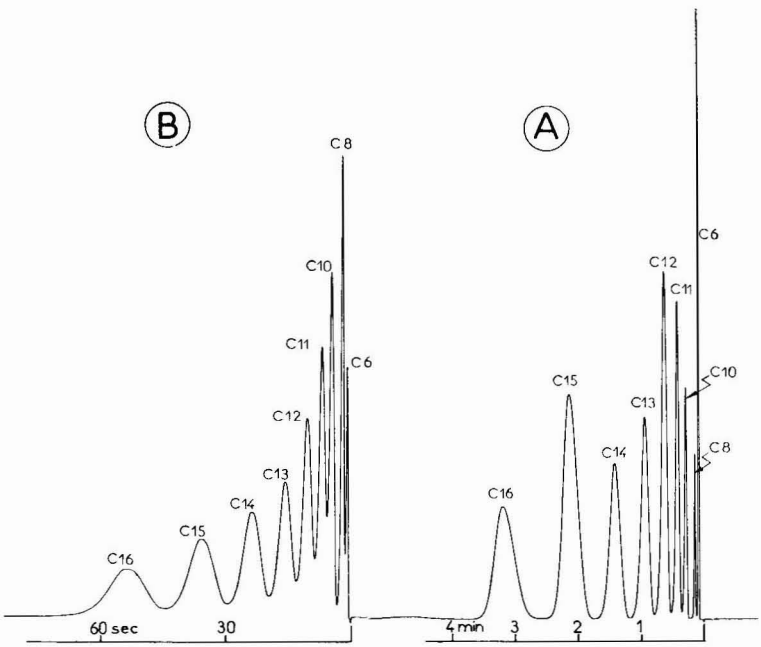

Fig. 5. Separation of saturated C_6–C_{16} aliphatic hydrocarbons. (A) Rapid analysis. Column, 35 cm × 1 mm I.D. Spherosil (93 m^2/g, d_p = 150–160 μm) + Carbowax 20M (20 g per 100 g); temperature, 180°; flow-rate (N_2), 0.25 l/h; Δp, 0.8 bar; injection, 0.1 μl of liquid. (B) Ultra-rapid analysis. Column, 35 cm × 1 mm I.D. Spherosil (200 m^2/g, d_p = 100–125 μm) + Carbowax 20M (40 g per 100 g); temperature, 180°; flow-rate (N_2), 2 l/h; Δp, 4.8 bar; injection, 0.1 μl of liquid.

l/h; in Fig. 5B, the separation was achieved in 65 sec with a 17-cm column of Spherosil (200 m^2/g) coated with Carbowax 20M (40 g per 100 g) in order to obtain the same film thickness. In this instance the carrier gas flow-rate was increased to 2 l/h in order to decrease the analysis time. It must be pointed out that in Fig. 5B the first peaks emerge very rapidly (*ca.* 1 sec) and good quantitative results can be obtained only with a computerized data reduction system.

A complex mixture of nine alcohols, the composition of which is given in Fig. 6, was separated in 4 min on a 70-cm, 1-mm I.D. column of Spherosil (200 m^2/g) coated with Carbowax 20M (14 g per 100 g) at 160°. In spite of the high specific surface area of the support, the peaks are well shaped; this result is probably due to a homogeneous coating of stationary phase.

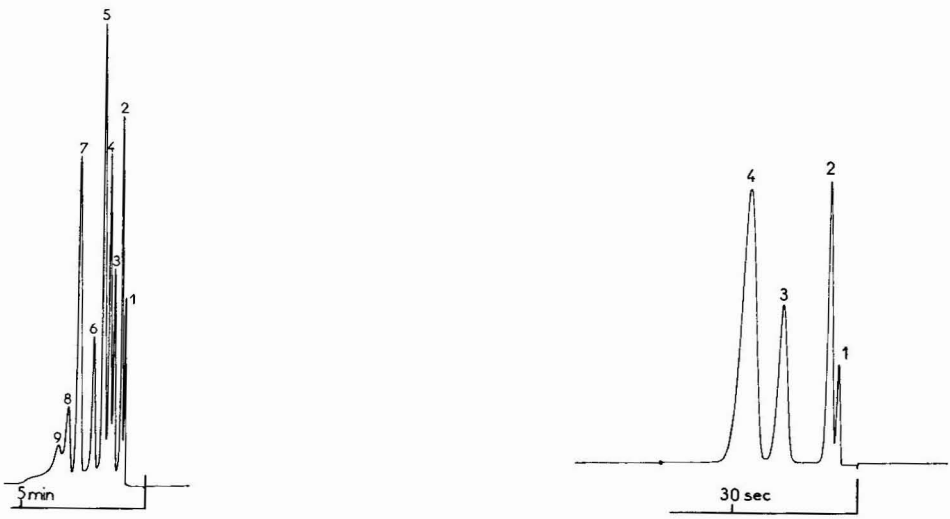

Fig. 6. Separation of a mixture of alcohols. Column, 70 cm × 1 mm I.D. Spherosil (200 m^2/g, d_p = 80–90 μm) + Carbowax 20M (14 g per 100 g); temperature, 160°; flow-rate (N_2), 0.2 l/h; Δp, 1.6 bar; injection, 0.05 μl of liquid. Peaks: (1) ethanol; (2) isopropanol; (3) 1-propanol; (4) 2-butanol; (5) 2-methyl-2-butanol; (6) 3-methyl-2-butanol; (7) 2-methyl-1-pentanol; (8) 2-methyl-1-butanol; (9) 3-methyl-1-butanol.

Fig. 7. Separation of acetates. Column, 17 cm × 1 mm I.D. Spherosil (93 m^2/g, d_p = 80–90 μm) + Carbowax 20M (20 g per 100 g); temperature, 130°; flow-rate (N_2) 0.2 l/h; Δp, 1 bar; injection, 0.05 μl of liquid. Peaks: (1) methyl acetate; (2) ethyl acetate; (3) butyl acetate; (4) isoamyl acetate.

Fig. 7 illustrates the separation of four acetate esters in 35 sec with a 17-cm, 1-mm I.D. column packed with Spherosil (93 m^2/g) coated with Carbowax 20M (20 g per 100 g) at 130°. The resolution of the peaks is satisfactory, and a faster analysis could easily be achieved by increasing the carrier gas flow-rate.

An air-pollution analysis of four components (vinyl acetate, ethyl acrylate styrene and butyl acrylate) is carried-out in 40 sec by using an 8-cm, 1-mm I.D. column of Spherosil (200 m^2/g) coated with triscyanoethoxypropane (25 g per 100 g)

(Fig. 8). In this instance, an efficiency of 400 theoretical plates in 8 cm was obtained (5,000 plates/m or 10 N_{eff}/sec) owing to the use of a small particle size (20–25 μm).

Trace analysis is possible in high-speed GC with short columns: impurities at concentrations below 200 ppm in 1,2 dichloroethane have been separated easily on an 8-cm, 1-mm I.D. column of Spherosil (200 m^2/g) coated with β,β'-oxydipropionitrile (14 g per 100 g) at 85° (Fig. 9). In spite of the small column dimensions, no overloading was observed; the major peak showed no tailing and the chromatogram was satisfactory.

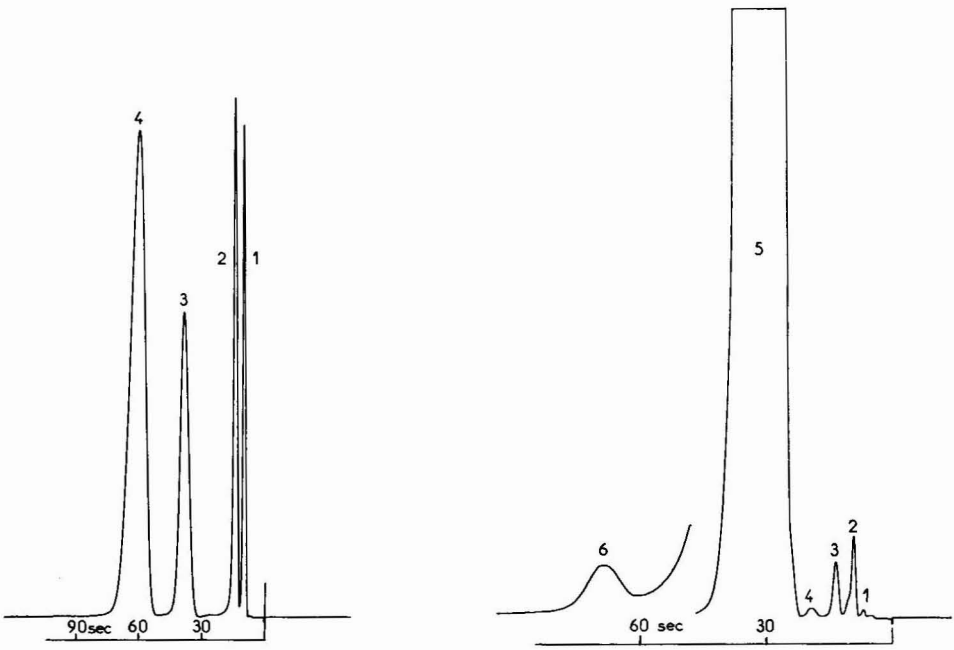

Fig. 8. Separation of airborne pollutants. Column, 8 cm × 1 mm I.D. Spherosil (200 m^2/g, d_p = 25–40 μm) + triscyanoethoxypropane (25 g per 100 g); temperature, 100°; flow-rate (N_2), 1 l/h; Δp, 4.75 bar; injection, 0.05 μl of liquid. Peaks: (1) vinyl acetate; (2) ethyl acrylate; (3) styrene; (4) butyl acetate.

Fig. 9. Analysis of trace impurities in 1,2-dichloroethane. Column, 8 cm × 1 mm I.D. Spherosil (200 m^2/g, d_p = 25–40 μm) + β,β'-oxydipropionitrile (14 g per 100 g); temperature, 85°; flow-rate (N_2), 0.2 l/h; Δp, 1 bar; injection, 0.04 μl of liquid. Peaks: (1) vinyl chloride; (2) vinylidene chloride; (3) methylene chloride; (4) carbon tetrachloride; (5) 1,2-dichloroethane; (6) 1,1,2-trichloroethane.

CONCLUSION

The characteristics of Spherosil (specific surface area and particle size) have been shown to allow tailor-made chromatographic performances from classical to ultra-rapid. Owing to the straightforward rules developed for this support in modified gas–solid chromatography, high-speed PGC analysis becomes economically feasible in comparison with other techniques such as the use of capillary columns. However, interest in high-speed analyses with Spherosil lies not only in a simple comparison with other techniques; a new trend in PGC can be foreseen. The process GC analyser

could be miniaturized as a result of the use of short columns, would be more reliable owing to the deferred standard technique, and its response more credible by increasing the number of analyses averaged by the incorporated miniprocessor. By setting it close to the sample injection point in the process, the sample line would be shortened and simplified and the total response time of the analysis would be considerably reduced. With such a process gas chromatograph, a further step towards process automation could be made.

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CHROM. 10,056

GAS CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF FREE AND TOTAL SOLANESOL IN TOBACCO

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SUMMARY

Solanesol, a trisesquiterpenoid alcohol in tobacco leaf, has been shown to be an important precursor of the tumorigenic polynuclear aromatic hydrocarbons of smoke. Thus, a rapid, reproducible method for the determination of leaf solanesol levels is desirable. We developed procedures based on high-temperature gas chromatography for the analyses of free and total solanesol. The alcohol, as its trimethylsilyl derivative, was separated and quantified on a short Dexsil 300 GC column, with 1,3-dimyristin as an internal standard. The free alcohol was determined by direct derivatization of ground tobacco and its hexane extract with N,O-bis(trimethylsilyl)-acetamide–dimethylformamide reagents. For total solanesol, the ground tobacco or its hexane extract was saponified with ethanolic potassium hydroxide. Acidification and hexane extraction yielded samples suitable for silylation and gas chromatographic analysis. Evaluation of the various methods indicated that free solanesol was best determined in the hexane extract of tobacco, and total solanesol after saponification of ground tobacco. Total solanesol levels in commercially important tobacco types were determined.

INTRODUCTION

The identification of polynuclear aromatic hydrocarbons (PAH) as major contributors to the tumorigenicity of cigarette smoke condensate[1–3] has raised the question of their origin. To determine the PAH precursors in tobacco leaf, several pyrolytic or thermal decomposition studies of tobacco leaf extracts have been conducted[4]. The results indicated that hexane extracts produce disproportionately large yields of PAH and benzo[α]pyrene, a most potent member of the group. The results of a subsequent study strongly suggested[5] that terpenoid components of the hexane extract are the potent precursors of smoke PAH and that the reduced tumorigenicity of the smoke from reconstituted tobacco sheet[6] is due to the partial removal of terpenoid compounds during the sheet manufacture. More recently, a study of the pyrolytic formation of PAH from the light petroleum-extractable constituents of

flue-cured tobacco leaf[7] found that the terpene, solanesol (I; 3,7,11,15,19,23,27,31,35-nonamethyl-2,6,10,14,18,22,26,30,34-hexatriacontanonaen-1-ol; Fig. 1), may produce more than 30% of the total PAH in the pyrolyzate of the total extract. This indicated that solanesol is a major precursor of the tumorigenic PAH of tobacco smoke and that utilization of tobacco with low solanesol content would lead to safer smoking products.

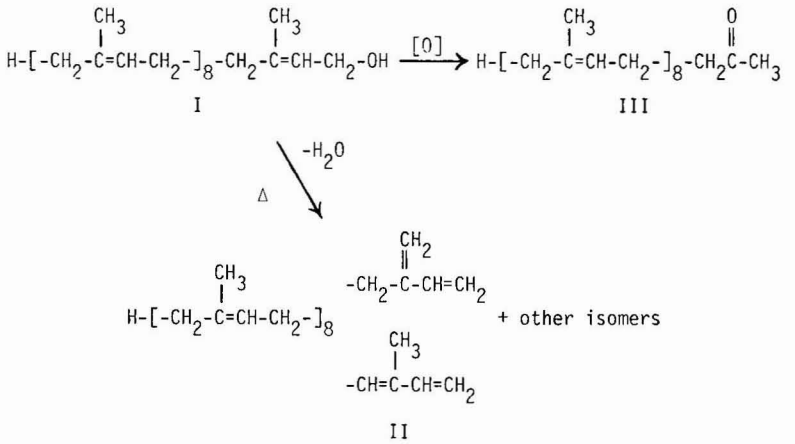


Fig. 1. Thermal conversion of solanesol (I) to solanesenes (II).

Solanesol was first isolated from flue-cured tobacco by Rowland *et al.*[8] in 1956 in quantities corresponding to 0.4% by dry weight of leaf. Other researchers[9,10] reported solanesol levels to be between 1 and 2% of dry weight of tobacco leaf. Thus, this C_{45} terpenoid is the most abundant component in the lipid fraction of tobacco and because of its co-relation to smoke PAH solanesol levels will have to be determined in all tobacco varieties.

To evaluate solanesol contents of different tobacco varieties, we required a rapid, quantitative method. A review of methods for the gravimetric determination of solanesol by column chromatography on silicic acid (SA), alumina, or Florisil showed that, generally, low and variable values were obtained[9,10]. The thin-layer chromatographic (TLC) densitometric method of Woolen and Jones[10] was considered undesirable since we had observed considerable decomposition on the plates. However, a report by Welburn and Hemming[11] on the gas chromatographic analysis of acetates and trimethylsilyl (TMS) derivatives of long-chain isoprenoid alcohols suggested that short-column, high-temperature gas chromatography (GC) could be used for analysis of solanesol. We adopted this approach and now describe the GC methods we developed for analyzing both free and total solanesol in tobacco leaf.

EXPERIMENTAL

Materials

All solvents (Burdick and Jackson*, Muskegon, Mich., U.S.A.; distilled-in-

* Reference to a company or product name does not imply approval or recommendation by the USDA.

glass) were redistilled according to the procedure described by Schepartz *et al.*[12]. Dimethylformamide (DMF) and N,O-bis(trimethylsilyl)acetamide (BSA) were silylation grade (Analabs, North Haven, Conn. U.S.A.). Stock solanesol was obtained from Hoffmann-LaRoche (Nutley, N.J., U.S.A.). The dimyristin internal standard was used as obtained from Analabs. The following tobacco samples were analyzed:

Flue-Cured (1968) —commercial Eastern Carolina type, flue-cured, redried, aged.

Burley I (1975) —United States Department of Agriculture, Agricultural Research Service (USDA-ARS), experimental, air-cured.

Burley II (1971) —commercial, air-cured, redried.

Cigar Filler (1972) —commercial, Pennsylvania, air-cured.

Maryland (1971) —commercial, air-cured, redried.

Turkish (1967) —commercial, Samsum, sun-cured.

Tobacco sample preparation

Tobacco samples were equilibrated at laboratory conditions for two days and then ground in a Wiley Mill to pass through a 32-mesh screen.

Determination of dry tobacco weight

For moisture determination, 200 mg of ground sample was heated for 3 h at $95 \pm 0.5°$ in a vented oven[13]. Moisture was determined before each extraction or hydrolysis.

Gas chromatography

GC analyses were performed on a Hewlett Packard Model 5750 gas chromatograph equipped with a 18 in. × 1/8 in. stainless-steel column containing 5% Dexsil 300 GC on 100–120 mesh Chromosorb W-AW (temperature program was 210° for 4 min, 210–330° at 6°/min, and 330° for 6 min; helium flow-rate, 50 ml/min; injector temperature, 300°; and flame ionization detector temperature, 350°). Peak areas were measured with an Autolab Systems IV integrator. (The above temperature program was required to obtain satisfactory baseline tracking of the integration system. During developmental work, other GC conditions were used and they are listed in the text as discussed.)

For preparative GC, the columns were switched from the flame ionization detector to a thermal conductivity detector (maintained at 330°). The components were collected in melting point capillary tubes under conditions identical to those used for analytical GC.

To obtain several columns with essentially identical resolution and retention characteristics, the following procedure for column preparation was used. A 15 ft. × 1/8 in. portion of stainless-steel tubing was washed consecutively with about 300 ml each of benzene, acetone, chloroform and acetone. The column was dried by air pulled through it for about 45 min and filled with 5% Dexsil 300 GC on 100–120 mesh Chromosorb W-AW by the gravity-vertical drop method[14]. The column was conditioned under helium flow (50 ml/min at room temperature) by repeated (about 10 times) heating from 90 to 330° at 2°/min, with a 1-h hold at 330°. To extend column life, the carrier gas was passed through a molecular sieve trap, followed by an oxygen trap. The ends of the column (about 6 in.) were discarded and the remainder

was cut into 18-in. sections. About 0.5 in. of the packing from each end was carefully removed and replaced with silanized glass wool. A glass liner was placed in the injection port and the 18-in. column was conditioned by two injections of about 25 μl of BSA–DMF (1:1) followed by temperature programming under conditions listed above. The glass liners were changed periodically to prevent excessive build-up of non-volatile material.

Column chromatography

About 0.5 g of hexane extract or an equivalent amount of hydrolyzed hexane extract as described below was deposited on 20 g of pre-washed, activated SA and placed on a 100-g SA column as described by Severson *et al.*[15]. The column was eluted with 1-l portions of light petroleum, benzene–light petroleum (1:3, v/v), benzene, diethyl ether and methanol. Eluate was collected in 100-ml fractions and reduced in volume prior to GC analysis. Fractions containing similar components were combined and subjected to preparative and analytical GC analyses.

Purification of solanesol

About 1 g of stock solanesol (80% purity by GC), dissolved in benzene, was placed on a 100-g SA column. The column was eluted with 1-l portions of benzene and diethyl ether. The ether fraction was evaporated to dryness. GC analysis of its TMS derivative showed that the ether fraction consisted of low-molecular-weight impurities and solanesol at a purity of about 89%. The ether fraction was subjected to preparative high-pressure liquid chromatography (HPLC) on a Varian 8500 liquid chromatograph on a 50-cm, 10-μm silica column. The eluate was monitored with a Varian Aerograph refractive index detector. The center cut of the major component, which was eluted after about 11 min with methylene chloride–hexane (1:4) at a flow-rate of 90 ml/h, was collected in a screw top test tube. The solvent was removed by a stream of nitrogen. After repeated collections and solvent removal, the test tube was placed in a vacuum desiccator under nitrogen and the residue dried under reduced pressure for several hours.

Free solanesol determination via hexane extract

A 80 × 25 mm cellulose extraction thimble containing 7–9 g of ground tobacco and a glass wool plug was placed in a small Soxhlet extractor fitted with an Allihn condenser and a 300-ml flat-bottom boiling flask containing boiling stones and 250 ml of hexane. (The hexane was distilled[12] from potassium hydroxide and purged with nitrogen before use.) The tobacco was extracted under a blanket of nitrogen for 16–18 h with rapid recycling of the hot hexane[16]. After the extract had cooled, the hexane was removed on a roto-evaporator. The residue was dried by azeotropic distillation *in vacuo* with benzene (thrice 10 ml) and quantitatively transferred with hexane to a 10-ml volumetric flask. From 0.1–0.2 ml of the extract solution and exactly 1.0 ml of the 1,3-dimyristin internal standard solution (1 mg/ml in benzene) were quantitatively transferred to a tapered test tube. After removal of the solvent under a stream of nitrogen, 35 μl each of BSA and DMF were added to the residue. The test tube was sealed with a PTFE-lined cap and heated for 10 min at 76°. An aliquot (1 to 5 μl) was analyzed by GC.

Total solanesol determination via hydrolyzed hexane extract

An aliquot (1 to 2 ml) of the volumetrically diluted hexane extract solution, described above, was quantitatively transferred to a 300-ml saponification flask, containing 40 ml of 2 *N* ethanolic potassium hydroxide and fitted with a 24/40 reflux condenser. The mixture was refluxed under nitrogen for 2.5 h and then cooled. Saturated potassium chloride solution (5 ml) was added and the solution was acidified to pH 2 with concentrated hydrochloric acid. Then, 10 ml of hexane was added, and the mixture was shaken. If necessary, small portions of water were added until a clear meniscus was obtained. The mixture was quantitatively transferred to a 125-ml separatory funnel and extracted with 10-ml portions of hexane until two successive hexane extracts were colorless. The combined hexane extracts were quantitatively diluted to 100 ml. This solution (1 to 2 ml) and 1.0 ml of the internal standard solution were quantitatively placed in a tapered test tube and the solvent removed. The residue was treated with BSA and DMF as described above and the silylated mixture was analyzed by GC.

Total solanesol determination via direct hydrolysis of ground tobacco

About 2 g of ground tobacco and 40 ml of 2 *N* ethanolic potassium hydroxide were placed in a saponification flask and the sample was hydrolyzed and processed for GC analysis for its solanesol content by the procedures described above.

Free solanesol via direct BSA–DMF extraction of tobacco

About 50 mg of ground tobacco and 1.0 ml of internal standard solution were placed in a tapered test tube or Reacti-Vial. After removal of the solvent under a stream of nitrogen, 100-μl portions of BSA and DMF were added. The vial was sealed with a PTFE-lined cap and vigorously agitated on a Super-Mixer (Lab-Line Instruments). The sample was heated for 30 min at 76° with frequent agitation. The tobacco was allowed to settle and 1 to 5 μl of the solution were analyzed by GC.

Spectral analysis

The components collected in melting point capillary tubes by preparative GC were removed with hexane and slowly deposited as thin films on potassium bromide plates. After the solvent was allowed to evaporate, IR analysis was done using a Beckman IR 4230 spectrophotometer. The samples were washed from the potassium bromide plates with cyclohexane into cuvettes and UV data were obtained using a Beckman Acta C-III spectrophotometer. Small portions of the capillary tubes containing GC preparative material were placed in the direct insertion probe and were analyzed using a DuPont 21-492 spectrometer.

RESULTS AND DISCUSSION

To develop a successful GC method for solanesol, we have applied the short GC column methodology of Parkin and Schuller[17]. Stock solanesol was analyzed by GC on the short column using a temperature program from 160 to 330° for 6°/min. Its chromatogram showed four major GC peaks (Fig. 2). Comparison of the IR and UV data of the major components, isolated by preparative GC, with data reported by Rodgman *et al.*[18] showed that the first three peaks in Fig. 2 were a mixture of

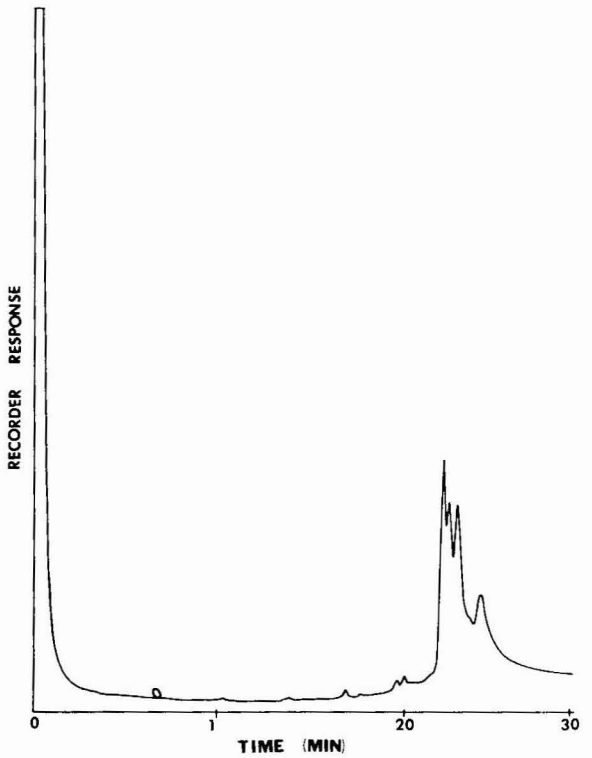


Fig. 2. Direct GC of solanesol on Dexsil 300 GC.

solanesenes (II, Fig. 1), formed by the thermal dehydration of solanesol. Therefore, direct GC analysis of the C_{45} alcohol was not feasible.

Because Welburn and Hemming[11] reported successful chromatography of the trifluoroacetate of solanesol, we reacted solanesol with excess trifluoroacetic anhydride in a sealed vial at 60° for 15 min. IR analysis of the product after removal of the excess anhydride showed that the alcohol had been quantitatively converted to the acetate. However, the solanesyl trifluoroacetate appeared to decompose almost quantitatively to II during GC analysis (Fig. 3). The formation of II during GC was confirmed by IR analysis after preparative GC. Pyrolysis of solanesol acetate has been shown to produce a hydrocarbon fraction (II) identical to that obtained from the dehydration of the parent alcohol[18].

The next attempt to volatilize solanesol was the preparation of the TMS derivative by reaction of the alcohol with BSA reagent in DMF. This attempt was successful and yielded only one GC peak, eluting in 23 min (320°) using an oven temperature program of isothermal hold at 210° for 5 min followed by a 6°/min increase to 330°. For confirmation of its identity the peak was collected by preparative GC and analyzed by IR (Fig. 4). Characteristic silyl ether absorption bands at 1250, 1065, 845 and 750 cm^{-1} (ref. 19) confirmed it to be the trimethylsilyl ether of solanesol (TMS-I).

This derivatization–GC approach was next applied to the determination of

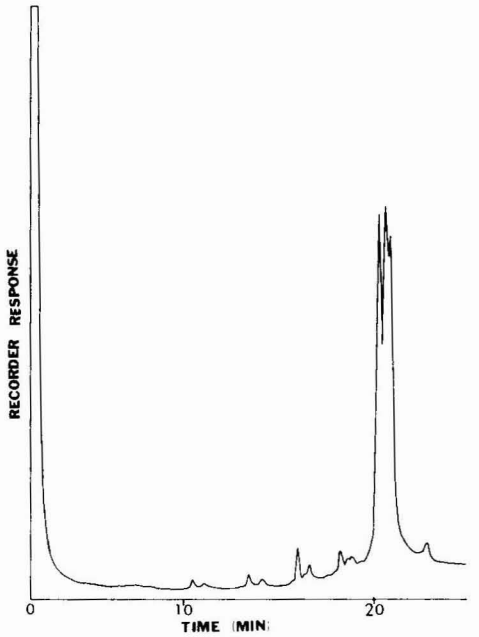


Fig. 3. Gas chromatogram of solanesol trifluoroacetate, showing thermal decomposition to solanesenes.

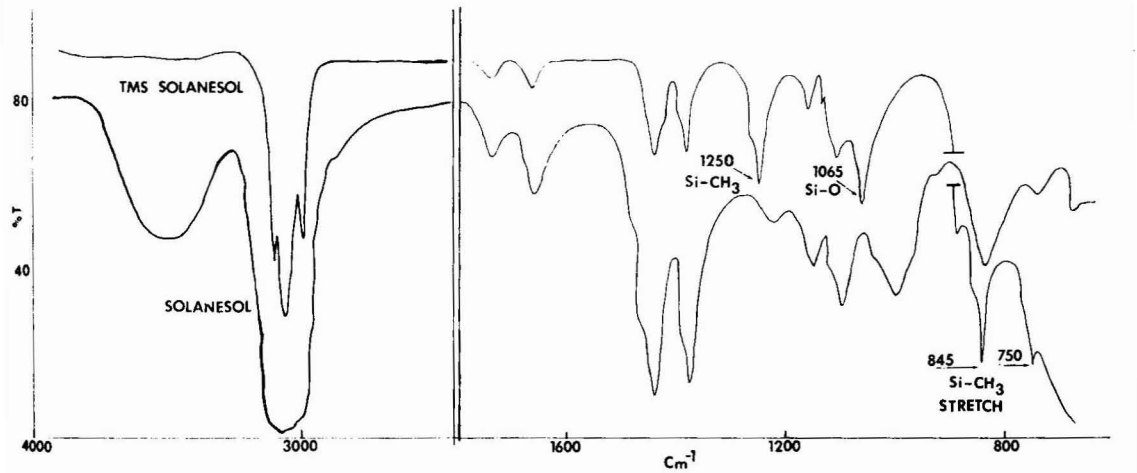


Fig. 4. IR spectrum of TMS derivative of solanesol.

solanesol in the hexane extract of flue-cured tobacco. The resulting gas chromatogram is shown in Fig. 5. Preparative GC cuts that corresponded to peaks 1, 2, 3, and 4 were obtained. The IR spectrum of peak 4 was identical to TMS-I. Spectral analysis of the triplet preceding TMS-I showed that 1 and 3 were identical to solanesenes. Peak 2 yielded an IR spectrum identical to solanesenes, except for a carbonyl ab-

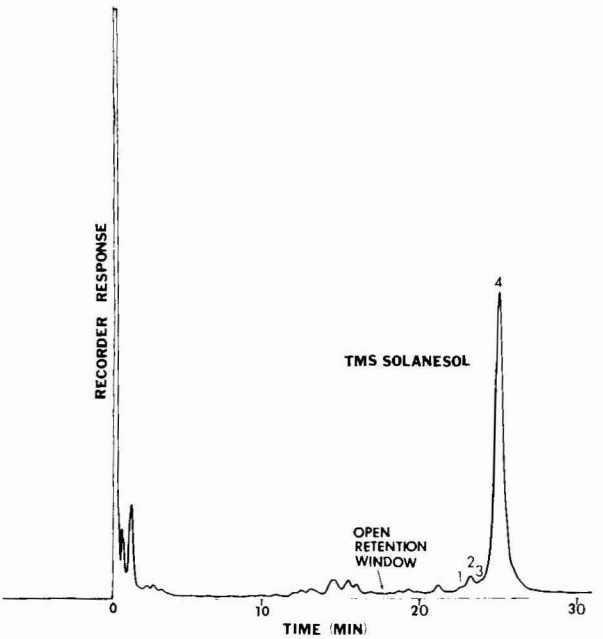


Fig. 5. Gas chromatogram of the TMS derivatives of the hexane extract of flue-cured tobacco.

sorption at 1720 cm^{-1}. The spectrum of the material in the tailing portion of peak 4 contained ester absorption bands. Comparison of GC retention data of known compounds in the hexane extract of tobacco[8] indicated that these bands were due to very low levels of steryl esters.

Since solanesyl esters have been reported in flue-cured tobacco[8,20], it was necessary to determine the quantity of bound solanesol, in addition to the free solanesol analyzed by the above procedure. Accordingly, the hexane extract was saponified with ethanolic potassium hydroxide, and the recovered organics were derivatized. The gas chromatogram of the TMS derivatives is shown in Fig. 6. Preparative GC cuts were obtained for peaks 1–4. The IR spectrum of the material corresponding to the back portion of peak 4 showed no ester absorption, indicating the absence of steryl esters. IR and UV spectra showed that peaks 1, 2, and 3 were identical to those labeled correspondingly in the hexane extract.

To determine whether solanesenes (peaks 1, 2, and 3) and the carbonyl compound in peak 2 were formed during the derivatization and/or GC, we separated portions of the starting and hydrolyzed hexane extracts by SA column chromatography. Continuous monitoring of the eluant by GC revealed the majority of solanesenes (characterized after preparative GC by IR, UV) eluted from the column with light petroleum–benzene (1:3). Mass spectral data of this preparative material revealed that co-eluting with the solanesenes (*m/e* 612) by both SA chromatography and GC were other similar hydrocarbons with masses of 614, 616 and 618 a.m.u. The steryl esters, present only in the starting hexane extract, also eluted in this fraction. In good agreement with our previous assignments, these esters began to

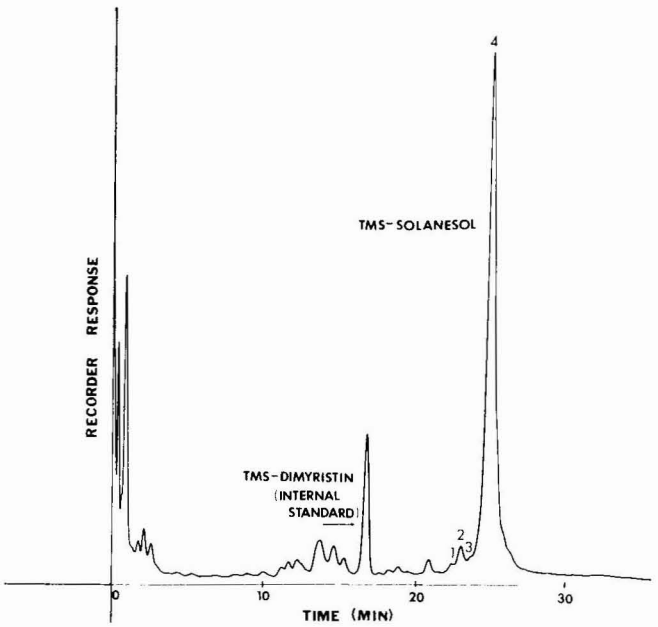


Fig. 6. Gas chromatogram of the TMS derivatives of base hydrolyzed hexane extract of flue-cured tobacco.

elute from the GC column on the backside of the TMS-I peak. Benzene eluted a carbonyl component whose GC retention time was identical to that of peak 2 (Figs. 5 and 6). After preparative GC, the UV, IR and mass (*m/e* 602) spectra of this compound were identical to the lipid component, bombiprenone (III; 6,10,14,18,22,26,30,34-octamethyl-5,9,13,17,21,25,29,33-pentatriacontaen-2-one), isolated from flue-cured tobacco by Irwine *et al.*[21]. They postulated that bombiprenone is formed during the biochemical breakdown of plastoquinone-A in the ripe leaf. Since bombiprenone likely derives from a C_{45} isoprenoid moiety, we included it in quantitating the C_{45} terpenes. Diethyl ether eluted residual traces of bombiprenone with solanesol from the SA column. Mass spectrometry of TMS-I obtained by preparative GC showed a molecular ion at *m/e* 702 and the usual fragmentation patterns of silyl ethers[19]. The levels of solanesenes and bombiprenone determined in this manner were in good agreement with those in total extract. These findings showed that little, if any, solanesol was decomposed during hydrolysis, derivatization, and/or GC. Analyses of the SA fractions showed that with the hydrolyzate the GC retention window for TMS-I was essentially free of other GC volatile material and that in the GC of the hexane extract less than 2% of the apparent peak area assigned to TMS-I was due to steryl esters. Thus, solanesol, solanesenes and bombiprenone can be quantitated by direct GC analyses of both the total and hydrolyzed tobacco hexane extract.

The success of the potassium hydroxide hydrolysis of the hexane extract in determining the total solanesol content spurred us to abbreviate the procedure by direct potassium hydroxide hydrolysis of ground tobacco. The resulting gas chromatogram of the TMS derivatives of the tobacco hydrolyzate products was identical

to that of the hexane extract hydrolyzate. In this way, extraction with hexane could be eliminated and total solanesol analyzed by a rapid, two-step procedure —the first step being direct ethanolic potassium hydroxide extraction hydrolysis of a tobacco sample and the second step GC analysis.

Because the presence of glucosidated sterols has been confirmed in both tobacco leaf and smoke[22], it appeared possible that a small amount of solanesol also exists in leaf as a glucoside. Since base hydrolysis would not cleave glycosidic linkages, the hexane extract was hydrolyzed by both sulfuric acid and potassium hydroxide[23]. Fig. 7 shows the disappointing results of this treatment. Solanesol was almost completely destroyed by the acid. Thus, in the following discussion, "bound solanesol" refers to that obtained by base hydrolysis of solanesyl esters.

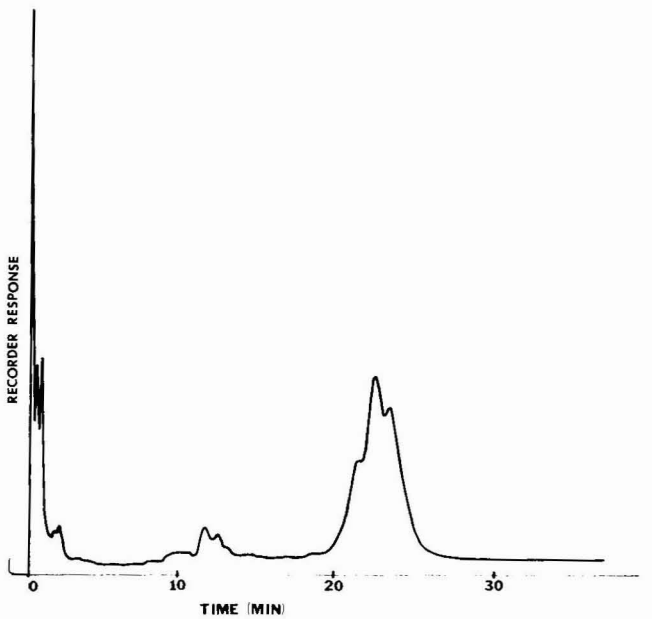


Fig. 7. Gas chromatogram of the TMS derivatives from acid and base hydrolyzed hexane extract of flue-cured tobacco.

A one-step analysis for free solanesol was also attempted by direct derivatization of ground tobacco. GC of the derivatives yielded a chromatogram identical to that obtained from the tobacco hexane extract.

To obtain a detector response factor for solanesol, we needed pure solanesol. However, solanesol purified by TLC, recrystallization, or column chromatography, still showed carbonyl impurities when analyzed by IR or the presence of solanesenes and/or bombiprenone when analyzed by GC. Column chromatography on silicic acid and then HPLC with 10-μm silica, yielded acceptably pure solanesol (94% by GC).

We were able to quantitate the method when we found that the TMS derivative of 1,3-dimyristin fitted perfectly into an open retention window in the chromatogram of both the hexane extract and saponified extract (Fig. 5 and 6). Solanesol levels could now be quantitated with this internal standard. The gas chro-

matogram of the HPLC purified solanesol and dimyristin is shown in Fig. 8. The other peaks surrounding the TMS-I peak were assumed to be related isoprenoids; consequently, the total area was used for the calculation of response data.

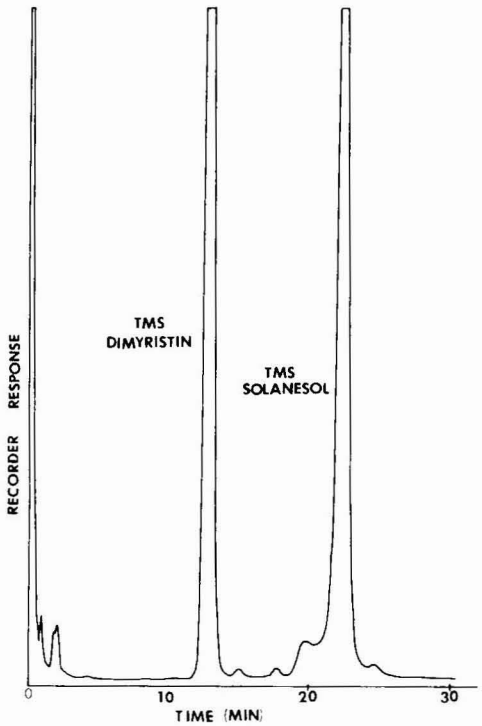
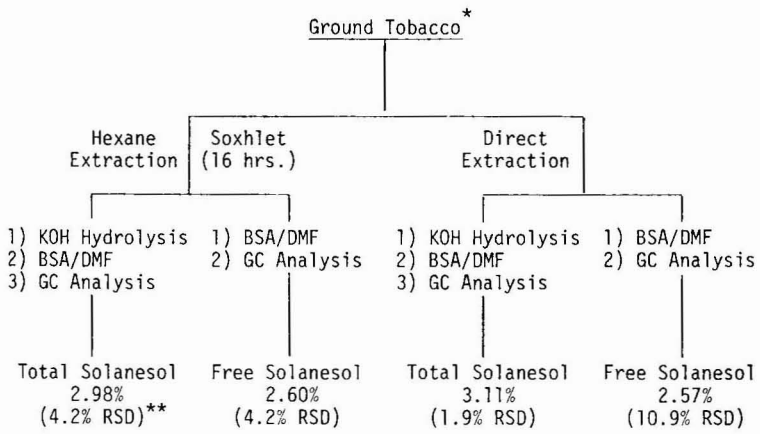


Fig. 8. Gas chromatogram of the TMS derivative of "pure" solanesol.



*Eastern Carolina, flue-cured, redried, and aged tobacco.

**RSD = Relative Standard Deviation from the mean.

Fig. 9. Summary of solanesol methodology.

TABLE I

COMPARISON OF METHODS FOR THE DETERMINATION OF FREE AND TOTAL SOLANESOL IN TOBACCO LEAF (EASTERN CAROLINA FLUE-CURED)

	Method	*Isoprenoid*	*Run number*								*Mean ± S.D.*
			1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	
			% Weight of dry leaf								
Free isoprenoids	Hexane extraction of tobacco	Solanesol	2.74	2.61	2.44						2.60 ± 0.11
		Solanesenes, Bombiprenone	0.18	0.17	0.16						0.17 ± 0.08*
	BSA–DMF extraction of tobacco	Solanesol	2.52	2.46	2.53	2.73	3.10	2.62	2.08	2.54	2.57 ± 0.28
		Solanesenes, Bombiprenone	0.09	0.10	0.14	0.17	0.21	0.16	0.11	0.16	0.14 ± 0.04*
Total isoprenoids	KOH hydrolysis of tobacco hexane extract	Solanesol	3.00	2.84	2.88	3.14	2.99	3.02			2.98 ± 0.05
		Solanesenes, Bombiprenone	0.28	0.21	0.22	0.26	0.26	0.25			0.24 ± 0.01*
	KOH extraction of tobacco	Solanesol	3.11	3.12	2.93	3.10	3.29				3.11 ± 0.06
		Solanesenes, Bombiprenone	0.30	0.29	0.25	0.24	0.16				0.25 ± 0.03*

* Calculated assuming a detector response identical to that for TMS-Solanesol.

All of the attempted solanesol methods are summarized in Fig. 9, and the quantitative aspects of the determination for free and total solanesol, or more accurately isoprenoids, are given in Table I. The table lists the data for an Eastern Carolina, flue-cured, redried tobacco analyzed by the various methods. Both procedures for the analysis of the free isoprenoids yielded essentially identical values. However, the direct silylation procedure yielded data with a much larger deviation. Comparison of the two methods for total solanesol showed that the average value was about 4% higher by the direct hydrolysis method. However, levels for solanesenes and bombiprenone were the same for both methods. The direct hydrolysis procedure, with only a 1.9% relative standard deviation from the mean, appeared to be the better method for determining total solanesol. By difference, about 14–19% of the C_{45} isoprenoids were bound by base-hydrolyzable linkages.

Subsequently, we analyzed a series of tobacco samples by the two potassium hydroxide hydrolysis methods for total solanesol and compared the results (Table II). Except for the Burley II sample, the direct potassium hydroxide hydrolysis of ground tobacco gave a higher value for total solanesol than the potassium hydroxide hydrolysis of the hexane extract.

TABLE II

DETERMINATION OF TOTAL SOLANESOL

Tobacco type	*% Dry Leaf*					
	KOH hydrolyzed hexane extract method			*KOH hydrolyzed tobacco method*		
	Solanesenes, bombiprenone (%)	*Total solanesol (%)*	*Total isoprenoids (%)*	*Solanesenes, bombiprenone (%)*	*Total solanesol (%)*	*Total isoprenoids (%)*
Flue-Cured*	0.24	2.99	3.23	0.25	3.11	3.36
Burley I**	0.33	2.07	2.40	0.18	2.14	2.32
Maryland***	0.22	2.04	2.26	0.13	2.09	2.22
Turkish§	0.06	0.87	0.93	0.07	1.20	1.27
Burley II§§	0.17	0.88	1.05	0.10	0.81	0.91
Cigar Filler§§§	0.13	0.78	0.91	0.09	0.98	1.18

* 1968, Commercial Eastern Carolina, flue-cured, aged, and redried.
** 1975, ARS Experimental, air-cured.
*** 1971, Commercial, air-cured, and redried.
§ 1967, Samsun.
§§ 1971, Commercial, air-cured, and redried.
§§§ 1972, Commercial, Pennsylvania, air-cured.

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PURIFICATION OF α-L-FUCOSIDASE FROM VARIOUS SOURCES BY AFFINITY CHROMATOGRAPHY

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(Received April 12th, 1977)

SUMMARY

An affinity column for α-L-fucosidases was constructed by linking *p*-aminophenyl 1-thio-α-L-fucopyranoside to Sepharose 4B through linkers of succinyl 3,3′-diamino-dipropylamine. Excellent purification of α-L-fucosidase from rat epididymis, *Clostridium perfringens* and *Limulus polyphemus* (horse shoecrab) could be effected in one step with good yield. An affinity column purification step can be introduced at any point in published purification procedures. The purified enzyme is essentially free of other glycosidases and proteolytic enzymes. The column material is stable and can be reused for at least two years.

INTRODUCTION

The carbohydrate components of glycoproteins and glycolipids play an important part in the functioning of the cell and in the interaction of the cell with its environment[1]. The detailed structural and functional analyses of bound oligosaccharides have been facilitated by various glycosidases[2]. One of these, α-L-fucosidase, has been studied in mammals[3–5], abalone[6,7], *Helix pomatia*[8], Limpet[9] and in *Trichomonas foetus*[10], *Clostridium perfringens*[11], *Aspergillus niger*[12], *Rhodopseudomonas palustris*[13] and *Klebsiella aerogenes*[14]. The enzyme has been extensively purified by classical methods from rat epididymis[15], *A. niger*[12] and *C. perfringens*[11]. Generally the methods of purification are somewhat difficult and time-consuming.

Recently an α-fucosidase has been purified from human placenta[16], liver[17] and rat epididymus[18] by affinity chromatography using a column of agarose–ε-aminocaproyl fucosamine. Shah and Bahl[19] as well as Mega and Matsushima[20] have described the synthesis of a number of *p*-aminophenyl 1-thio glycosides including *p*-

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aminophenyl 1-thio-α and β-L-fucopyranosides[21]. The α form was found to compete with *p*-nitrophenyl-α-L-fucoside for the active site of clam α-L-fucosidase[21]. Although Chawla and Bahl synthesized the thio-fucoside for construction of affinity columns, they have provided no information on the exploitation of the thiofucoside derivative for enzyme purification[21]. Independently, we synthesized *p*-aminophenyl 1-thio-α-L-fucopyranoside. Despite the fact that our yield was relatively low, the properties of our products were comparable to those of Chawla and Bahl[21]. We have found that when the final product of our synthesis was linked to Sepharose 4B through succinyl 3,3′-diamino-dipropylamine, an effective, specific affinity column for α-L-fucosidase from a number of biological sources was produced.

EXPERIMENTAL

All materials were reagent grade commercial samples, unless otherwise specified. α-L(—)-fucose and the *p*-nitrophenyl glycoside, *p*-nitrophenyl α-L-fucoside, *p*-nitrophenyl α-D-galactoside, *p*-nitrophenyl α-D-mannoside, *p*-nitrophenyl N-acetyl β-D-glucosamine, and *p*-nitrophenyl α-D-fucoside used in routine assays were purchased from Sigma (St. Louis, Mo., U.S.A.). Sepharose 4B was purchased from Pharmacia (Piscataway, N.J., U.S.A.). Azocoll for proteinase assay was purchased from Calbiochem (Los Angeles, Calif., U.S.A.). 3,3′-Diamino-dipropylamine was obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.). Melting points were observed without corrections on compounds between glass slides with a Fisher-Johns apparatus. The rats used in these experiments were supplied by Dr. D. Kritchevsky of the Wistar Institute. A culture of *C. perfringens* was obtained from D. Aminoff and was grown according to Aminoff and Furukawa[11].

Thin-layer chromatography (TLC) was carried out using Eastman Chromatogram sheets 6060 or Baker sheet IBF (silica gel with fluorescent indicator); solvent, ethyl acetate–acetic acid–water (3:1:1) for deacetylated compounds. The spots were visualized by short wave ultraviolet light or with iodine vapor.

Synthesis of p-aminophenyl 1-thio-α-L-fucopyranoside

Of the several methods available for glycosidation[22–28], a slightly modified procedure of Levvy and McAllan[28] was used for the synthesis of the 1,2-*cis* anomer (Fig. 1) in which 1,2,3,4-tetra-O-acetyl-L-fucopyranose was condensed with *p*-nitrothiophenol in the presence of anhydrous zinc chloride (Helferich reaction). The product was purified, deacetylated and the *p*-nitro derivative was obtained; slightly yellow crystals (m.p. 218°–226° [lit.[20] 224°–226°]; $(\alpha)_D$ —343° (methanol) [lit.[20] —382°; R_F 0.85 [lit.[20] R_F 0.88]). Reduction of the deacetylated product with hydrogen gas and platinum oxide yielded a material which, upon TLC yielded one spot with an R_F of 0.65. The *p*-aminophenyl 1-thio α-fucopyranoside synthesized by Chawla and Bahl[21]

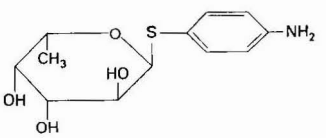

Fig. 1. Structure of *p*-aminophenyl 1-thio α-L-fucopyranoside.

had an R_F of 0.66. Since our synthetic method was unsatisfactory with regard to yield, it has not been reported in detail.

Preparation of affinity columns

Sepharose 4B was activated with cyanogen bromide and coupled with 3,3′-diamino-dipropylamine according to the method of Cuatrecasas[29]. Succinylation of the 3,3′-diamino-dipropylamine Sepharose 4B was also done by the method of Cuatrecasas. To couple *p*-aminophenyl 1-thio α-L-fucopyranoside to the succinyl derivate, 0.068 g of the fucoside was dissolved in 1 ml of dimethylformamide and added to 10 ml of the Sepharose derivative suspended in water. The pH was adjusted to 4.7 with 0.5 g of 1-ethyl 3-(3-dimethylaminopropyl) carbodiimide in 1 ml of water added dropwise. The pH was maintained at 4.7 for 1 h. The suspension was stirred at room temperature overnight and was then washed with 4 l of 0.1 *N* NaCl solution in a büchner funnel (coarse disc) without using suction. Columns were stored in 0.1 *N* NaCl solution containing 0.1% sodium azide at 4°.

Preparation of enzymes

Glycosidases from rat epididymis were partially purified according to Carlsen and Pierce[15] until the 35–50% ammonium sulfate precipitation step, and stored at −20° until purified further by affinity chromatography.

C. perfringens was cultured and the enzymes in the medium were prepared by the method of Aminoff and Furukawa[11]. Preliminary purification consisted of two ammonium sulfate precipitations and chromatography on a large column of Sephadex G-150[11]. The starting material was stored at −20° until used. Serum obtained from *Limulus polyphemus* was allowed to clot. Clots were removed by filtration and the serum was stored at −20°.

Columns (8.5 × 1.2 cm) were run at room temperature since the α-L-fucosidases being studied were reasonably stable at that temperature. The column was equilibrated and developed with 0.2 *M* sodium citrate buffer (pH 6.0) containing 0.02% NaN_3. The flow-rate was 25 ml/h, and 1.4-ml fractions were collected. Aliquots of 0.05 ml were routinely assayed for α-L-fucosidase, α-D-mannosidase, β-D-galactosidase and N-acetyl β-D-glucosaminidase with the appropriate *p*-nitrophenyl glycoside as substrate. Usually 5.0 ml of enzyme solution, prepared in 0.2 *M* sodium citrate (pH 6) containing approximately 12 mg of protein, were placed on the column. After washing the column with the same buffer until no enzyme activities were detectable and no protein was present as measured by light absorption at 280 nm, 10 ml of α-L-fucose solution (40 mg/ml) in the developing buffer were added to the column.

Glycosidases were assayed essentially by the method described by Bosmann[30]. A typical incubation mixture contained 0.3 ml of 0.05 *M* sodium citrate (pH 4.5), 0.3 μmoles in 0.3 ml water of *p*-nitrophenylglycoside substrate and 0.05 ml solution of enzyme. Where α-mannoside was assayed, 1.2 μmole of the *p*-nitrophenyl α-mannoside substrate was used. After 20 min of incubation at 37° the vessels were chilled in ice and 0.6 ml of 0.2 *M* potassium borate buffer (pH 9.8) was added and the light absorbance was determined at 400 nm. A unit of enzyme can cleave 1 μmole of substrate in 1 min under the conditions of the assay. Specific activities are expressed as units per mg of protein.

To assay α-L-fucosidase from *C. perfringens* which does not cleave *p*-nitrophenyl

α-L-fucoside, 0.05 ml of enzyme solution was incubated with 2 mg hog gastric mucin and 0.05 ml of 1 *M* sodium acetate buffer (pH 6) in a final volume of 0.3 ml for 25 min at 37°. Free L-fucose was assayed by an enzymatic method[31].

Purified α-L-fucosidase was electrophoresed in 5.6% polyacrylamide gels at 10° by the method of Davis[32]. The gels were run at 2.5 mA per tube (80 V) for 2 h with bromophenol blue as tracer dye. In order to locate the enzyme activity, gels were sliced into 1-mm pieces; each slice was placed in a tube containing 0.3 ml of sodium citrate buffer (pH 6) and kept overnight at 4°. Aliquots were assayed for enzyme activities as described previously. Before cutting, gels were scanned at 280 nm in a Gilford spectrophotometer.

Slab gels were also run in the presence of 0.1% sodium dodecyl sulfate (SDS) using the discontinuous system of Laemmli[33]. The separating gel was 10% acrylamide and the stacking gel 5%. Electrophoresis was conducted at a current of 20 mA for 4 h. Pyronine Y was used as a marker. Gels with and without SDS were stained according to Fairbanks *et al.*[34].

RESULTS

α-Fucosidase from rat epididymis

As seen from Fig. 2, α-L-fucosidase of rat epididymis is weakly but competitively inhibited by *p*-aminophenyl 1-thio α-L-fucoside (K_I 12.5 × 10^{-4} *M*); L-fucose itself is more inhibitory (K_I = 2.85 × 10^{-4} *M*). The K_m of *p*-nitrophenyl α-L-fucoside is 2.77 × 10^{-4} *M*[37]. Chawla and Bahl[21] found a K_I of 7.1 × 10^{-4} *M* for *p*-aminophenyl 1-thio α-L-fucoside and a K_m of 0.85 × 10^{-4} *M* for *p*-nitrophenyl α-L-fucoside for the α-L-fucosidase of clam under somewhat different assay conditions. D-fucose, D-galactose, L-arabinose and D-arabinose at 1-m*M* concentration did not inhibit α-L-fucosidase.

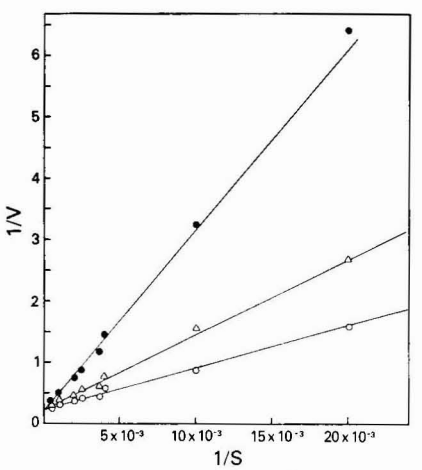

Fig. 2. Lineweaver and Burk[37] plots of the reaction catalyzed by α-fucosidase of rat epididymis with and without inhibitors. Various amounts of substrate (0.05 × 10^{-3} *M* to 0.5 × 10^{-3} *M*) were incubated with the enzyme purified on the affinity column under conditions explained in Experimental. ●, α-Fucosidase; △, α-fucosidase + 1 m*M* *p*-aminophenyl 1-thio α-L-fucopyranoside; ○, α-fucosidase + 1 m*M* α-L(−)-fucopyranose.

In Fig. 3 is seen the results of a typical run of the affinity column using enzyme from rat epididymis. Virtually all of the α-L-fucosidase is retained and essentially all of the activity is recovered (Table I) with a 59-fold purification in the one step and a final purification of over 400-fold. The specific activity is 15.6 which compared favorably with the value of 12.8 obtained by Carlsen and Pierce[15] after chromatography on columns of CM-cellulose and DEAE-cellulose and entailing considerable loss of activity.

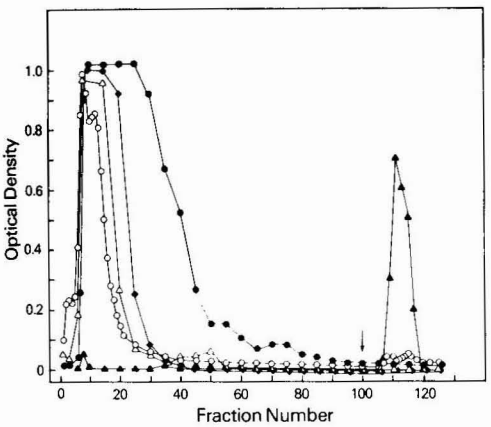

Fig. 3. Affinity chromatography of the 35–50% ammonium sulfate fraction of rat epididymis extract. The column (1.2 × 7.5 cm) was equilibrated and developed with 0.2 *M* sodium citrate buffer (pH 6) containing 0.02% sodium azide, and run at room temperature at a flow-rate of 33 ml/h. Samples 105–120 were dialyzed to remove the sugar before being assayed for enzyme activities. At fraction 101 (arrow), 10 ml of 40 mg/ml L-fucose were added to the column. Fractions (2 ml) were collected and aliquots were assayed for absorbance at 280 nm (●) and for α-L-fucosidase (▲), α-D-mannosidase (△), β-D-galactosidase (○), and β-N-acetyl-D-glucosamine (◆).

When the dialyzed, concentrated eluate containing α-L-fucosidase was electrophoresed on polyacrylamide gel[32] only one band could be detected by absorption at 280 nm and by staining with Coomassie blue. This band contained the α-L-fucosidase activity. Upon electrophoresis in an SDS gel system[33] two bands were seen which when compared to standards had molecular weights of 50,000 and 57,000. Carlsen and Pierce[15] have reported the presence of two subunits of molecular weights 47,000 and 60,000 for α-L-fucosidase of rat epididymis.

The effectiveness of the column depends on the presence of the *p*-amino 1-thio-α-L-fucopyranoside residues. The enzyme is not retained on columns of Sepharose 4B to which are attached succinyl 3,3′-diamino-dipropylamine groups alone nor is it taken up by the column if *p*-aminophenyl 1-thio α-L-fucopyranoside residues are substituted for by the anomeric β compound. Details of the synthesis of *p*-aminophenyl 1-thio β-L-fucopyranoside and the construction of affinity columns with it are described elsewhere[35].

Once the enzyme is retained by the column it cannot be removed by shifting the pH values between 6 and 4, nor by an NaCl gradient up to 2 *M* nor by passing a solution of D-fucose or D-galactose (40 mg/ml) through the column.

While the original eluate contained more than 90% of the α-mannosidase,

TABLE I

PURIFICATION OF α-L-FUCOSIDASE FROM RAT EPIDIDYMIS

Fraction	*Volume (ml)*	*Concentration (mg/ml)*	*Total (mg)*	*Sp. activity μmole/min per mg protein)*	*Total activity*	*Purification of step*	*Total purification*	*Yield (%)*
Homogenate after heating at 37°	545	3.86	2104.0	0.037	77.9	1		100
Homogenate after heating at 60°	540	3.16	1708.0	0.046	78.6	1.2	1.2	100
$(NH_4)_2SO_4$ ppt. (35–50%)	7	20.3	142.0	0.264	37.5	5.7	6.8	48.1
After elution with L-fucose and dialysis	172	0.014	2.4	15.57	37.4	59.0	420.8	48.0

β-galactosidase and N-acetyl β-D-glucosaminidase activities applied, these could not be detected in the pooled fractions eluted by L-fucose. Further, this fraction contained no detectable protease (azocoll assay), β-L-fucosidase or α-D-fucosidase activities. The recovery of protein as assayed by the method of Lowry *et al.*[36] was quantitative (102%).

As much as 1.03 units of α-fucosidase of rat epididymus in 2.11 mg of protein in 2.5 ml of buffer of the 35–50% ammonium sulfate fraction[15] could be applied to 1 ml of column material without overloading it. This is about 2.5 times more than was usually used in routine runs. The purified material off the column retained full activity for 2 months when stored at 4° while material frozen at −20° retained only half of its activity after 6 months. The enzyme could be concentrated without appreciable loss of activity after 6 months. The enzyme was concentrated by dialyzing against a solution of 20% polyethylene glycol in 0.2 *M* sodium citrate solution (pH 6).

Excellent purification of α-L-fucosidase from *C. perfringens* and *Limulus polyphemus* were effected on the same affinity column by the same procedure used for the enzyme from rat epididymis (Table II). For unknown reasons the yield of enzyme of *Limulus* was variable although purification was always good. The enzyme from *C. perfringens* clearly differs from the others in that it does not cleave *p*-nitrophenyl α-L-fucoside and yet behaves similarly on the affinity column. Assay of this enzyme was accomplished by measuring the L-fucose released from hog gastric mucin[31].

TABLE II

PURIFICATION OF α-L-FUCOSIDASE FROM VARIOUS SOURCES

	Rat epididymis	*C. perfringens*	*Limulus polyphemus**
Preparation for column	30–50% $(NH_4)_2SO_4$ fraction	Eluate from Sephadex G-50 column	Serum
Specific activity (μmole/min per mg protein)			
before column	0.26	0.014	0.001
after column	15.57	2.61	0.394
Purification	59	186	394
Yield on step (%)	100	100	24
Ref. for preliminary preparation	15	11	

* The serum was obtained at the Marine Biology Laboratory (Woods Hole, Mass., U.S.A.). Some of the work using serum of *Limulus* was carried out at the M.B.L.

DISCUSSION

It is apparent that α-L-fucosidases from a variety of sources (bacteria, invertebrate, mammal) can be specifically bound to an affinity column and extensively purified and freed of other glycosidases and proteases in one step. The column binds α-fucosidases of different specificities; that from *C. perfringens* which does not cleave *p*-nitrophenyl α-L-fucopyranoside and the enzyme from other sources which does split this substrate.

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TRENNUNG DER HALOGENOHYDROBORATE DES TYPS $B_{10}H_{10-n}X_n^{2-}$ DURCH HOCHSPANNUNGSIONOPHORESE

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(Eingegangen am 14. März 1977)

SUMMARY

Separation of halohydroborates of the type $B_{10}H_{10-n}X_n^{2-}$ by high-voltage ionophoresis

The separation of mixtures of halohydroborates $B_{10}H_{10-n}X_n^{2-}$; X = Cl, Br, I; n = 1–9 is possible because the ion mobilities decrease stepwise with increasing halogenation. The zones are rendered visible by spraying the pherograms with a solution of acridine hydrochloride, and the members of the homologous series are isolated in pure form. With some iodohydroborates the ionophoretical separation of geometrical isomers is successful owing to the different molecular shapes.

EINLEITUNG

Bei der Umsetzung des Dekahydroborats $B_{10}H_{10}^{2-}$ mit Halogenen bilden sich Halogenohydroborate des Typs $B_{10}H_{10-n}X_n^{2-}$, X = Cl, Br, I; n = 1–9 bzw. 10. Dabei handelt es sich um Verbindungen, die für die Untersuchung von Substituenteneffekten und von Reaktionsmechanismen von Interesse sind. Die Bemühungen um die gezielte Darstellung sind bisher an der aussergewöhnlichen chemischen und physikalisch-chemischen Ähnlichkeit zwischen den einzelnen Gliedern dieser homologen Verbindungsreihen gescheitert. Bei den wenigen bisher isolierten Zwischenprodukten, wie z.B. $B_{10}H_2Cl_8^{2-}$, $B_{10}H_7Br_7^{2-}$ und $B_{10}H_8I_2^{2-}$, wurde nicht ausgeschlossen, dass es sich um Gemische mehrerer Komponenten handelt[1].

Mit Hilfe der Hochspannungspapierionophorese, die sich bereits bei der Trennung von Gemischtligandkomplexen des Typs $[MX_nY_{6-n}]^{2-}$, M = Re, Os, Ir, Pt; X ≠ Y = Cl, Br, I, n = 1–5 vielfach bewährt hat[2,3], gelang jetzt die systematische Isolierung der Halogenohydroborate[4]. Die Trennung beruht auf der mit steigendem Halogenierungsgrad schrittweise abnehmenden Ionenbeweglichkeit. Im Falle der Jodohydroborate wurde auf Grund der etwas unterschiedlichen Molekülgestalt zusätzlich die Auftrennung in mehrere geometrische Isomere erreicht. Die ionophoretische Isolierung und die Eigenschaften einer ganzen Reihe von Halogenohydroboraten werden beschrieben.

EXPERIMENTELLES

Herstellung der Ausgangsgemische

Zur Darstellung von $B_{10}H_{10}^{2-}$ geht man vom Dekaboran $B_{10}H_{14}$ aus, das mit Triäthylamin oder Dimethylsulfid umgesetzt wird[5,6]. Auch bei der Pyrolyse von $[(C_2H_5)_4N]BH_4$ entsteht es in guter Ausbeute[7]. Zur Halogenierung wird durch eine eisgekühlte Lösung von 1,5 g $(NH_4)_2B_{10}H_{10}$ in 50 ml Wasser ein mit sehr wenig Cl_2 bzw. Br_2 beladener Stickstoff-Strom geleitet. Für die Jodierung schüttelt man die wässrige Lösung mit einigen Millilitern CCl_4, in dem I_2 gelöst ist, bis die Jodfarbe verschwindet. Je nach der Menge des eingesetzten Halogens entstehen Gemische mit verschiedenem Halogenierungsgrad. Stets enthalten die Lösungen drei bis fünf benachbarte Komponenten, die nach dem Abfiltrieren geringer Mengen harziger Nebenprodukte mit Tetramethylammoniumionen ausgefällt werden. Durch Behandeln mit einem Kationenaustauscher werden die in Wasser sehr gut löslichen NH_4^+-bzw. Na^+-Salze hergestellt und der hochspannungsionophoretischen Auftrennung unterworfen. Zu Gemischen mit den entsprechenden markierten Verbindungen kommt man, indem $B_{10}H_{10}^{2-}$ mit radioaktiven Halogenen umgesetzt wird.

Hochspannungspapierionophorese

Die Auftrennung der Gemische, die mehrere Spezies der Reihen $B_{10}H_{10-n}X_n^{2-}$ enthalten, erfolgt nach dem Prinzip der Zonenionophorese. Die Konzentration der zu trennenden wässrigen Lösung, die mit einer Kunststoffpipette auf den Papierstreifen aufgetragen wird, darf nicht höher sein als die des Grundelektrolyten, weil sich sonst überladene breite Zonen ausbilden[8]. Die verwendete Apparatur ist mehrfach beschrieben worden[2]. Die besten Trennungen werden unter folgenden Versuchsbedingungen erreicht: Grundelektrolyt: 0.2 *M* Trichloroessigsäure–Kaliumhydroxyd (pH = 2.5); Elektrodenabstand: 110 cm; Spannung: 6–7 kV; Kühltemperatur: 1–5°; Trägermaterial: Filterpapier, B mgl, 120 × 15 cm (Schleicher & Schüll, Dassel, B.R.D.); Trenndauer: 1–3 h.

Nachweis und Isolierung der Halogenohydroborate

Da alle Hydroborate und deren Halogenierungsprodukte farblos sind und auch keine UV-Absorption zeigen, ist es schwierig, geringe Mengen auf den Pherogrammen nachzuweisen. Bei Verwendung radioaktiv markierter Substanzgemische kann durch Registrierung der Aktivitätsverteilung mit einem Papierchromatogramm-Scanner die Lage der Halogenohydroborationen ermittelt werden.

Auch die Suche nach einfachen Entwicklungsverfahren zum visuellen Nachweis war erfolgreich. Besprüht man die feuchten Ionophoresestreifen mit 1%iger wässriger $AgNO_3$-Lösung und setzt sie dem Licht aus, so zeichnen sich nach längerer Zeit auf dem Papier Zonen ab. Der Vorgang wird durch gleichzeitiges Erwärmen auf 50° und in Gegenwart von Reduktionsmitteln, z.B. in H_2S-Atmosphäre beschleunigt. Neben den Halogenohydroboraten lassen sich so auch die Zonen der freien Halogenid ionen auf den Pherogrammen erkennen. Für präparative Zwecke ist die Methode nicht geeignet, weil Ag^+ mit allen Anionen $B_{10}H_{10-n}X_n^{2-}$ schwerlösliche Niederschläge bildet.

Mit salpetriger Säure reagiert $B_{10}H_{10}^{2-}$ zu einer instabilen explosiven roten Verbindung[9]. Auch die ersten Halogenierungsprodukte ($B_{10}H_9X^{2-}$ und $B_{10}H_8X_2^{2-}$)

ergeben auf dem Pherogramm die entsprechenden Farbreaktionen. Höher halogenierte Spezies sind so nicht nachweisbar.

Grosse organische Kationen wie Pyridinium-, α,α'-Dipyridinium-, Chinolinium- und Acridiniumionen bilden mit den Halogenohydroboraten schwerlösliche Niederschläge, die als Folge von Charge-Transfer-Übergängen farbig sind. Als Sprühreagenz eignet sich vor allem Acridiniumhydrochlorid (0.5 g/l), das mit $B_{10}H_{10}^{2-}$ eine rötliche, mit den halogenierten Spezies gelbe Zonen ergibt. Besser als in sichtbarem Licht lassen sich die Zonen auf den feuchten Pherogrammen unter einer UV-Lampe erkennen. Auch die Zonen der freien Halogenide sind dann gut sichtbar.

Für die präparative Isolierung ist es wichtig, dass sich die Acridiniumsalze der Halogenohydroborate durch Zugabe von Natriumhydroxyd in Acridin und die entsprechenden Na-Salze umwandeln. Beim Auspressen der aus mehreren Pherogrammen ausgeschnitten und gesammelten Zonen mit 0.01 *N* Natriumhydroxyd bleibt das schwerlösliche Acridin zurück, so dass man eine alkalische Lösung der Halogenohydroborate erhält, aus der die farblosen feinkristallinen Tetramethylammoniumsalze ausgefällt werden.

ERGEBNISSE UND DISKUSSION

Sukzessive Bildung der Halogenohydroborate

Die Änderung der Zusammensetzung der Lösungen mit den verschiedenen Halogenohydroboraten in Abhängigkeit von der zugesetzten Mol-Menge an Halogen bzw. von der Reaktionszeit erfolgt bei der Chlorierung, Bromierung und Jodierung in gleicher Weise. Das Fortschreiten der Umsetzung lässt sich ionophoretisch gut verfolgen, wenn ein Trägergasstrom, der nur sehr wenig Cl_2 enthält, langsam durch eine $B_{10}H_{10}^{2-}$-Lösung geleitet wird. Die Pherogramme von Proben, die in bestimmten Zeitabständen genommen wurden, zeigen, dass stets mehrere Spezies nebeneinander entstehen und dass die einzelnen Verbindungen durch Folgereaktionen auseinander hervorgehen (Fig. 1).

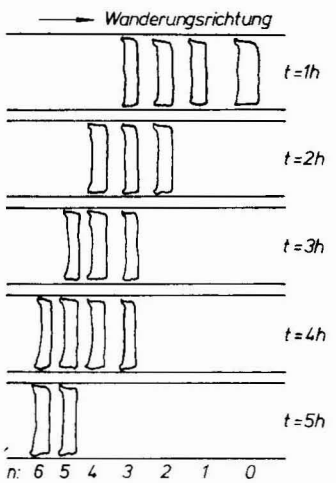

Fig. 1. Zeitabhängigkeit der sukzessiven Bildung von $B_{10}H_{10-n}Cl_n^{2-}$, $n = 0$–6.

Abhängigkeit der Beweglichkeit von der Ionenmasse

Die gute Qualität, aber auch die Grenzen der ionophoretischen Trennungen lassen sich am besten an Gemischen, die möglichst viele radioaktiv markierte Halogenohydroborate enthalten, demonstrieren. In den Fig. 2–4 sind jeweils die mit Acridiniumhydrochlorid sichtbar gemachten Zonen und darunter die mit dem Scanner gemessenen Aktivitätsverteilungskurven wiedergegeben. Die Wanderungsgeschwindigkeit nimmt erwartungsgemäss mit steigender Ionenmasse ab. Die nach der Massebeziehung

$$\frac{u_1}{u_2} = \sqrt{\frac{M_2}{M_1}}$$

berechneten relativen Beweglichkeiten u bezogen auf $B_{10}H_{10}^{2-}$ (100%) sind für die Chlorohydroborate in Fig. 5 den gemessenen Werten gegenübergestellt. Während die theoretischen und experimentellen Werte für die Verbindungen mit niedrigem Halogenierungsgrad gut übereinstimmen, wandern die höheren Spezies langsamer als erwartet, so dass sie nicht mehr einwandfrei trennbar sind. Der Grund dürfte in der stärkeren Wechselwirkung der grösseren Ionen mit dem Trägermaterial zu suchen sein. Entsprechend stärkere Abweichungen treten bei den noch grösseren bromierten und jodierten Verbindungen auf.

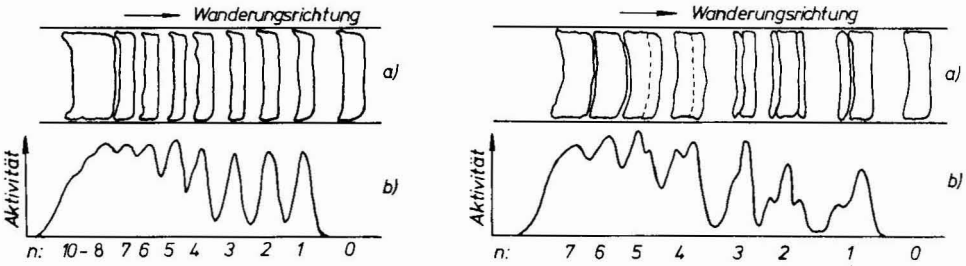

Fig. 2. Pherogramm (a) und Aktivitätsverteilungskurve (b) der mit ^{36}Cl markierten Verbindungen $B_{10}H_{10-n}Cl_n^{2-}$.

Fig. 3. Pherogramm (a) und Aktivitätsverteilungskurve (b) der mit ^{82}Br markierten Verbindungen $B_{10}H_{10-n}Br_n^{2-}$.

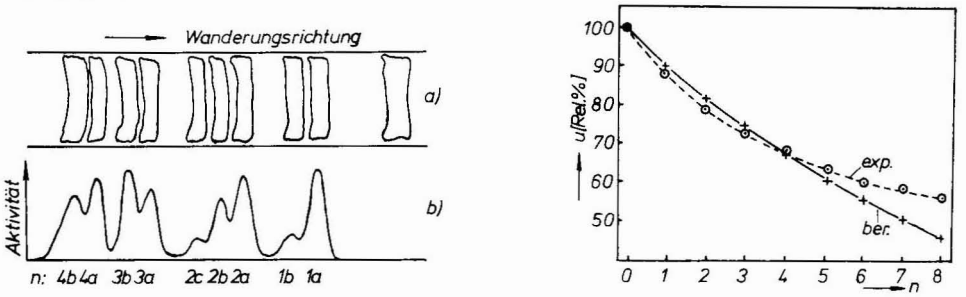

Fig. 4. Pherogramm (a) und Aktivitätsverteilungskurve (b) der mit ^{131}I markierten Verbindungen $B_{10}H_{10-n}I_n^{2-}$ mit Auftrennung in geometrische Isomere.

Fig. 5. Abhängigkeit der relativen Ionenbeweglichkeit u von der Ionenmasse in der homologen Reihe $B_{10}H_{10-n}Cl_n^{2-}$.

Ionophoretische Isomerentrennung

Nach längerer Ionophoresedauer beobachtet man bei den leichteren Bromierungsprodukten zunächst Zonenverbreiterungen und danach Andeutungen für Aufspaltungen (Fig. 3). Bei den Jodohydroboraten, die bis zu 4 I-Atome enthalten, bilden sich mehrere gut aufgetrennte Unterzonen aus (Fig. 4), die sich separat ausschneiden lassen. Die daraus isolierten Verbindungen ergeben jeweils die gleichen Analysenwerte, so dass es sich um geometrische Isomere handelt. Die genaue Untersuchung der einzelnen Substitutionsschritte hat das bestätigt. Über den Bildungsmechanismus und die Zuordnung der Isomeren wird an anderer Stelle[10] berichtet.

Der Trennerfolg wird mit der unterschiedlichen Molekülgestalt der Isomeren erklärt. Die Abhängigkeit der Ionenbeweglichkeit von der Ionenform ist an organischen Verbindungen genauer studiert worden[11]. Kugelförmige Ionen wandern im elektrischen Feld schneller als gleich schwere Teilchen mit länglich ellipsoider Gestalt. 1-$B_{10}H_9I^{2-}$ (Zone 1b, Fig. 4) ist im Vergleich zu $B_{10}H_{10}^{2-}$ stabförmig um 20% verlängert, während 2-$B_{10}H_9I^{2-}$ (Zone 1a, Abb. 4) eine kompaktere Gestalt aufweist und deshalb erwartungsgemäss etwas schneller wandert. Mit zunehmendem Jodierungsgrad wird der Einfluss der Substituenten auf die Molekülgeometrie zwar geringer, die experimentell gefundenen Trenneffekte lassen sich aber ebenfalls aus der Ionenform erklären.

DANK

Der Deutschen Forschungsgemeinschaft und dem Fonds der chemischen Industrie danken wir für die Unterstützung mit Sachmitteln.

ZUSAMMENFASSUNG

Die hochspannungsionophoretische Trennung von Gemischen der Halogenohydroborate $B_{10}H_{10-n}X_n^{2-}$; X = Cl, Br, I; n = 1–9, gelingt, weil die Ionenbeweglichkeiten mit steigendem Halogenierungsgrad schrittweise abnehmen. Auf den Pherogrammen lassen sich die Zonen durch Besprühen mit Acridinhydrochloridlösung sichtbar machen, und die einzelnen Spezies der homologen Reihen werden in reiner Form isoliert. Bei einigen Jodohydroboraten gelingt auf Grund der unterschiedlichen Molekülgestalt die ionophoretische Trennung der geometrischen Isomeren.

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CHROM. 10,112

DIRECT AQUEOUS INJECTION GAS CHROMATOGRAPHY–MASS SPECTROMETRY FOR ANALYSIS OF ORGANOHALIDES IN WATER AT CONCENTRATIONS BELOW THE PARTS PER BILLION* LEVEL

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SUMMARY

A rapid and precise method for the determination of organohalides in the concentration range of 0.1–50 ppb in water samples is described. The method involves the use of mass fragmentographic gas chromatography–mass spectrometry and direct aqueous injection of a large sample (100 μl) on the column (diglycerol as a liquid phase); no concentration or extraction is required. Tap water samples from five locations in Japan were found to contain numerous organohalides, the concentrations of which were determined.

INTRODUCTION

The determination of mixtures of organic compounds in water is a common analytical problem in studies of environmental contamination. The primary requirement is that detection should be sensitive and stable at maximum sensitivity, and gas chromatography–mass spectrometry (GC–MS) is a suitable technique. However, when the concentration of the individual substance to be determined in the practical water sample is low, GC–MS usually requires a concentration step. The concentration procedures now in use or reported in the literature, such as liquid–solid adsorption[1,2], batchwise or continuous liquid–liquid extraction[3], head-space[4,5], vacuum evaporation and gas-phase stripping[6,7], are laborious and inconvenient.

Recently, Harris *et al.*[8] proposed, to water analysis, a new approach of direct aqueous injection GC–MS which requires no pretreatment. However, the detection limit is not sufficient for relatively clean water, *i.e.*, drinking water or surface water, as the method involves no concentration step. Mass fragmentography meets the sensitivity requirements, surpassing the performance of the electron capture detector, and providing maximum sensitivity of the detectors currently in use.

This paper reports the extension of the direct aqueous injection GC–MS

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

method to the use of large volume water samples and operation in the mass fragmentography mode, and provides a quantitative survey of organohalides in the Tokyo region drinking water.

EXPERIMENTAL

Apparatus

All analyses were performed on a Finnigan 3300F gas chromatograph–mass spectrometer equipped with a multiple ion detector and operated in the electron impact mode. The interface between the gas chromatograph and the mass spectrometer was an all-glass jet-type enrichment device. The mass spectrometer was set to unit resolution (10% valley between adjacent nominal masses). The resulting ion currents were recorded on a multichannel strip chart recorder. Other conditions held constant throughout the analysis were: helium carrier gas flow-rate (30 ml/min); temperature of the gas chromatograph injection port (200°); pressure in the mass spectrometer ($6 \cdot 10^{-6}$ Torr); ionizing voltage (70 eV); emission current (320 μA).

Column

A 90 cm × 2 mm I.D. metal straight main column was used, in simple conjunction with a 70 cm × 2 mm I.D. metal straight precolumn. The precolumn contained 10% diglycerol on 60/80 mesh Chromosorb W NAW (Johns-Manville, Denver, Colo., U.S.A.) and the main column 5% SE-30 on 60/80 mesh Chromosorb W AW DMCS (Johns-Manville) to allow each organohalide to appear before an overload water peak due to aqueous injection and to achieve the required separation. This situation was made possible by the very long elution time of water by the diglycerol precolumn in comparison to the elution time of the examined organic substances. The longer diglycerol precolumn should be selected in order to elute the higher boiling materials in the particular water sample before the water. Other columns capable of performing the required separation could be used for the main column, depending on whether any interfering substances are present in the sample.

A venting valve (three-way valve, Hoke 316SST), which was connected to the outlet of the main column, was positioned in the column oven to prevent high volume effluent water in the sample (eluting after informative components) from entering the mass spectrometer. Venting of the water allows continuous MS operation without the possibility of damage to the filament or electron multiplier.

The column temperature was maintained isothermally.

Standardization

A series of $CHCl_3$ standard concentrations from 0.1 to 50 ppb was made by successive dilutions into interfering organics-free water with a reagent-grade $CHCl_3$. A series of standard solutions of each organohalide was also made for calibration work. Water used as the diluent was prepared with the Milli-Q water purification system (Millipore, Bedford, Mass., U.S.A.) and then distilled twice. The CH_2Cl_2, $CHCl_3$, $CHBr_3$, CCl_4, $CHCl{=}CCl_2$, $CCl_2{=}CCl_2$, CH_2ClCH_2Cl, CH_3CCl_3, and $CH_2ClCHClCH_3$ used were reagent-grade chemicals (Wako, Osaka, Japan). The $CHClBr_2$ and $CHCl_2Br$ were purchased from Tokyo Kasei (Tokyo, Japan).

Procedure

Water analysis was performed as follows. A 100 μl water sample was injected directly with a 100 μl Hamilton syringe (Model 710). Positive identification of organohalides in the tap water samples is supported not only from known retention times of the standards but also from the selectivity afforded by selected ion monitoring. As the selected ion contains chlorine and/or bromine, isotope clusters were checked to confirm the absence of interferences. Quantitative information was obtained from peak heights.

Although all examined organohalides eluted in less than 6.5 min, this analysis normally required *ca.* 60 min, as the column was maintained at 100° for at least 50 min so that water and less volatile materials would be removed before the next analysis. The short- and long-term stability of the GC–MS system was good throughout the analysis period.

Fig. 1 illustrates typical mass fragmentograms of specific ions of $CHCl{=}CCl_2$

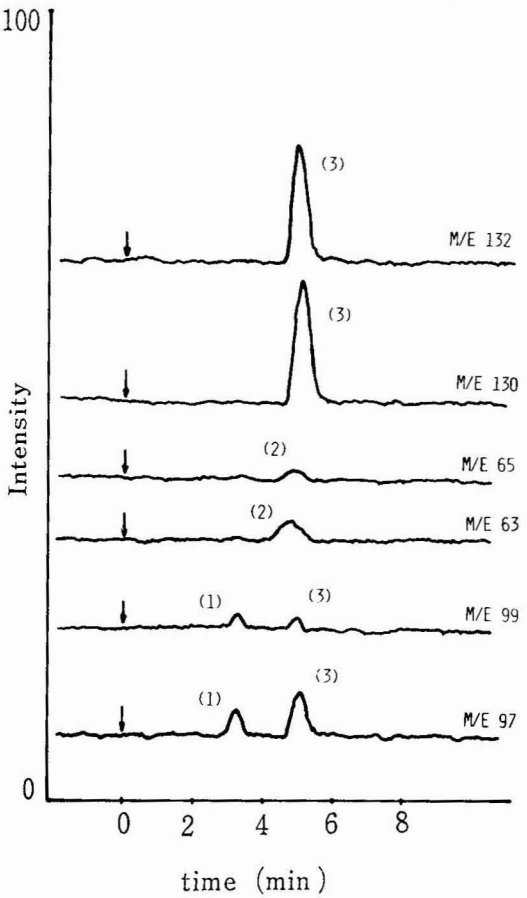

Fig. 1. Six-channel mass fragmentograms obtained from a 100 μl sample of the tap water collected at Tokorosawa near Tokyo. The gas chromatographic effluent is analysed by the mass spectrometer operated in the mass fragmentography mode. The two specific ions in the mass spectrum of each organohalide were monitored. Peaks are: (1) CH_3CCl_3, 0.5 ppb; (2) $CH_2ClCHClCH_3$, 0.8 ppb; $CHCl{=}CCl_2$, 0.9 ppb.

CH_3CCl_3 and $CH_2ClCHClCH_3$ in a certain tap water, showing no interference from other organics.

RESULTS AND DISCUSSION

Detection limit

Trace components are not easily determined when they appear on the tail of an overload water peak. However, use of a column from which water elutes much later than the organic substances of interest, and injection of very large (100 μl) samples, lead to an extension of the detection limit. As 100 μl of standard solution was injected, it was possible to obtain a clean (signal to noise ratio 3 or greater) mass fragmentogram from each organohalide standard solution of the less than ppb level. Table I summarizes the examined organohalides, the GC–MS conditions, the retention times and the detection limit of each (concentration of single substance producing a peak three times higher than noise level).

TABLE I

ORGANOHALIDES DETECTED IN THE TAP WATER SAMPLE

Organohalide	*Retention time (min)*	*Column temperature (°C)*	*Masses monitored*	*Detection limit (ppb)*
CH_2Cl_2	2.4	50	84, 86	0.2
$CHCl_3$	4.1	50	83, 85	0.1
$CHCl_2Br$	1.8	70	83, 85, 127, 129	0.2
$CHClBr_2$	3.1	70	127, 129	0.1
$CHBr_3$	6.5	70	171, 173	0.2
CCl_4	4.3	50	117, 119	0.8
$CHCl{=}CCl_2$	5.0	55	130, 132	0.2
$CCl_2{=}CCl_2$	3.4	70	164, 166	0.1
CH_2ClCH_2Cl	4.8	50	62, 64, 98, 100	0.5
CH_3CCl_3	3.2	55	97, 99	0.4
$CH_2ClCHClCH_3$	4.8	55	63, 65, 97, 99	0.8

Linear concentration range

Detection response was linear over the chosen range of 0.1–50 ppb standard solutions for the organohalides. A plot of six concentrations of each substance in distilled water ranging from 0.1 to 50 ppb against their corresponding peak heights produced a straight line with an intercept at the origin.

Accuracy and precision

The accuracy of the method was determined by making measurements on spiked tap water samples at various concentrations of $CHCl_2Br$ (Table II).

The precision of the method was determined from replicate analyses (five times) of the standard sample at 1 ppb concentration of $CHCl_2Br$, when the standard deviation was 0.14 and the coefficient of variation 4.2% using peak height (mm). These figures are close to the generally accepted best performance of a microlitre

TABLE II

ACCURACY OF MEASUREMENTS ON SPIKED TAP WATER SAMPLES WITH VARYING AMOUNTS OF $CHCl_2Br$ ADDED

$CHCl_2Br$ added (ppb)	*$CHCl_2Br$ found (ppb)*	*$CHCl_2Br$ recovered (ppb)*
0	4.0	0
4	8.1	4.1
6	9.8	5.8
10	14.2	10.2

syringe in the hands of an experienced operator. Similar results were obtained for all compounds examined, although a decrease in precision was observed at near the detection limit. Direct aqueous injection of relatively large quantities on the diglycerol precolumn had no significant effect on the precision.

Application to tap water sample

Numerous organohalides have been discovered in the drinking water supplies of many major U.S. cities[2,3,6,7,9,10]. As the method described has proved to be sensitive, accurate and precise for the analysis of organohalides in water samples, it was applied to the analysis of organohalides in the Tokyo region tap water.

Table III summarizes the substances determined in the drinking tap water of five locations near Tokyo. $CHCl_3$ was the major component with concentrations in the low ppb range which represented a very large peak in the mass fragmentograms. The profiles obtained from various tap water samples showed similar patterns, although variations in the total concentration were large. Other researchers[3,11] have confirmed the hypothesis that nearly all of these compounds are generated only during water treatment, and possible mechanism for their formation are being studied. As

TABLE III

ORGANOHALIDE CONCENTRATIONS (ppb) IN JAPANESE TAP WATER SAMPLES (11 DECEMBER 1976)

Organohalide	*Tokorosawa**	*Fussa***	*Tsuchiura****	*Urawa†*	*Hanamuro††*
CH_2Cl_2	0.3	—	—	—	—
$CHCl_3$	10.2	2.6	13.0	2.7	17.2
$CHCl_2Br$	6.4	1.6	10.5	2.3	4.0
$CHClBr_2$	3.2	0.6	4.0	1.4	0.6
$CHBr_3$	0.5	—	0.4	0.3	—
CCl_4	1.2	—	—	—	—
$CHCl=CCl_2$	0.9	—	0.7	—	—
$CCl_2=CCl_2$	0.6	0.2	0.2	0.2	0.2
CH_2ClCH_2Cl	0.9	—	—	—	—
CH_3CCl_3	0.5	—	—	—	—
$CH_2ClCHClCH_3$	0.8	—	—	—	—

* Located 30 miles NE of Tokyo.
** Located 30 miles E corner of Tokyo.
*** Located 40 miles W of Tokyo.
† Located 20 miles N of Tokyo.
†† Located in Tsukuba Research Center.

the above method is capable of monitoring the products, these mechanisms will be studied successively.

CONCLUSION

This study has demonstrated that direct aqueous injection GC–MS is an effective and practical method for the measurement of organohalides in water samples. High sensitivity (less than 1 ppb detectable) and precision were afforded by the large water sample injection and operations in the mass fragmentography mode. The diglycerol precolumn has an extremely long retention time for water in comparison to that of many organic substances, so that the latter appear before a large peak due to water in the sample. This method is not applicable to all trace organic compounds in water; those which elute later than water cannot be determined. However, it is versatile and convenient and can be applied to relatively volatile compounds, such as phenols, alcohols, ketones, hydrocarbons and amines. Vinyl chloride is one compound of considerable interest that can be determined by this method.

Direct aqueous injection GC–MS could be further modified, perhaps by the aqueous injection of large quantities on cross-linked porous polymer packed columns, from which water elutes very quickly, with concentrated significant organics retained for measurement.

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CHROM. 10,131

A SIMPLE PROCEDURE FOR COMBINED GAS CHROMATOGRAPHIC ANALYSIS OF NEUTRAL SUGARS, HEXOSAMINES AND ALDITOLS

DETERMINATION OF DEGREE OF POLYMERIZATION OF OLIGO- AND POLYSACCHARIDES AND CHAIN WEIGHTS OF GLYCOSAMINOGLYCANS

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SUMMARY

A reliable and reproducible method that allows the combined, simultaneous gas chromatographic (GC) determination of neutral sugars, hexosamines, alditols, identification and quantitation of the reducing aldose end-group in oligo- and polysaccharides and glycosaminoglycans has been described. It involves the following steps: release of the reducing end-group from its protein linkage in glycosaminoglycans and reduction of this reducing end-group into alditol, release of the components of the reduced polymer by resin-catalysed hydrolysis, nitrous acid deamination of the resin-bound hexosamines in this hydrolysate into anhydroaldoses and a combined derivatization and GC determination of the neutral sugars as aldononitrile acetates, anhydroaldoses as peracetylated oximes and alditols as alditol acetates. Application of the method to determination of degree of polymerization of oligo- and polysaccharides and chain weights of proteoglycans has been described. This method has several advantages over the previous methods.

INTRODUCTION

Gas–liquid chromatography (GLC) is widely used for determination of neutral sugars and hexosamines, which are important constituents of glycosaminoglycans and glycoproteins. The determination of the terminal reducing end-group has been used for calculating the degree of polymerization of a polysaccharide and the chain weights of proteoglycans. Among the various chemical end-group analysis methods[1] reduction of the end-group sugar with $NaBH_4$ or tritiated borohydride[2] and determination of the alditol formed gives a more sensitive analysis of the end-group.

In the case of proteoglycans it is necessary to release the reducing sugar end-group of its polysaccharide portion from its covalent linkage to protein prior to any reducing end-group determination. The alternate approach in these cases has been determination of the neutral sugar components of the linkage region of the intact

proteoglycan[3,4]. GLC analyses give more accurate determinations of the end-groups than the majority of the chemical end-group methods. While a number of different derivatives[5,6] have been used for GLC analyses of neutral sugars in such situations, their determination as aldononitrile acetates[7–9] and peracetylated oximes of anhydrosugars[10] formed by deamination of hexosamines has several advantages including their easy preparation, lower retention times, single peak per sugar and greater stability of the derivatives. In the case of trimethylsilyl (TMS) derivatives of the sugars, multiple peaks are obtained due to anomerization and ring isomerization making the chromatogram difficult to interpret. Also, xylans cannot be analyzed when TMS derivatives are used, since one of the xylose peaks and xylitol overlap. In the analysis of alditols as TMS derivatives or acetates a single peak is obtained per alditol, but arabinose and lyxose give the same alditol. Also, there are problems in the simultaneous resolution of the sugar pairs: fucose–rhamnose, arabinose–ribose, lyxose–arabinose, glucose–galactose and glucosamine–galactosamine[5,6]. The use of the aldononitrile acetate derivatives helps identification and determination of the parent sugar with greater reliability and overcomes the aforementioned problems of the resolution.

In previous publications we reported the resolution of twelve neutral sugars as their aldononitrile acetates on a single column and its application to polysaccharides[7], and analysis of neutral sugars[8] and hexosamines[9] from glycoproteins and mucopolysaccharides. In a recent publication[11] we reported the simultaneous determination of neutral sugars and hexosamines from glycoproteins and mucopolysaccharides of tissues and biological fluids. Baird *et al.*[12] have reported the applicability of aldononitrile acetates to the analysis of Smith degradation products. Morrison[13] has recommended the use of these derivatives to the analysis of the degree of polymerization of oligo- and polysaccharides. This paper reports the simultaneous GLC resolution of neutral sugars, hexosamines and alditols and its application to the determination of the degree of polymerization of oligosaccharides and polysaccharides and the chain weights of the glycosaminoglycans.

EXPERIMENTAL

Materials

The materials were obtained or prepared as described previously[11].

Alkaline-borohydride reduction

A 2–5-mg sample of chondroitin-4-sulfate was dissolved in 1.75 ml of 0.2 *N* NaOH and treated with 11–15 mg of $NaBH_4$ for 48 h at 25° in a tightly stoppered 25-ml evaporating flask[14]. In the case of oligosaccharides 1–3-mg samples were dissolved in 0.5 ml water or 0.5 ml of 0.2 *N* NaOH and treated with $NaBH_4$ (10 moles per mole of reducing sugar) at room temperature for 1 h[13]. The solution was neutralized carefully to pH 5 with 3 *M* acetic acid and evaporated to dryness in a rotary evaporator at room temperature. The excess of boric acid was removed by repeated evaporations with methanol. The solid was dissolved in a small quantity of water and transferred quantitatively into a 10-ml glass ampoule containing 20–50 μg of a neutral sugar (as an internal standard) not present in the test material and freeze dried.

Resin-catalysed hydrolysis of reduced material

The residue in the ampoule was dissolved in 0.1 ml of water and to this 0.6–0.8 ml of a 40% (w/v) suspension of AG 50W-X8 (H^+) resin in 0.02 *N* HCl was added. The tip of the ampoule was sealed carefully and left in the oven at 100° for 48 h.

Nitrous acid deamination of resin-bound hexosamines

This step was omitted for the oligosaccharides, since they did not contain the hexosamines. The ampoule containing the glycosaminoglycan hydrolysate was cooled to room temperature, the tip was gently broken and after adding 0.15 ml of a solution containing 35 mg of $NaNO_2$, the tip of the ampoule was quickly and carefully resealed avoiding any heating of the solution. The cooled ampoule was subjected to intermittent vortexing for 30 min for completion of deamination[11].

Isolation of the mixture of aldoses, anhydroaldoses and alditols

The ampoule was opened and a pinch of AG 50W-X8 (H^+) resin was added. The contents of the ampoule were transferred with water washings to a tandem arrangement of two columns made of two 10-ml Kimble glass disposable serological pipettes (Owens-Illinois, Toledo, Ohio, U.S.A.) plugged with glass wool and packed to the 5-ml capacity with resins. The upper column containing AG 50W-X8 (H^+) drained into the lower column packed with AG 1-X2 (HCO_3^-). A 30–40 ml of eluate and washings with deionized water were collected in a 50-ml evaporating flask and evaporated to dryness in a rotary evaporator at room temperature.

Derivatization and washing of the derivatives

The solid in the evaporating flask was transferred to a 10-ml glass ampoule with 0.5–1 ml of dry pyridine. The excess pyridine was removed with a stream of nitrogen, leaving 8–10 drops of pyridine in the ampoule. To this 6–7 mg of dry hydroxylamine hydrochloride were added and further processed for derivatization as described previously[11]. After derivatization, the residue obtained by evaporation with nitrogen can be dissolved in dry chloroform and gas chromatographed. However, we have found that washing of the derivatives prior to GLC prevents any possible tailing of peaks and also prolongs the life of the column. For washing, the evaporated derivatives were transferred with 1 ml chloroform into a centrifuge tube fitted with a stopper. The chloroform solution was washed successively with 1 ml of each of cold 3 *M* HCl, deionized water, 0.5 *M* $NaHCO_3$ solution and deionized water. At each step extraction was done in the original centrifuge tube by shaking, centrifugation and careful removal of the top aqueous layers (which were discarded) from the lower chloroform layer. The final chloroform solution was dried by adding a pinch of anhydrous sodium sulfate. The dried solution and the chloroform washings were transferred to a vial, evaporated to dryness with nitrogen, dissolved in 25–50 μl of chloroform and gas chromatographed.

Preparation of standards

Standards containing 100 μg of each of the neutral aldoses, 150 μg of each of hexosamines and 100–150 μg of each alditol were prepared as described previously[11].

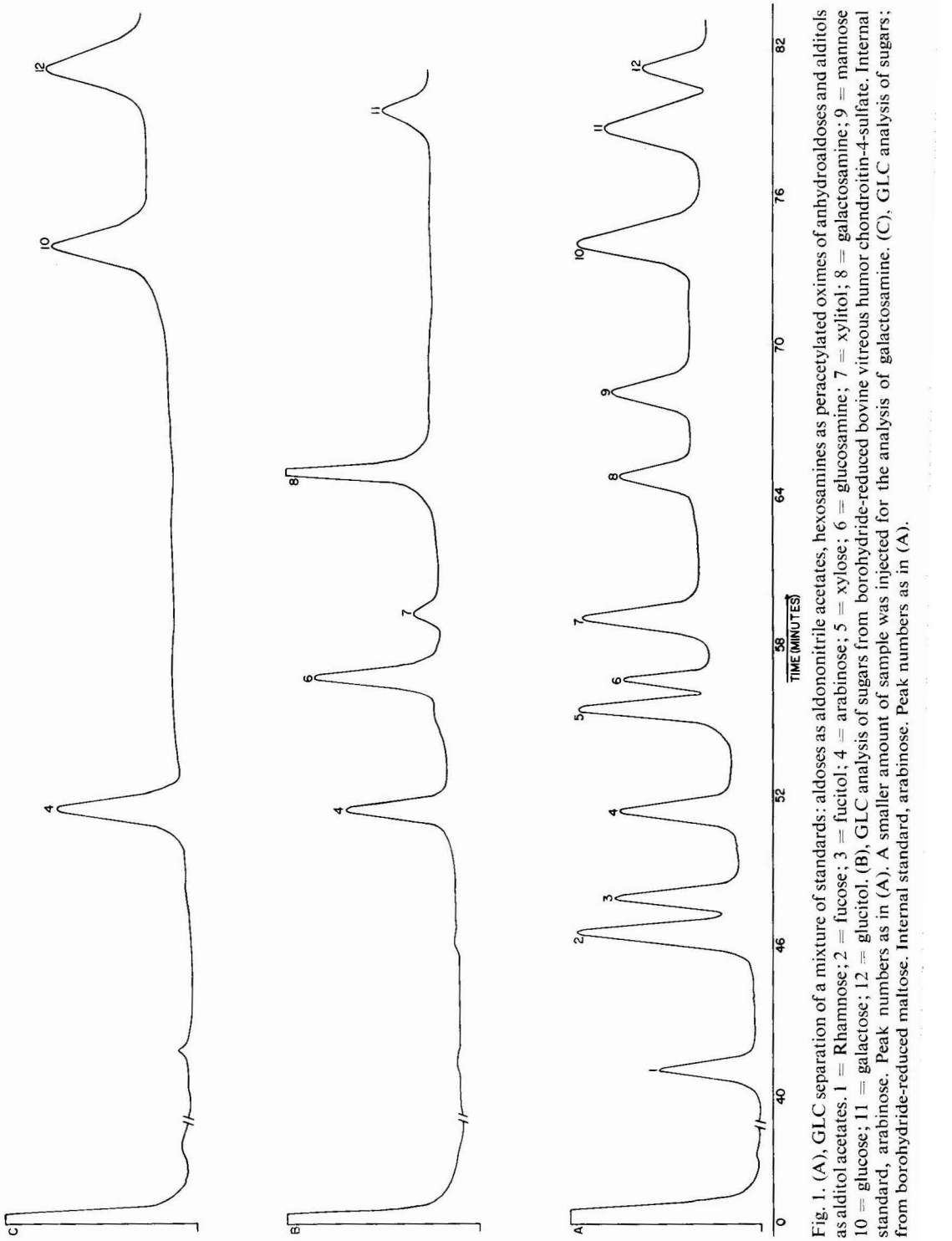

Fig. 1. (A), GLC separation of a mixture of standards: aldoses as aldononitrile acetates, hexosamines as peracetylated oximes of anhydroaldoses and alditols as alditol acetates. 1 = Rhamnose; 2 = fucose; 3 = fucitol; 4 = arabinose; 5 = xylose; 6 = glucosamine; 7 = xylitol; 8 = galactosamine; 9 = mannose 10 = glucose; 11 = galactose; 12 = glucitol. (B), GLC analysis of sugars from borohydride-reduced bovine vitreous humor chondroitin-4-sulfate. Internal standard, arabinose. Peak numbers as in (A). A smaller amount of sample was injected for the analysis of galactosamine. (C), GLC analysis of sugars; from borohydride-reduced maltose. Internal standard, arabinose. Peak numbers as in (A).

Gas chromatography

GLC analyses were carried out on a Series 1200 Varian Aerograph equipped with a flame ionization detector and a Model SRG Sargent-Welch recorder. A stainless-steel column (9 ft. × 1/8 in.) packed with 3% poly(neopentylglycol succinate) on Gas-Chrom W AW (100–120 mesh) was used. The gas chromatograph was initially programmed from 130°–195° at 1°/min and later operated isothermally till the last component (sorbitol) had eluted. Nitrogen was used as the carrier gas at a flow-rate of 36 ml/min. The injector and detector temperatures were maintained at 130° and 230°, respectively.

RESULTS

Fig. 1A illustrates the resolution of a standard mixture of aldononitrile acetates of neutral aldoses, alditol acetates and peracetylated oximes of anhydrosugars formed by nitrous acid deamination of hexosamines. The retention times for all these derivatives are given in Table I. Fig. 1B shows a typical chromatogram for the separation of arabinose (internal standard) and the interior chain components (galactose, glucosamine and galactosamine) of bovine vitreous humor chondroitin-4-sulfate and xylitol formed by reduction of its terminal reducing end-group xylose exposed upon β-elimination reaction in the alkaline medium. A similar chromatogram was obtained for the veal brain chondroitin-4-sulfate.

TABLE I

RETENTION TIMES AND RECOVERIES OF NEUTRAL SUGARS, HEXOSAMINES AND ALDITOLS

Sugar	*Retention time (min)*	*% Recovery (mean ± S.D., n = 7)*
Rhamnose	41.1	93 ± 5
Fucose	46.6	92 ± 5
Fucitol	47.9	94 ± 5
Arabinose	51.4	93 ± 5
Xylose	55.4	94 ± 6
Glucosamine	56.6	93 ± 5
Xylitol	59.0	94 ± 5
Galactosamine	64.7	93 ± 5
Mannose	68.1	94 ± 5
Glucose	74.0	94 ± 4
Galactose	78.8	93 ± 6
Glucitol	81.1	92 ± 5

Fig. 1C shows the separation of arabinose (internal standard), glucose and sorbitol obtained from the hydrolysate of borohydride reduced maltose. Maltotriose gave a similar chromatogram. The values obtained by the present method were in close agreement with the theoretical values for maltose and maltotriose (Table II). The percentage of each sugar component was calculated using the equation given elsewhere[11]. The procedure for polysaccharide is the same as for oligosaccharides and the applicability to polysaccharides is not given here since it has been given earlier[13]. The degree of polymerization of an oligosaccharide or a polysaccharide can be deter-

mined from the ratio of the interior chain residues of the homopolymer to the terminal reducing sugar group measured in the present method as aldononitrile acetates and alditol acetates, respectively. The ratios of the percentages of glucose and glucitol obtained by this method agree with the calculated theoretical values for maltose and maltotriose (Table II).

TABLE II

CARBOHYDRATE COMPOSITION (%) OF CHONDROITIN-4-SULFATES FROM VEAL BRAIN AND BOVINE VITREOUS HUMOR, MALTOSE AND MALTOTRIOSE

Sugar	*Veal brain chondroitin sulfate*		*Vitreous humor chondroitin sulfate*		*Maltose*		*Maltotriose*	
	Previous method[11]	*Present method*	*Previous method*[11]	*Present method*	*Theoretical value*	*Present method*	*Theoretical value*	*Present method*
Xylose	0.52	–	0.86	–	–	–		
Xylitol	–	0.50	–	0.83	–	–		
Galactose	1.30	1.28	1.99	1.90	–	–		
Glucosamine	3.76	3.43	5.28	5.33	–	–		
Galactosamine	17.6	17.1	21.39	20.93	–	–		
Glucose	–	–	–	–	52.63	47.37	71.42	67.50
Glucitol	–	–	–	–	53.21	50.72	36.11	33.73

The values of xylitol determined in the borohydride-reduced chondroitin-4-sulfates from veal brain and vitreous humor are close to the values for the terminal neutral sugar xylose in the unreduced chondroitin-4-sulfates (Table II). The number-average chain weights (C_n) of chondroitin sulfates were calculated from its content of linkage region terminal neutral sugar xylose or its reduction product xylitol as the weight of chondroitin sulfate in grams, which will contain 1 mole (150.13 g) of xylose or 152.13 g (1 mole) of xylitol. The chain weight values calculated for chondroitin sulfates from veal brain and vitreous humor from xylitol values by this method were 30,487 and 18,329, respectively, which agree well with the values of 29,151 and 17,457 calculated from xylose content of the intact unreduced chondroitin sulfates.

DISCUSSION

Proteoglycans, *e.g.* chondroitin sulfates, are covalently linked protein–polysaccharide complexes. The alkali-borohydride reduction brings about the cleavage of the covalent linkage between the hydroxyamino acid serine in the protein and the terminal neutral sugar xylose in the polysaccharide chain by β-elimination reaction[15] and the xylose residue so exposed is reduced to xylitol simultaneously. In case of oligosaccharides and polysaccharides any free reducing end-group is reduced to the corresponding alditol. The resin-catalysed hydrolysis quantitatively releases[16] the hexosamines, aldoses and alditol from the reduced material. The hexosamines and amino acids in this hydrolysate stay bound to the cationic resin and upon nitrous acid deamination glucosamine and galactosamine yield 2,5-anhydromannose and 2,5-anhydrotalose, respectively, which are released from the resin[11]. Deamination of the

amino acids present in this hydrolysate gives the corresponding carboxylic acids[9] which, along with any hexuronic acid from mucopolysaccharide hydrolysis, are removed by the anionic resin AG 1-X2 (HCO_3^-) during passage through the tandem columns. Treatment of the aldoses, anhydroaldoses and alditols, isolated from the eluate, with hydroxylamine hydrochloride in pyridine converts the aldoses and anhydroaldoses into their oximes. Subsequent acetylation of the mixture yields aldononitrile acetates from aldose oximes, peracetylated anhydroaldose oximes from anhydroaldose oximes and alditol acetates from alditols. All these derivatives are determined simultaneously by GLC.

Mannosamine is of a limited occurrence[17,18] and upon deamination forms glucose[19]. If and when the pair mannosamine–glucose is, rarely, present, the resin-bound hexosamines may be separated from the neutral sugars in the hydrolysate by two slightly different approaches and determined after deamination as described previously[11].

In proteoglycans a number of polysaccharide chains may be present covalently linked to protein as complexes. The number-average chain weight (C_n), which is the molecular weight of the individual polysaccharide chain is a meaningful and useful parameter in addition to the molecular weight of the proteoglycan *per se*, which offers information on the structure of the proteoglycan on a more detailed level[20]. Bollet and Nance[21] applied this concept of the chain weight for understanding the changes in chondroitin sulfate in osteoarthritis. The determination of the xylitol following alkali-borohydride reduction gives the positive identification and a reliable quantitative value of xylose that was involved in alkali-labile protein–carbohydrate linkage. The agreement between the chain weights for chondroitin sulfates from vitreous humor and veal brain calculated from xylitol value following alkali-borohydride reduction and that calculated from xylose content of the intact unreduced samples suggests that all of the xylose present in these samples is involved in an alkali-labile covalent linkage.

The present method has several advantages over the other methods. It allows the determination of the neutral sugars, hexosamines, alditols and identification and determination of the terminal reducing aldose and the number-average chain weight (C_n) in a single GLC analysis. Since the complete analysis is carried out in the same run, more accurate and reproducible results are obtained. Recoveries of various neutral sugars, hexosamines and alditols carried through the entire experimental procedure as described earlier are shown in Table I. Reproducibility of the present method was determined by carrying out analyses on several different starting concentrations of maltose, since this disaccharide was available in abundance, and each analysis done in duplicate. The mean and standard deviation of the difference between each pair of values was 0.28 ± 0.21 for glucose and 0.23 ± 0.18 for glucitol. The aldononitrile acetates, peracetylated oximes of anhydrosugars and alditol acetates are stable derivatives, give single peaks and a simple, easily-interpretable chromatogram. Also, the present method overcomes the difficulties encountered with the other derivatives[5,6] in the resolution of the pairs: fucose–rhamnose, arabinose–ribose, lyxose–arabinose, glucose–galactose, glucosamine–galactosamine, xylose–xylitol, xylitol–2,5-anhydromannitol.

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QUANTITATIVE DETERMINATION OF THE HERBICIDE PARAQUAT IN HUMAN PLASMA BY GAS CHROMATOGRAPHIC AND MASS SPECTROMETRIC METHODS

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SUMMARY

The gas chromatographic (GC) determination of the herbicide paraquat, the 1,1′-dimethyl-4,4′-dipyridyl cation in human plasma is described. In poisoning cases, plasma concentrations provide a necessary index of the severity of intoxication and a means of monitoring subsequent therapy. The methods may be extended to the specific trace analysis of paraquat in body fluids or *post-mortem* tissue. Reduction of fully ionised paraquat salts with sodium borohydride yields a hexahydro derivative, a diene, amenable to solvent extraction and GC. Employing 1,1′-diethyl-4,4′-dipyridyl dichloride as the internal standard, plasma concentrations of 0.1 μg/ml (± 6% S.D.) may be determined with flame ionisation detection and 0.025 μg/ml with nitrogen-selective flame ionisation. Further enhancement of specificity is achieved using selected ion monitoring mass spectrometry and the value of this technique in forensic analysis is illustrated.

INTRODUCTION

Paraquat, the 1,1′-dimethyl-4,4′-dipyridyl cation (I), is widely accepted as an effective contact herbicide[1]. As the dichloride salt, it is marketed in Great Britain under the trade names Gramoxone**, a 20% aqueous solution, and Weedol**, a granular preparation composed of 2.5% paraquat and 2.5% diquat (III). Paraquat does not represent a serious poisoning risk to man in the normal process of application or subsequently as an environmental contaminant. However, in the period from 1964 to 1974, there have been over 200 recorded deaths resulting from ingestion of paraquat in all countries of use. All aspects of paraquat poisoning have recently been reviewed[2]. While deaths are in part attributable to such misadventures as swallowing of concentrated solutions stored in unlabelled soft drink bottles, more than half appear to have

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** Trademark of Imperial Chemical Industries Ltd., Macclesfield, Great Britain.

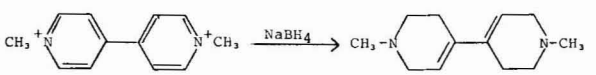
been with suicidal intent. Paraquat causes widespread organ damage, but typically where this phase is survived, death results from effects upon the lung. Our interests are in possible therapeutic approaches in paraquat poisoning[3] and in the mechanisms of its toxic effects. For studies in man, we required a reliable index of the severity of intoxication based on measurements made in plasma during several days after ingestion. New methods of analysis were required for this purpose and for related studies of the pharmacokinetics of the chemical in man.

The most widely used technique for the detection of paraquat is colorimetry after concentration by cation-exchange chromatography. This was originally developed for residue analysis in food crops[4] and is readily adapted for urine and tissue samples (*e.g.*, refs. 2 and 5). However, with a limit of detection of 5 μg per aliquot, it is inappropriate for serial determinations in blood. In our observations, even in severe poisoning cases, concentrations rapidly fall to the 0.1–0.5 μg/ml range and sample size is necessarily limited. High-speed liquid chromatography with UV spectrophotometric detection has been used for paraquat analysis in urine, but the limit of detection is around 100 μg paraquat per 1 urine[6].

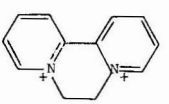
Several groups have reported gas chromatographic (GC) detection methods for paraquat. Thus, pyrolysis to 4,4′-dipyridyl in the injection port of the gas chromatograph has been introduced as a rapid technique for urine analysis in the 1-ppm range, with a probable limit of 0.1 μg/ml in 5-ml samples[7]. Mass spectrometry (MS) and nitrogen-selective flame ionisation have now also been used to determine the pyrolysis product and the method can be used for blood analyses in the range 0.02–0.5 μg/ml[8]. Reduction of both paraquat and diquat to volatile diamines amenable to GC has been applied to water and soil samples[9–11].

Of particular interest has been the reduction of paraquat in aqueous solutions of sodium borohydride to the diene (II)[10]. In our laboratory, the observation that this reduction procedure could be applied to biological extracts led us to explore the reaction as the basis of a quantitative GC assay from plasma. In this communication, we describe the use of flame ionisation (FID) and nitrogen-selective flame ionisation detection (NFID) suitable for routine analysis to 0.025 μg/ml. The necessary precision is achieved by the use of 1,1′-diethyl-4,4′-pyridyl dichloride as an internal standard. Extension to selected ion monitoring (SIM), MS in GC detection (otherwise "mass fragmentography") provides additional sensitivity and the specificity required for forensic analysis.

The methods described have been evaluated during a three year period and are now also in use in other centres in Great Britain. They have previously been reported only in the form of an abstract[12].



(I) (II)



(III)

EXPERIMENTAL

Gas chromatography and gas chromatography–mass spectrometry

For routine GC assay, a Pye model 104 gas chromatograph (dual FID head) was employed with 5 ft. × 2 mm I.D. glass columns. For NFID, the Hewlett-Packard model 5750 G gas chromatograph with a model 15161 B nitrogen detector was used. The glass columns were 5 ft. × 2 mm I.D. Combined GC–MS was carried out with an AEI (Manchester, Great Britain) Model MS12 mass spectrometer interfaced via a silicone membrane separator with a Varian Model 1400 gas chromatograph. The glass columns were 6 ft. × 2 mm I.D. This instrument had been modified for SIM analysis based on accelerating voltage switching[13]. Reference mass spectra were recorded from the gas chromatograph employing a Digital Equipment Corporation PDP 8/I computer for data reduction and background substraction (AEI, data system DS 30).

The GC packing employed routinely in FID and NFID assay was 0.75% Carbowax 20M, 5% KOH on Chromosorb G (80–100 mesh) (Phase Separations, Queensferry, Great Britain). Slight inter-batch differences in relative retention times were observed with this packing material and were inferred to be due to variation in the nominal KOH load. Within-batch variation was not encountered. The packing 3% OV-1 on Gas-Chrom Q (80–100 mesh) was used in GC–MS assay.

Reagents and reference compounds

Sodium borohydride and 4,4′-dipyridyl were obtained from BDH (Poole, Great Britain) and methyl iodide, ethyl iodide and methyl-d_3 iodide ($\geqslant$ 99 atom % D) from Koch-Light (Colnbrook, Great Britain). Solvents were AnalaR grade and were not redistilled. Paraquat dichloride (methyl viologen hydrate) was obtained from Aldrich (Milwaukee, Wisc., U.S.A.) and was dissolved in methanol, filtered, reprecipitated with acetone and dried before use as a reference standard.

[Methyl-^{14}C] paraquat dichloride (30 mCi/mmol) was supplied by the Radiochemical Centre (Amersham, Great Britain).

The diene II was synthesised by the following procedure. Sodium borohydride (100 mg) was added to a continuously stirred solution of paraquat dichloride (0.5 g) in 10 ml of water. An intense blue colouration due to the paraquat radical ion persisted for several minutes. After 30 min, the pH was adjusted to 12 and the reaction mixture extracted with three aliquots of 10 ml ether. The combined ether extracts were dried (sodium sulphate) and evaporated to dryness under nitrogen, yielding 297 mg of solid material (79% yield based on paraquat ion). Concentrated solutions rapidly darkened on standing. Recrystallisation was effected from chilled hexane after brief treatment with silicic acid to decolourise the solution. The diene II [m.p. 100.5° (uncorrected); $C_{12}H_{20}N_2$: calculated C 74.95%, H 10.48%, N 14.57%; found C 74.85%, H 10.35%) was characterised by NMR, UV and mass spectrometry.

1,1′-Diethyl-4,4′-dipyridyl dichloride and 1,1′-di(trideuteromethyl)-4,4′-dipyridyl dichloride (paraquat-d_6) were prepared from the appropriate alkyl halides and 4,4′-dipyridyl. The following procedure was used for the diethyl derivative. Ethyl iodide (1 g) and 4,4′-dipyridyl (0.5 g) were dissolved in 6 ml of dimethylformamide. After standing for 18 h at room temperature, the resulting red precipitate was filtered and washed with dimethylformamide and acetone. The diiodide was dissolved in 5 ml of water and an excess of a saturated aqueous solution of picric acid added. The

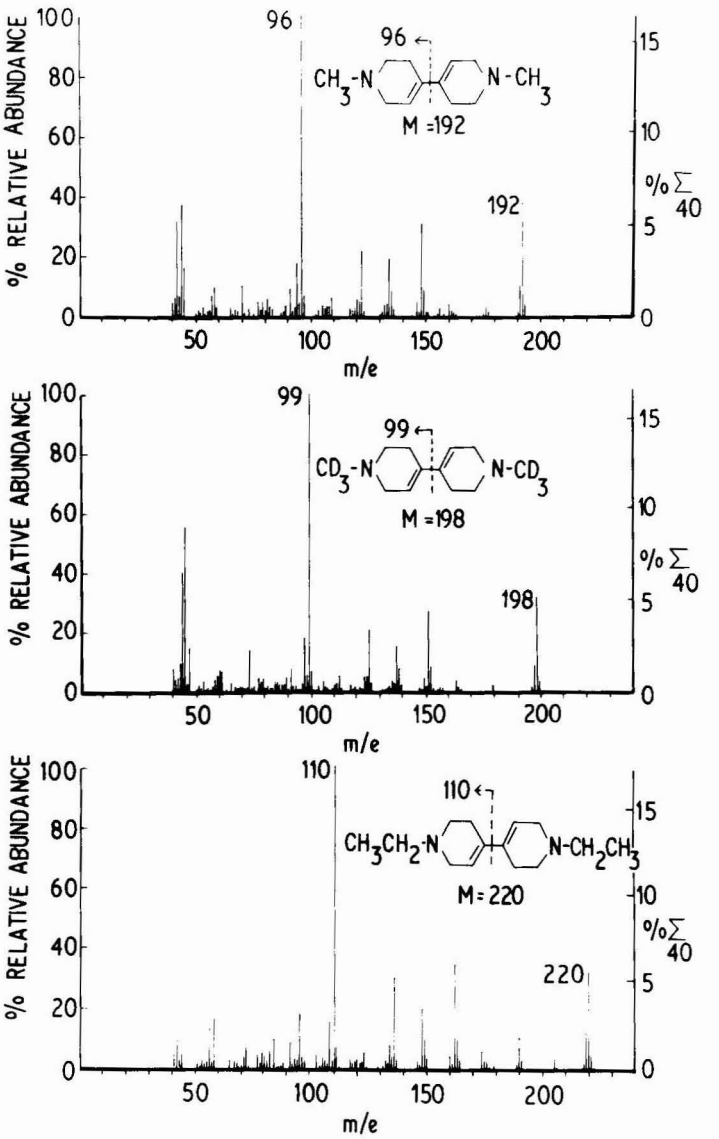


Fig. 1. Electron impact mass spectra of dipyridyl reduction products at 70 eV.

resulting fine light yellow precipitate was filtered and washed consecutively with water and acetone. The picrate salt was suspended in 100 ml of acetone, and 1 ml of concentrated HCl added with stirring, resulting in conversion to the colourless chloride. After 30 min, the precipitate was filtered, washed with acetone and dried *in vacuo*. A further purification could be obtained by solution in a minimum volume of methanol and reprecipitation by the slow addition of acetone. The product 1,1′-diethyl-4,4′-dipyridyl dichloride was characterised by GC–MS after sodium borohydride reduction. Paraquat-d_6 was similarly synthesised (from methyl iodide-d_3) and characterised by GC–MS; mass spectra are presented in Fig. 1.

Analytical procedures

3 ml of plasma, 1 ml of an aqueous solution of 1,1′-diethyl-4,4′-dipyridyl (1 μg ion per ml for FID and 0.1 μg ion per ml for NFID) and 1 ml of a 25% aqueous solution of trichloroacetic acid were thoroughly mixed and centrifuged (2500 rpm). The protein precipitate was resuspended in 5 ml of a 5% aqueous solution of trichloroacetic acid, mixed and centrifuged. The combined supernatant was placed in a 50-ml tube, adjusted to pH 10 with 1 ml of 5 *N* NaOH and 150 mg of $NaBH_4$ added. The mixture was left at room temperature with intermittent shaking for a period of 60–90 min. (The reaction time to produce approximately 90% conversion to II was checked for individual batches of commercial reagent). Sodium acetate (8 g) was added to the reduction mixture which was then extracted twice with 9 ml of ether. The ether layers were back-extracted in turn into 0.5 ml 1 *N* HCl contained in a 10-ml tapered tube. The ether layers were discarded and the acid transferred to a 3-ml Reacti-vial (Pierce, Rockford, Ill., U.S.A.) and traces of ether were removed under a stream of nitrogen.

For FID analysis, the solution was adjusted to alkaline pH with 0.15 ml of 5 *N* NaOH and thoroughly mixed with 30 μl of dichloromethane. After centrifuging, a 3-μl aliquot of the organic layer was used for GC. The chromatographic conditions were: oven 180°, injection block 210°, detector 250°, and nitrogen flow-rate 20 ml/min. Retention data are noted in Table I. Quantification was based upon peak height ratio of II to the internal standard with interpolation of unknowns on a standard curve for paraquat ion in plasma in the range 0.1–1.0 μg/ml. (See Fig. 2.)

For analysis by NFID, dichloromethane could not be used as a solvent. Following extraction into 0.5 ml of 1 *N* HCl and readjustment to basic pH, the reduction products were recovered into ether (2 × 1 ml). The ether was evaporated under nitrogen and the residue reconstituted in 20 μl of methanol. A 2-μl aliquot was used for GC. The chromatographic conditions were: oven 200° and detector 400°; helium (carrier gas) flow-rate 60 ml/min, hydrogen 28 ml/min and air 180 ml/min. Adjustment of the crystal position for maximum selectivity in response to nitrogen was carried out daily prior to paraquat analysis and was most conveniently effected by injecting 10 ng of diphenylamine and 5 μg of n-C_{20} alkane in 1 μl of hexane. The crystal was adjusted until peaks of equal height were obtained. The calibration range for paraquat ion in plasma was 0.025–0.2 μg/ml.

TABLE I

GAS CHROMATOGRAPHIC RETENTION DATA FOR SODIUM BOROHYDRIDE REDUCTION PRODUCTS OF DIPYRIDYL DERIVATIVES

Conditions: 5 × 2 mm I.D. glass column of 0.75% Carbowax 20M 5% KOH on Chromosorb G (80–100 mesh) at 180°; Pye Model 104 instrument; detection, FID.

Dipyridyl derivative	*Reduction product*	*Retention time (min)*	*Relative retention time*
Paraquat (I)	diene (II)	3.55	1.00
	monoene	1.50	0.40
1,1′-Diethyl-4,4′-dipyridyl	diene	5.90	1.66
	monoene	2.50	0.70
Diquat (III)	diene	3.10	0.87

Aliquots of tissue (1–3 g) after homogenisation in 5 volumes of 10% trichloroacetic acid could be treated in a similar manner. For certain urine and bile samples, protein precipitation could be omitted and it was possible to proceed to reduction with sodium borohydride. Quantitative methods have been evaluated fully in terms of the extraction sequence and possible endogenous substance interference only for plasma.

RESULTS AND DISCUSSION

Sodium borohydride reduction

Sodium borohydride reduction of paraquat dichloride yielded an ether-soluble crystalline solid of molecular formula $C_{12}H_{20}N_2$ corresponding to a hexahydro derivative. The base peak in the mass spectrum at *m/e* 96, shifted 3 a.m.u. in the spectrum of the trideuteromethyl analogue and 14 a.m.u. in the diethyl analogue (Fig. 1) was attributed to cleavage of the 4,4′-bond and indicated a bis(dehydropiperidine) structure.

The course of reduction of paraquat was examined in aqueous solution with 10 mg of the dichloride salt and a substantial excess of sodium borohydride (20 mg). [^{14}C-Methyl] paraquat was used as a tracer and the products were examined by GC–MS. Reaction to ether extractable radioactivity was 30% at 1 min, 80% at 5 min and complete at 10 min. A by-product of higher volatility than the diene was formed and identified by GC–MS as a monoene. Retention data are discussed below. This material accounted for 7% of the reduction product at 5 min and 9% at 10 min based on integration of GC peak areas. Reduction and subsequent extraction prior to GC was conveniently standardised by incorporating 1,1′-diethyl-4,4′-dipyridyl (II) as the reference. Reduction followed an analogous course to that for paraquat, the major product being the diene.

The related herbicide diquat (III) combined with paraquat in the commercial preparation Weedol and frequently encountered in poisoning cases, was similarly reduced to a hexahydro product.

Extraction from biological fluid

Protein precipitation using trichloroacetic acid could be applied to plasma and to tissue homogenates and was adopted as the initial clean-up step in the recovery of paraquat from biological material. Yields at each stage in the extraction sequence were checked with [^{14}C-methyl] paraquat at a concentration of 1 μg per 3 ml of plasma. Separation from protein into the trichloroacetic acid supernatant could be made quantitatively. The rate of reduction in this medium following readjustment to basic pH was slower than in water. Reaction to ether extractable radioactivity was 60% after 40 min, 90% at 70 min and complete at 90 min. Typically, the reduction was allowed to proceed for 60–90 min. The subsequent stages of ether extraction and back-extraction into HCl resulted in quentitative recoveries of radioactivity. In the final partition between dichloromethane (0.03 ml) and the basic aqueous phase (0.65 ml), 60–65% of the radioactivity was concentrated in the organic layer. One tenth of the organic extract was used for FID analysis.

The reduction products were found to be markedly adsorbed on glass surfaces. Thus, for NFID assay, where concentration by solvent evaporation was substituted

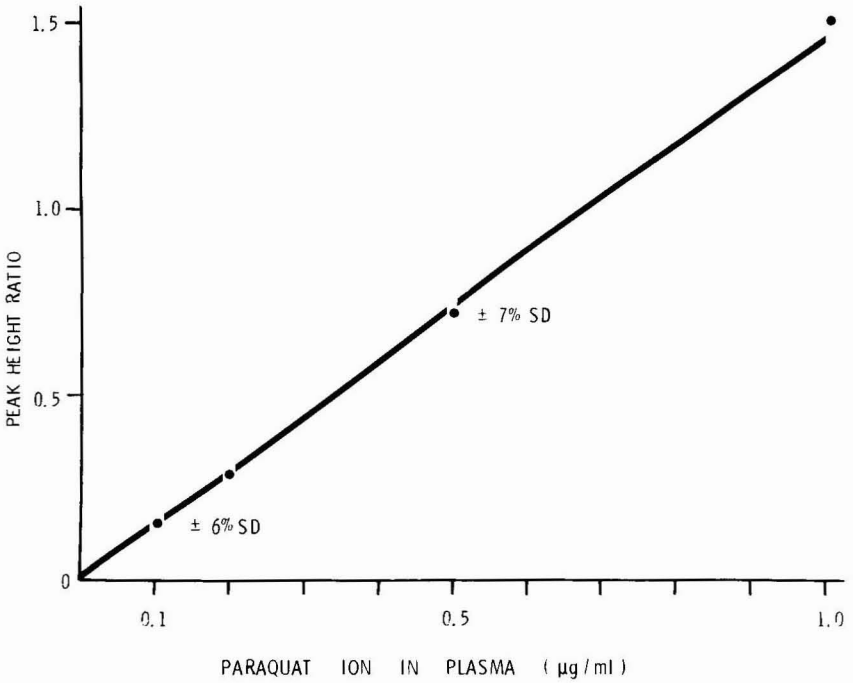


Fig. 2. Calibration and precision of measurement (as % relative standard deviation on the mean) for paraquat in plasma using FID. Response ratio of II to the internal reference is plotted against paraquat ion concentration.

for back-extraction into dichloromethane, care had to be taken to wash the walls of the tube with methanol in reconstitution of extracts for chromatography. Dispersal on glass surfaces during the removal of large volumes of ether was avoided where possible.

GC conditions for plasma assay

The reduction products of paraquat and its diethyl analogue could be chromatographed on SE-30, OV-1, OV-17, carbowax 20 M and on Carbowax coated on base-treated supports. The combination of 0.75% Carbowax 20M and 5% KOH on Chromosorb G provided good peak shape, and negligible memory effects or adsorptive loss at low concentration of sample. Retention data for dipyridyl reduction products are reported in Table I.

With FID, 5 ng of the diene II could be detected and with NFID the detection limit was approximately 0.1 ng. In FID analysis, calibration was linear in the range 0.1–1.0 μg/ml for paraquat ion based on the extraction of 3 ml of plasma (Fig. 2). The precision of measurement at 0.1 μg/ml was $\pm$ 6% (S.D., $n = 6$) and at 0.5 μg/ml was $\pm$ 7% (S.D., $n = 6$). The NFID procedure was used chiefly in the range 0.025–0.2 μg/ml. Fig. 3 shows a chromatogram obtained using NFID from plasma containing 0.025 μg/ml of paraquat and 0.1 μg/ml of the internal standard. Control extracts showed no interference from endogenous substances.

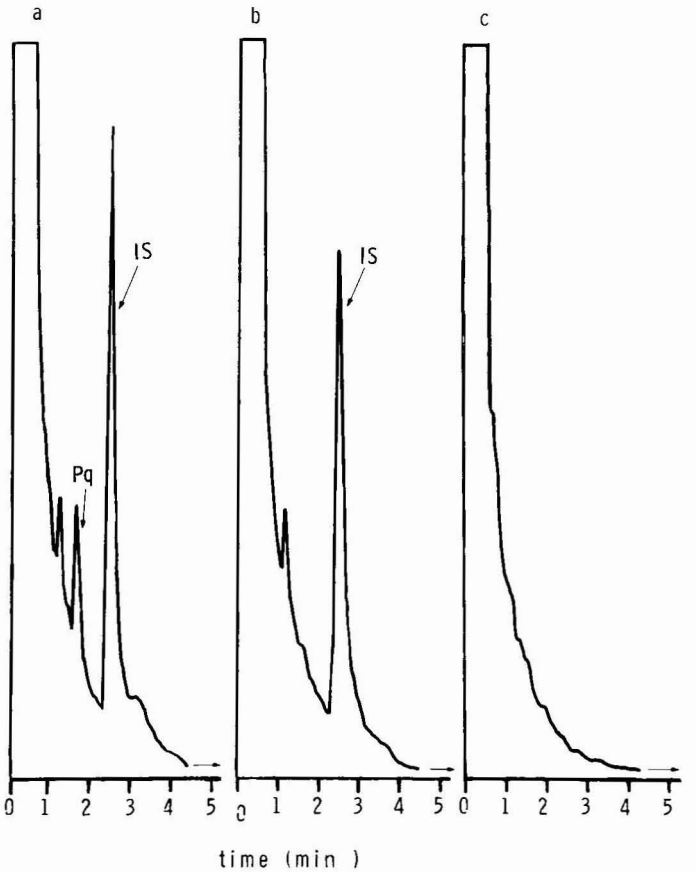
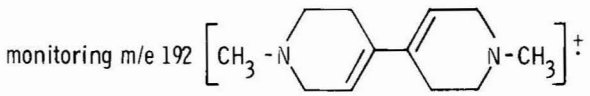


Fig. 3. NFID analysis after sodium borohydride reduction of plasma extracts: (a) 0.025 μg/ml of paraquat ion (Pq) and 0.1 μg/ml of 1,1′-diethyl-4,4′-dipyridyl (internal standard, IS), (b) internal standard only and (c) control plasma.

GC–MS analysis

Enhancement in selectivity and sensitivity was obtainable using SIM mass spectrometry as the GC detection technique. Fig. 4 illustrates the detection of paraquat at the molecular ion of the reduction product, in the plasma of a patient on the 10th day after ingestion of an unspecified quantity of weed killer. Based, in this example, on external standardisation, the level was estimated to be between 5 and 10 ng/ml. Quantitative SIM could be achieved using the diethyl analogue as the internal standard or by stable isotope dilution employing paraquat-d_6. However, the principal use of GS–MS has been in the positive identification of paraquat at trace levels in forensic analysis. Fig. 5 shows the determination of paraquat in the *post-mortem* bile of a murder victim, monitoring the response ratio at *m/e* 192–190. Advantage was taken of a characteristic effect of source temperature on M−2/M ratio which ranged from 0.12 at 205° to 0.30 at 280°. The identity of retention time and of response ratio under two sets of operating conditions with the values for the authentic substance provided convincing evidence for the presence of paraquat in the victim. In this case,



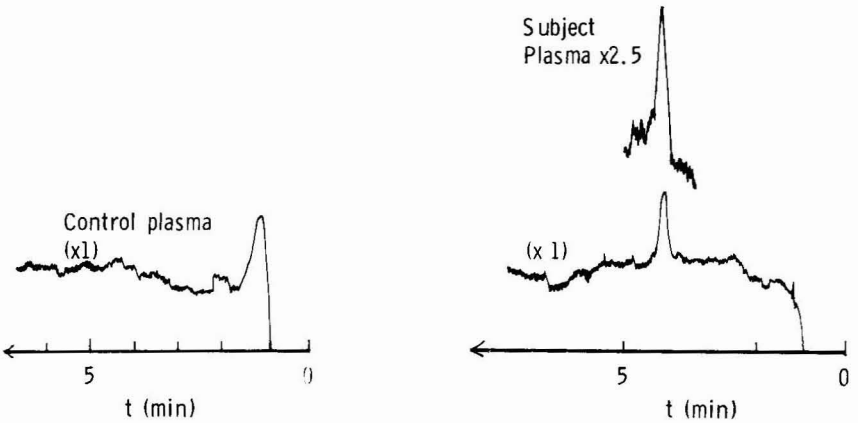


Fig. 4. Selected ion monitoring detection at the molecular ion (*m/e* 192) of II in extracts of plasma 10 days after ingestion of paraquat in man.

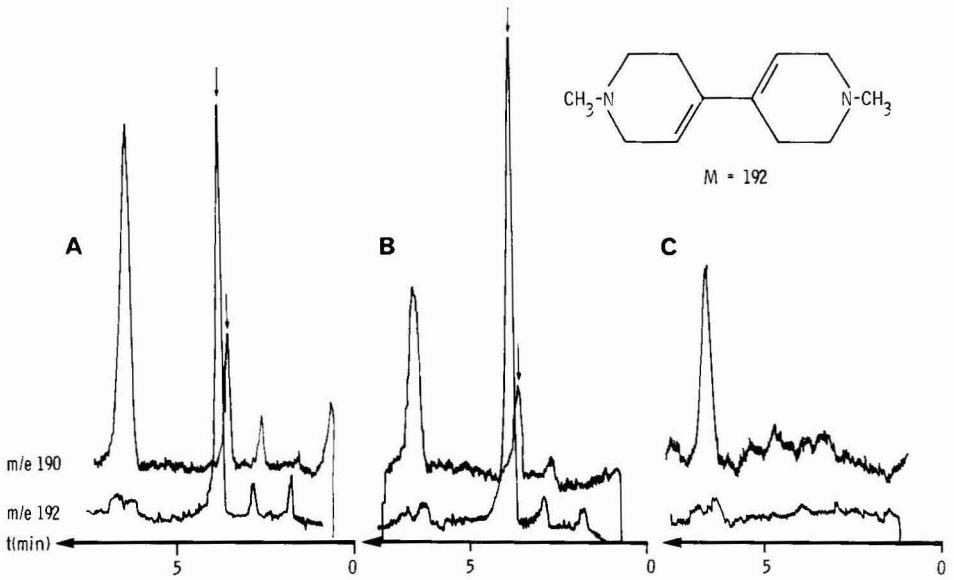


Fig. 5. The identification of paraquat as its reduction product II in *post-mortem* bile. Arrowed points indicate channel offset and correspond to the elution time of authentic II. M/M − 2 (*m/e* 192/190) ratios at source temperatures of (A) 280° and and (B) 205° were consistent with the values for the authentic substance, II; C, control bile extract.

poisoning had not been suspected until some 8 days after admission to hospital. At death on the 12th day the residue in the body was close to the limits of determination by conventional techniques.

CONCLUSIONS

An accurate and sensitive method has been developed to measure paraquat in plasma. With this technique, we have shown that excretion of paraquat in urine is not a reliable guide to the severity of intoxication[14,15]. Paraquat can cause severe renal failure such that even in the presence of high systemic concentrations of the chemical only small amounts are excreted in the urine. Further, we have found that patients with plasma concentrations in excess of 0.25 μg/ml from 12 to 68 h after ingestion, with associated renal failure, usually do not survive. Our views on the pharmacokinetics of paraquat and the treatment of paraquat poisoning have now been presented in detail[15].

NOTE ADDED IN PROOF

The analytical procedures described have now been further developed with enhanced speed of analysis by Dr. G. Steele, ICI Central Toxicology Laboratories, Great Britain and by Professor Maes, University Toxicological Centre, Vondellaan 14, Utrecht, The Netherlands. (Personal communications to the authors.)

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DETERMINATION OF THE RODENTICIDE DIFENACOUM IN BIOLOGICAL MATERIALS BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY WITH CONFIRMATION OF IDENTITY BY MASS SPECTROMETRY

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SUMMARY

A method for determining difenacoum in liver, plasma, urine and feedingstuffs by high-pressure liquid chromatography is described. Samples are cleaned up by molecular exclusion chromatography on porous glass. In some cases this also serves for determination; if not, the separated difenacoum is determined on an adsorption column. Identity is confirmed by chemical ionisation mass spectrometry.

Recoveries at levels of 0.025–5 ppm from plasma were 101–113% by exclusion chromatography alone and 93–101% after adsorption chromatography. Recoveries from liver after both chromatographic steps were 62–86%. Reasons for the lower recoveries from liver are suggested.

INTRODUCTION

Difenacoum is the common name** for 3-(3-biphenyl-4-yl-1,2,3,4-tetrahydro-1-naphthyl)-4-hydroxycoumarin. The usual product is a mixture of isomers thought to be *cis* and *trans*[1]: this is supported by the nuclear magnetic resonance spectra of the separate isomers, where the 1-proton of the tetrahydronaphthalene ring resonates at δ 4.90 or 4.75. It is an anticoagulant rodenticide which is effective against warfarin-resistant rats[2,3]. A method of identifying and determining it was required as an aid to diagnosis in cases of suspected poisoning of farm animals and wildlife. Recently developed methods of analysis for other coumarin anticoagulants, including warfarin and its metabolites, at residue levels have been based on gas chromatography[4–7] or high-pressure liquid chromatography (HPLC)[8–12]. Difenacoum is too involatile for convenient determination by gas chromatography but has been separated from warfarin and some other anticoagulants by HPLC[8]. The clean-up of biological samples by the pyrophosphate procedure used for warfarin residues[8,13] would not be effective

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** Recommended by the British Standards Institution; a draft common name of the International Organisation for Standardization (ISO).

however, because difenacoum is insufficiently polar. Preliminary attempts to analyse tissue extracts by adsorption HPLC without prior clean-up gave good results with new columns, but contamination by co-extractives was so rapid as to make the procedure impracticable for regular use. Clean-up by molecular exclusion chromatography, which has been used successfully for other pesticides (see Masud *et al.*[14], Pflugmacher and Ebing[15] and Johnson *et al.*[16], where other references are given), seemed likely to be successful, particularly as difenacoum would probably be eluted before rather than after its co-extractives.

This communication describes a method for the determination of difenacoum at residue levels in animals tissues, body fluids and feedingstuffs by HPLC. An extract of the sample is cleaned up by molecular exclusion chromatography on a column of porous glass. In some cases this suffices for determination: otherwise the difenacoum fraction is collected and analysed by HPLC on an adsorption column. Identity is confirmed by chemical ionisation mass spectrometry (CI-MS).

EXPERIMENTAL

Materials and apparatus

The sources of anticoagulant rodenticides were as previously specified[8]. Anhydrous sodium sulphate, chloroform, methanol, isopropyl alcohol and iso-octane (2,2,4-trimethylpentane) were of analytical reagent grade. In preliminary work, chloroform used as a minor component of eluent solutions was washed, dried and distilled before use: this procedure did not affect the chromatographic properties of the eluent and was subsequently omitted.

The liquid chromatograph with UV detector, mass spectrometer with CI source and homogeniser were previously described[8].

Extraction

Solid samples (10 g) were macerated with anhydrous sodium sulphate (20 g) and chloroform (30 ml). The extract was filtered through sintered glass, the residue re-extracted with chloroform (15 ml) and again filtered. Plasma and urine (10 ml) were extracted with 15 and 10 ml of chloroform; the extract was dried with sodium sulphate and filtered. The chloroform filtrates were evaporated to dryness under a stream of nitrogen and re-dissolved in methanol (0.5 ml).

Exclusion chromatography

The column was stainless steel, 2 m × 4 mm I.D., packed with Bio-Glas 200, 200–325 mesh (Bio-Rad Labs., Bromley, Great Britain), a porous glass with average pore diameter 200 Å. The eluent was methanol at a flow-rate of 2.5 ml/min and UV detection was at 260 nm. As a pneumatic amplifier solvent pump was used, a restrictor of high resistance in the solvent delivery tube was necessary to avoid an excessive flow-rate.

Replicate aliquots of the methanolic extract (100 μl, the largest volume that could be used without danger of overloading the column with co-extractives) were injected. The difenacoum content was estimated by comparing the mean peak heights with those produced by standard solutions. Normally four aliquots of each extract were injected and the eluate fractions containing difenacoum (eluted before the main

bulk of the UV-absorbing material) were combined for adsorption chromatography or mass spectrometry.

Adsorption chromatography

The column was stainless steel, 0.5 m × 1.5 mm I.D., packed with Corasil II (Waters Assoc., Stockport, Great Britain). The eluent was isopropanol–chloroform–isooctane (1:2:397) and the flow-rate 1 ml/min.

The combined difenacoum fractions from the exclusion column were evaporated to dryness under a stream of nitrogen and the residue re-dissolved in the eluent for the adsorption column (100 μl). Duplicate aliquots (10 μl) were injected. The isomers of difenacoum were eluted as two well-separated peaks. For quantitative measurement, the height of the earlier peak was compared with that produced by appropriate standards. To confirm identity the two fractions believed to contain the difenacoum isomers were collected, the remaining cleaned-up difenacoum extract was chromatographed and the combined difenacoum eluates were examined by MS.

Mass spectrometry

The solvent was evaporated from the difenacoum fractions and the residue transferred in acetone to a capillary tube. The acetone was evaporated and the capillary heated in the solid probe of the mass spectrometer with methane, at a pressure of about 1 torr, as reagent gas. The spectrum of difenacoum appeared at a temperature of 225°, reached after about 2½ min.

RESULTS AND DISCUSSION

In preliminary experiments the apparent recovery of difenacoum added to extracts of livers immediately before exclusion chromatography, at a level equivalent to 1 ppm in the liver, ranged from 104 to 114%. In later recovery experiments with liver and plasma, difenacoum was added to freshly thawed material, which had been stored at −20°, before extraction. Liver and plasma were chosen for detailed examination because they are usually the most useful materials for diagnosis and liver is particularly difficult to analyse.

Exclusion chromatography

Fig. 1 shows chromatograms of extracts of pig liver and plasma, with and without added difenacoum. Difenacoum is eluted as a single peak well before the bulk of the UV-absorbing co-extractives, although the chromatogram of unfortified liver extract shows a peak in the same position as difenacoum. When extracts of livers from 2 cows, a dog, fox and horse were chromatographed, all except that from the fox showed the interfering peak, but it was virtually absent from extracts of plasma from several other species, urine, oats and milk powder. It is clear that the extracts are effectively cleaned up by the porous glass column, at least for the purpose of subsequent adsorption chromatography with UV detection.

When the other commonly used anticoagulant rodenticides warfarin, coumatetralyl and chlorophacinone, and the main mammalian metabolites of warfarin, were chromatographed, all except coumatetralyl were eluted in the main co-extractive fraction but coumatetralyl was eluted at the same retention time as difenacoum. This

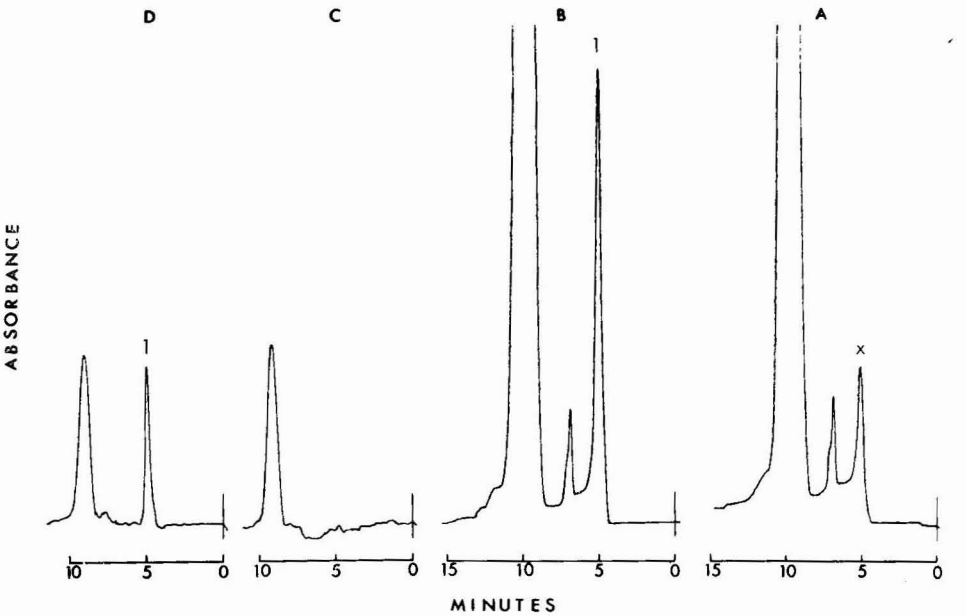

Fig. 1. Molecular exclusion chromatography of extracts from (A) 2 g unfortified liver, (B) 2 g liver + 1 ppm difenacoum, (C) 2 ml unfortified plasma, and (D) 2 ml plasma + 0.1 ppm difenacoum. 1 = Difenacoum; × = co-extractive at difenacoum retention time. A and B at 0.2, C and D at 0.05 absorbance units per mV.

was surprising, since the behaviour of coumatetralyl on an exclusion column might be expected to resemble that of warfarin rather than difenacoum. Clearly, however, difenacoum cannot be differentiated from coumatetralyl by this procedure.

For calibration, 100-μl aliquots of solutions containing 0.5–100 ppm of difenacoum in methanol were injected in triplicate. The response was linear over this concentration range (*i.e.*, the range 0.05–10 μg injected, corresponding to levels of 0.025–5 ppm in an original sample of 10 g or 10 ml) and was described by the equation:

$$h = (155.1 \pm 0.503)\, w + 15.9$$

where h represents the peak height (mm) at a detector sensitivity of 0.02 absorbance units for a sigmal of 1 mV (250 mm) and w the weight of difenacoum injected (μg). The coefficient of w is shown as mean $\pm$ standard error.

Difenacoum was added to pig plasma and liver over the range 0.025–5 ppm. Apparent recoveries from plasma are shown in Table I as means from five separate samples at each level with their 95% confidence limits, the result for each sample being the mean from quadruplicate injections. Results were slightly high and notably consistent over the range 0.05–5 ppm: at 0.025 ppm rather higher but still acceptable. Unfortified plasma gave a small peak in the difenacoum position, equivalent to about 0.005 ppm in the original plasma or 20% of the lowest level of difenacoum examined. For the purposes of residue analysis, contamination at this level can usually be ignored and it appears that difenacoum can be determined in plasma, at levels of 0.025 ppm and above, by exclusion chromatography of chloroform extracts.

TABLE I

RECOVERY OF DIFENACOUM FROM PLASMA BY EXCLUSION HPLC

Difenacoum was added to plasma before extraction with chloroform. Column, 2 m × 1.4 mm I.D., Bio-Glas 200; mobile phase, methanol; pressure, 45 kg/cm² (with flow restrictor); flow-rate, 2.5 ml/min.

Difenacoum added (ppm)	*Recovery (%), mean (95% confidence limits) from 5 separate samples of plasma*
0.025	113 (110–116)
0.05	104 (101–107)
0.1	106 (103–109)
1	101 (98–104)
2	105 (102–108)
5	105 (102–108)

The interfering peak from the liver used in the recovery experiments represented an apparent difenacoum content of about 0.4 ppm. Recoveries of difenacoum added over the range 0.025–1 ppm varied widely; at higher levels they were more consistent, but low. It appeared that exclusion chromatography alone would not suffice for determination in pig liver.

Adsorption chromatography

It had previously been established that warfarin, coumatetralyl, chlorophacinone and difenacoum could be separated from one another and from metabolites of warfarin by HPLC on a column of Corasil II, with 2% isopropyl alcohol in isooctane as eluent[8]. This solvent system was designed for the determination of warfarin and is too polar for determining difenacoum on the Corasil column. With the isopropanol–chloroform–isooctane eluent used in the present work, the two isomers of difenacoum were eluted as well-separated peaks, the first about three times the height of the second, and coumatetralyl was eluted slightly later than the first difenacoum isomer. Chromatography on columns of LiChrosorb SI-60 and Spherisorb ODS was also examined, but peaks from difenacoum tailed more than on the Corasil column when eluents giving convenient retention times were used. The separation of the difenacoum isomers and coumatetralyl on the Corasil column is illustrated in Fig. 2. Chlorophacinone, warfarin and its metabolites were eluted so slowly as to be undetectable at the levels likely to be encountered; they would in any case be separated from difenacoum on the exclusion column. The separation between the first difenacoum isomer and coumatetralyl is small, but sufficient for the two peak heights to be separately measured if the compounds are present at similar concentrations. If the extract contained only one of them, the presence or absence of a peak at the retention time of the second difenacoum isomer would be diagnostic.

For calibration, 10-μl aliquots of the isopropanol–chloroform–isooctane mixture containing 2.5–100 ppm (i.e., 0.025–1 μg) of difenacoum were injected in triplicate. These levels corresponded to 0.03–1.25 ppm in a 10-g sample. The response to the earlier isomer was linear according to the equation $h = (341 \pm 1.6)\, w - 1.86$,

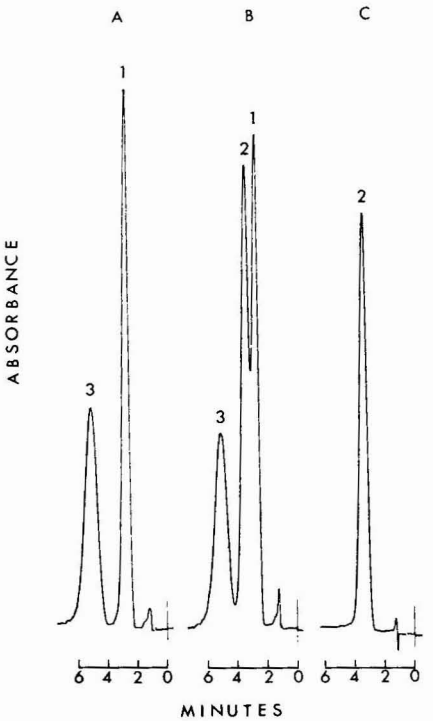

Fig. 2. Adsorption chromatography of solutions of (A) difenacoum, (B) difenacoum + coumatetralyl and (C) coumatetralyl. 1 and 3 = isomers of difenacoum; 2 = coumatetralyl.

where h and w have the same meanings as before. The sensitivity was higher than on the exclusion column because the peaks were narrower.

Recoveries of difenacoum from plasma and liver fortified at 0.025–5 ppm are given in Table II. They are based on the injections of duplicate 10-μl aliquots of collected eluates from the exclusion column and include losses incurred by both chromatographic steps. The result at each level is the mean from five separate samples with its 95% confidence limits.

Mean recoveries ranged from 93 to 101% for plasma and from 62 to 86% for liver. Since no response to unfortified plasma or liver was detected, there is scope for determining lower levels by increasing the sample size. Although recoveries from liver were low, their consistency indicated that the method was adequete for residue analysis.

Since recoveries were low when difenacoum was added directly to liver but not when it was added after extraction with chloroform and transfer to methanol, two possible sources of loss were suspected. One was a small waxy residue, insoluble in methanol, which remained after evaporation of chloroform from the initial extract and which might retain some of the difenacoum. The other was metabolic degradation. The possibility of appreciable metabolism had originally been discounted, partly because the conditions of fortification made it unlikely and partly because it had apparently not been a factor in similar experiments with the related compound warfarin[8].

TABLE II

RECOVERY OF DIFENACOUM FROM PLASMA AND LIVER BY ADSORPTION HPLC

Plasma fortified as in Table I. Liver fortified with difenacoum, then macerated with sodium sulphate and chloroform. Extracts chromatographed on exclusion column and eluate fractions therefrom on adsorption column. Exclusion chromatography as in Table I. Adsorption column, 0.5 m × 1.5 mm I.D., Corasil II; mobile phase, isopropanol–chloroform–isooctane (1:2:397); pressure, 20 kg/cm²; flow-rate, 1 ml/min.

Difenacoum added (ppm)	*Recovery (%), mean (95% confidence limits) of 5 separate samples*	
	Plasma	*Liver*
0.025	94 (90–97)	62 (52–71)
0.05	95 (91–98)	68 (59–78)
0.1	95 (92–98)	73 (63–83)
1	98 (95–101)	86 (76–95)
2	100 (97–103)	72 (63–82)
5	101 (98–104)	83 (74–93)

To examine these possibilities difenacoum was added to a chloroform extract of unfortified liver, to a blank extract after transfer to methanol and to portions of liver which were then allowed to remain at ambient temperature (18°) for varying periods before extraction. Recoveries of difenacoum are shown in Table III. It appears that some loss occurs during transfer from chloroform to methanol and some as a result of metabolism. In the original recovery experiments, the interval between fortification of the liver and its maceration with chloroform varied from about 2 to 15 min. The loss on transfer to methanol was not thought sufficient to necessitate modification of the method.

TABLE III

RECOVERY OF DIFENACOUM ADDED TO LIVER OR LIVER EXTRACT AT VARIOUS STAGES

Difenacoum added to liver at various intervals before extraction, and to extracts. All additions equivalent to 2 ppm in liver. Samples chromatographed on exclusion and adsorption columns. Conditions as in Tables I and II.

Difenacoum added to	*Recovery (%) (mean of duplicate injections)*
Extract after transfer to methanol	98
Initial extract	88
Liver, 30 sec before extraction	83
3 min before extraction	82
10 min before extraction	78
30 min before extraction	68

Mass spectrometry

The base peak of the CI spectrum of difenacoum was at *m/e* 163 and presumably represented the protonation of 4-hydroxycoumarin or an isomeric rearrangement product. At masses above 300 a.m.u. only the characteristic ions at *m/e* 445 (M + H,

41% of the base peak), 473 (M + C_2H_5, 9%) and 485 (M + C_3H_5, 3%) showed intensities above background levels. The low mass of the base peak makes it of little value for identification, but the peaks representing the molecular ion were above the mass of most contaminants and well suited for the confirmation of identity.

Difenacoum was much less sensitively detected by CI-MS than was warfarin[8], although its initial volatilization from the heated probe was sharp. After some 20 sec, the spectrum decreased rapidly to a much lower intensity, then faded slowly to an undetectable level during periods up to 30 min. It appeared that difenacoum which had evaporated from the heated probe condensed on the ion source of the spectrometer, from which it again slowly evaporated. Because of this slow evolution, about 200 ng was needed for a satisfactory spectrum. This quantity would be present in 10 g of material containing 0.02 ppm however, so the lower limit of detection is similar to that of the chromatographic procedures. For comparison, the lower limit of visual detection after thin-layer chromatography on fluorescent plates was about 1 μg.

The extent of clean-up needed for MS was examined. In the case of plasma, the combined exclusion column eluates from four successive 100-μl injections of the concentrated uncleaned blank extract gave a spectrun with no peaks above *m/e* 350. Bulked eluate fractions from the exclusion column would therefore be suitable for MS. Eluates from the exclusion chromatography of liver extracts sometimes showed interfering peaks when the entire fraction was examined, but not when the eluate from a single 100-μl injection was used. Since this represented one fifth of the total sample and 200 ng was required for an adequate spectrum, MS of the eluate from the exclusion column should be effective if the sample contained 0.1 ppm or more of difenacoum.

CONCLUSIONS

(1) Difenacoum residues in liver and plasma can be determined by succesive exclusion and adsorption HPLC. Recoveries are satisfactory at concentrations from 0.025 to 5 ppm.

(2) Since exclusion chromatography of extracts of urine, oats and milk powder showed no peaks near the retention time of difenacoum, the method should be suitable for an extensive range of tissues and feedingstuffs.

(3) The absence of interfering peaks in adsorption chromatograms of blank substrates implies that larger samples could be taken and lower levels determined if required.

(4) Exclusion chromatography alone was adequate for the analysis of plasma, and should be successful for other substrates free from UV-absorbing co-extractives eluted in the same position as difenacoum.

(5) Identity can be confirmed by CI-MS: the quantity needed would depend upon the equipment available, and was about 200 ng in the work described. The concentrated eluate from the exclusion column representing 10 ml of plasma or 2 g of liver is sufficiently clean for CI-MS.

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CHROM. 10,071

USE OF ω-AMINOHEXYL-SEPHAROSE IN THE FRACTIONATION OF *ESCHERICHIA COLI* B AMINOACYL-tRNA SYNTHETASES

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SUMMARY

The usefulness of aminohexyl-Sepharose in purification of *E. coli* B aminoacyl-tRNA synthetases is presented.

The purification factors for 14 synthetases lie in the range 3- to 94-fold and the recoveries of the enzymatic activity were 30–80%, depending on the enzyme.

INTRODUCTION

Classical procedures for the separation of proteins are usually based on differences in their solubility, charge, size and shape. Recently it was shown[1–4] that proteins can also be separated on the basis of their hydrophobicity, *i.e.*, the availability of their hydrophobic pockets. Thus, alkyl- and aminoalkyl-Sepharoses were recognized as useful adsorbents in protein separation and several enzymes were purified on these adsorbents[1–3,5–11]. One of these adsorbents, aminohexyl-Sepharose, has also been used for fractionation of transfer RNA[12]. In our hands, aminohexyl-Sepharose was superior to other adsorbents in the purification of plant aminoacyl-tRNA synthetases[3,11] and tRNA methyltransferases[9].

As *E. coli* is widely used as a source of many enzymes and since the purification of enzymes is often difficult, we felt that it was worthwhile to try to apply hydrophobic chromatography of aminohexyl-Sepharose for the purification of *E. coli* B aminoacyl-tRNAsynthetases. The resolution of 14 synthetases on aminohexyl-Sepharose columns is presented; purification factors for these enzymes were within the range 3–90-fold.

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EXPERIMENTAL

Materials

E. coli B was purchased from the Grain Processing Co., Muscatine, Iowa, U.S.A. and was stored at −20° until used. Sepharose 4B was purchased from Pharmacia (Uppsala, Sweden), CNBr from Pierce (Rockford, Ill., U.S.A.), 1,6-hexanediamine from Aldrich (Milwaukee, Wisc., U.S.A.), glass beads, streptomycin sulphate, ARP and mercaptoethanol from Sigma (St. Louis, Mo., U.S.A.) and *E. coli* B tRNA from Schwarz Bioresearch (Orangeburg, N.Y., U.S.A.). Uniformly labeled [^{14}C]-amino acids were purchased from the Radiochemical Centre (Amersham, Great Britain), and [^{3}H]amino acids from New England Nuclear (Boston, Mass., U.S.A.). Other reagents were of analytical-reagent grade.

Methods

Aminohexyl-Sepharose was prepared according to Cuatrecasas[13]; 100 mg of BrCN was used per millilitre of the gel. Protein was determined by the methods of Warburg and Christian[14] and Lowry *et al.*[15].

Assays of aminoacyl-tRNA synthetases were carried out in a reaction medium composed of 0.1 *M* tris–HCl (pH 7.8), 10 m*M* $MgCl_2$, 1 m*M* ATP, 1 m*M* mercaptoethanol, 4 mg/ml tRNA, 10^{-5}–$2.8 \cdot 10^{-4}$ *M* [^{14}C]- or [^{3}H]-amino acid and enzyme. After appropriate time intervals at 37°, radioactive aminoacyl-tRNA in 30- or 50-μl aliquots was determined by the filter-paper disc technique of Mans and Novelli[16]. One unit of enzyme activity is determined in the aminoacylation of 1 nmole of tRNA in 1 min at 36°.

The 40–60% ammonium sulphate saturation fraction was prepared from *E. coli* B as described by Lövgren *et al.*[17] and was dialysed against 60 m*M* potassium phosphate (pH 6.8), 7 m*M* mercaptoethanol and 10% (v/v) glycerol before application on to the aminohexyl-Sepharose column.

RESULTS AND DISCUSSION

In our earlier studies[3] we showed that plant aminoacyl-tRNA synthetases are not adsorbed on aminoethyl- and aminobutyl-Sepharose. However, these enzymes are adsorbed on an aminohexyl-Sepharose column and can be eluted from the column in a potassium chloride gradient. Our results also indicated that the main forces responsible for binding of aminoacyl-tRNA synthetases to aminohexyl-Sepharose were hydrophobic.

The successful application of aminohexyl-Sepharose in the purification of plant aminoacyl-tRNA synthetases led us to search for other applications of this adsorbent. We found that it was useful in the purification of tRNA methyltransferases[9] and in the fractionation of lupin tRNA[12].

Another application of aminohexyl-Sepharose columns is illustrated in this paper. *E. coli* B aminoacyl-tRNA synthetases are adsorbed on aminohexyl-Sepharose in 60 m*M* potassium phosphate buffer (pH 6.8) containing 10% of glycerol and 7 m*M* mercaptoethanol. The enzymes can be eluted in a linear potassium chloride gradient (Figs. 1 and 2). The elution profiles did not change when the size of the column was

increased 10-fold. The same order of elution of aminoacyl-tRNA synthetases was obtained on 50-ml (Fig. 1) and 500-ml (Fig. 2) columns.

The positions of some aminoacyl-tRNA synthetases were further confirmed by stepwise elution. For example, glutamyl-tRNA synthetase emerges in 0.1 *M* potassium chloride eluate, leucine enzyme in 0.2 *M* potassium chloride eluate and asparaginine enzyme in 0.3 *M* potassium chloride eluate. Thus, the order of elution of *E. coli* B aminoacyl-tRNA synthetases from the aminohexyl-Sepharose column was established as follows: 1, glutamyl-; 2, isoleucyl-; 3, aspartyl-; 4, tyrosyl-; 5, arginyl-; 6, glutaminyl-; 7, histidyl-; 8, valyl-, alanyl- and methionyl-; 9, phenylalanyl-; 10, leucyl-; 11, lysyl-; and 12, asparaginyl-tRNA synthetase. Yellow lupin aminoacyl-tRNA synthetases[3] are eluted under the same conditions in different positions on the gradient and the order of elution is 1, valyl- and lysyl-; 2, leucyl-; 3, tryptophanyl-; and 4, isoleucyl- and phenylalanyl-tRNA synthetase. Only phenylalanine enzymes from both sources are eluted in about the same position (0.25 *M* potassium chloride).

Comparison of the elution positions with the sizes of the enzyme reveals that aminoacyl-tRNA synthetases with more complex structures are eluted in higher ionic strength from the aminohexyl-Sepharose columns, with the exception of valyl-, leucyl- and isoleucyl-tRNA synthetases. It is conceivable that the larger the enzyme molecule the higher is the frequency of occurrence of hydrophobic pockets[3]. Thus we

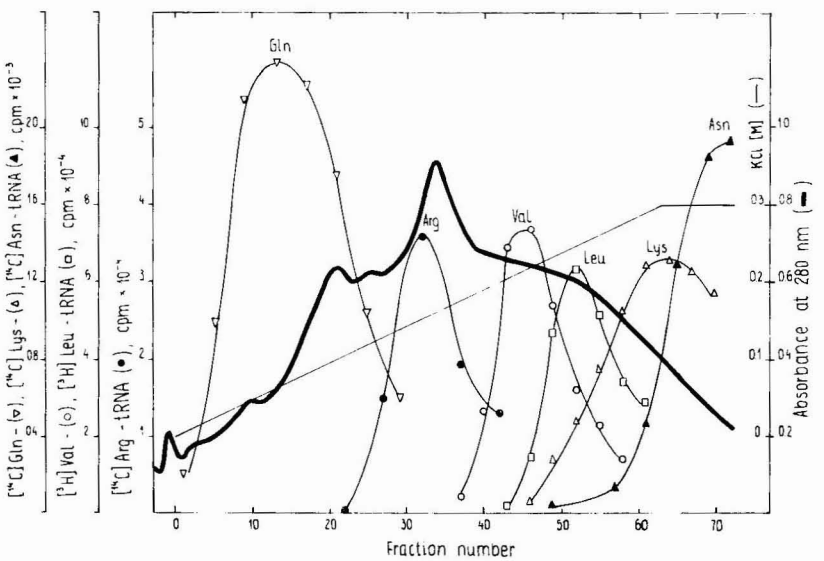

Fig. 1. Chromatography of *E. coli* B aminoacyl-tRNA synthetases on aminohexyl-Sepharose column. The column (1.5 × 25 cm) was equilibrated with 60 m*M* potassium phosphate (pH 6.8), 7 m*M* mercaptoethanol and 10% glycerol. Protein (15 ml; 900 mg) was applied on to the column. After washing with 100 ml of the buffer, the enzymes were eluted with a 0–0.3 *M* linear potassium chloride gradient in the buffer (total volume 512 ml) followed by 0.3 *M* potassium chloride. The flow-rate was 32 ml/h and 8-ml fractions were collected. The absorbance at 280 nm (thick curve) and the synthetase activities in 10-μl aliquots of fractions were determined: glutamyl- (▽), arginyl- (●), valyl- (○), leucyl- (□), lysyl- (△) and asparaginyl-tRNA synthetase (▲). Fractions 35–50, 45–55, 60–70 and 63–74 contain isoleucyl-, tyrosyl-, methionyl- and phenylalanyl-tRNA synthetase, respectively.

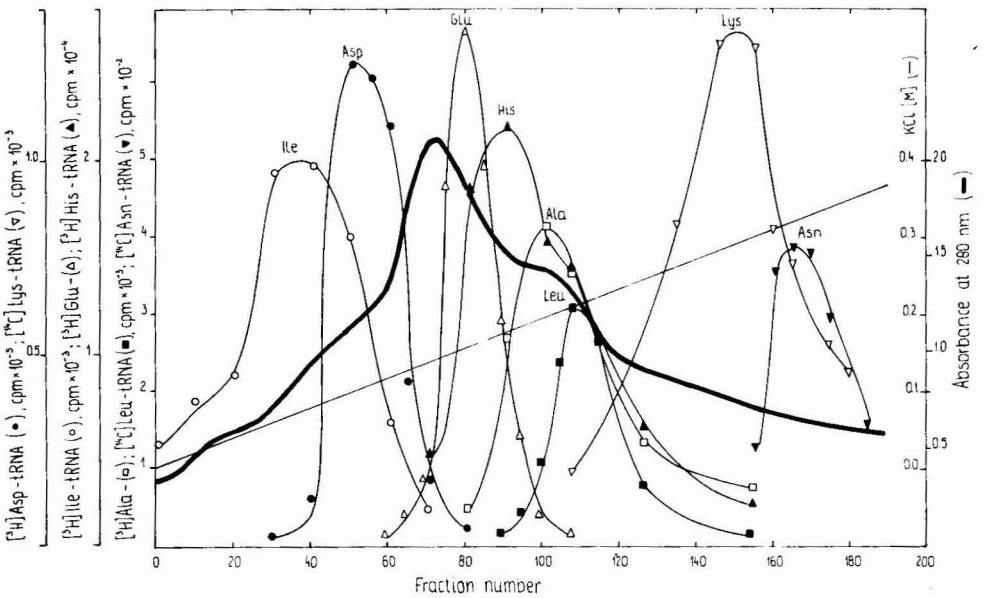

Fig. 2. Chromatography of *E. coli* B aminoacyl-tRNA synthetases on aminohexyl-Sepharose column. The column (5 × 25 cm) was equilibrated with 60 m*M* potassium phosphate (pH 6.8), 7 m*M* mercaptoethanol and 10% glycerol. Protein (190 ml; 13.3 g) was applied on to the column and the column was washed with 2900 ml of the buffer. The enzymes were eluted in a 0–0.4 *M* linear potassium chloride gradient in the buffer (total volume 5120 ml). The flow-rate was 320 ml/h and 25-ml fractions were collected. The absorbance at 280 nm (thick curve) and the synthetase activities in 10-μl aliquots of appropriate fractions were determined: isoleucyl- (○), aspartyl- (●), glutaminyl- (△), histidyl- (▲), alanyl- (□), leucyl- (■), lysyl- (▽) and asparaginyl-tRNA synthetase (▼).

could expect, at least with this class of enzymes, that an enzyme with a molecular weight above 150,000 daltons would be eluted from a hydrophobic column later than an enzyme with a molecular weight below 100,000 daltons. This was the case for yellow lupin enzymes[3] and also seems to be true for *E. coli* B enzymes: the aminoacyl-tRNA synthetases with molecular weights below 100,000 daltons are eluted below 0.2 *M* potassium chloride, whereas those with molecular weights above 150,000 daltons are eluted above 0.2 *M* potassium chloride (see Figs. 1 and 2; the molecular weights of *E. coli* aminoacyl-tRNA synthetases are listed in reference 18).

It was shown by Hofstee[19] that the binding behaviour of several proteins on DEAE-agarose is similar to that on certain-*n*-aminoalkyl-Agaroses. Indeed, aminohexyl-Sepharose resembles in some respects DEAE-cellulose (the order of elution of isoleucyl-, arginyl-, valyl- and leucyl-tRNA synthetases is the same for both adsorbents), but not in others (lysyl-tRNA synthetase is eluted after leucyl-tRNA synthetase from aminohexyl-Sepharose but before it on DEAE-cellulose). It is reasonable to propose that different features of proteins are engaged in binding to DEAE-cellulose (mainly ionic charges) and aminohexyl-Sepharose (mainly hydrophobic pockets).

The purification factors for the 14 aminoacyl-tRNA synthetases lie in the range 3–94-fold, and for 10 enzymes the purification factor is at least 10-fold (Table I). The recoveries of the enzymatic activity were 30–80%, depending on the enzyme. These results clearly illustrate the usefulness of aminohexyl-Sepharose in the purifica-

TABLE I

PURIFICATION OF AMINOACYL-tRNA SYNTHETASES FROM *E. COLI* B ON AMINOHEXYL-SEPHAROSE COLUMNS

*Aminoacyl-tRNA synthetase for**	*Specific activity of aminoacyl-tRNA synthetases (units/mg)*		*Purification factor*
	40–60% $(NH_4)_2SO_4$ satn.	*Aminohexyl-Sepharose*	
Gln	1.3	26	20
Ile	0.16	15	93.8
Asp	1.0	28	28
Tyr	1.2	7	5.8
Arg	3.4	18	5.3
Glu	0.13	2	15.4
His	0.2	2	10
Ala	0.7	2	2.9
Met	2.1	11	5.2
Val	4.2	53	12.6
Phe	1.6	16	10
Leu	2.6	55	21.2
Lys	5.2	130	25
Asn	1.2	15	12.5

* Aminoacyl-tRNA synthetases are listed in order of elution.

tion of *E. coli* B aminoacyl-tRNA synthetases. Combined with classical procedures for the separation of proteins, hydrophobic chromatography on aminohexyl-Sepharose could considerably improve the purification of aminoacyl-tRNA synthetases. The recovery of pure arginyl-tRNA synthetase from *E. coli* B was increased 4–5-fold by the introduction of aminohexyl-Sepharose column chromatography into the original purification procedure[20].

The operation of aminohexyl-Sepharose column is very convenient, and it can be regenerated simply by washing with 1 *M* sodium chloride solution. The flow-rates are about one column volume per hour.

In conclusion, we consider that aminohexyl-Sepharose is useful for procedures of the purification of aminoacyl-tRNA synthetases from many organisms.

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CHROM. 10,100

CHROMATOGRAPHIC BEHAVIOUR OF NUCLEIC ACID CONSTITUENTS AND OF PHENOLS ON CHITOSAN THIN LAYERS

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SUMMARY

The chromatographic characteristics of several nucleic acid constituents and of 36 phenols have been investigated on mixed layers of powdered chitosan and microcrystalline cellulose, with water, water–methanol mixtures and aqueous salt solutions at different pH values as mobile phases. The behaviour of the phenols was strongly correlated with the form in which these compounds were present in solution and therefore with the pH of the eluent. Chitosan was more effective than PEI- and DEAE-cellulose as adsorbent in separating phenols. Analytical applications of chitosan layers are reported.

INTRODUCTION

Chitosan is a β-1,4-linked D-glucosamine polymer prepared by deacetylating the natural polysacharide chitin; it was widely employed by Muzzarelli[1] in studies on inorganic ions by column chromatography. Recently, however, chitosan has been used for the separation of nucleic acid constituents by thin-layer chromatography[2,3], layers of microcrystalline cellulose impregnated with chitosan formate being used.

In this paper, layers of powdered chitosan directly mixed with microcrystalline cellulose have been used, and, in order to compare the characteristics of these layers with those of microcrystalline cellulose (alone or impregnated with chitosan formate), we investigated the behaviour of some nucleic acid constituents; the work was then extended to phenols.

EXPERIMENTAL

The layers of chitosan (300 μm thick) were prepared by mixing 4 g of finely powdered chitosan (100–200 mesh), prepared from chitin according to the deacetylation procedures of Broussignac[4], with 8 g of microcrystalline cellulose in 50 ml of water. Layers of microcrystalline cellulose alone were prepared from 9 g of cellulose in

50 ml of water, and those of microcrystalline cellulose impregnated with chitosan formate were prepared as described by Nagasawa *et al.*[2], using a 0.8% (w/v) solution of chitosan in 0.5% (w/v) formic acid.

The solutions of the test compounds were prepared in water or in water–organic solvent mixtures; such solutions, if required, were neutralized with ammonia in order to prevent gelation of the powdered chitosan layers, which occurs in acid solution. For this reason, acid solutions were not used as mobile phases; indeed, with such mobile phases, layers of microcrystalline cellulose impregnated with chitosan formate exhibit poor reproducibility and irregularities at the solvent front.

The chromatograms were developed by the ascending technique at 25°, and the migration distance was 11 cm unless otherwise stated. The nucleic acid constituents were detected by exposure to UV radiation; phenols were detected by the Boute reaction[5].

RESULTS AND DISCUSSION

Nucleic acid constituents

Table I shows the R_F values of many nucleic acid constituents on development with 0.25 *M* ammonia buffer and with water–methanol (1:1, v/v) on layers of (a) microcrystalline cellulose, (b) microcrystalline cellulose impregnated with chitosan formate, and (c) microcrystalline cellulose mixed with powdered chitosan. With the ammoniacal mobile phase, there is no important difference in the behaviour of the compounds on the three layers; this demonstrates that the addition of chitosan or chitosan formate does not appreciably affect the retention properties of microcrystalline cellulose. Under such conditions of development, the chromatographic data of Nagasawa *et al.*[2] for nucleic acid constituents must be ascribed to the presence on the layers of microcrystalline cellulose and not to chitosan formate.

During development with water–methanol, the nucleosides and nucleic acid bases behave similarly on the three layers, except for xanthine and xanthosine, which are retained to a greater extent on the layers containing chitosan. The behaviour of these two compounds is ascribed to the fact that their acid character is more marked than that of the parent compounds, as evidenced by the pK_a values shown in Table I.

With this developing solvent, the behaviour of the nucleotides on layers containing chitosan (R_F between 0.00 and 0.06) is very different from that on microcrystalline cellulose alone (R_F 0.81 to 0.85). Such behaviour is ascribed to the presence in nucleotide molecules of one or more phosphate groups, which react (probably through an ion-exchange mechanism) with the functional groups of the exchanger. The strong retention of these compounds on chitosan layers can be used analytically for their separation from nucleosides and nucleic acid bases.

The behaviour of xanthine and xanthosine on layers containing chitosan (with water–methanol as mobile phase) shows that layers with powdered chitosan exhibit stronger retention than those impregnated with chitosan formate. This different retention can be ascribed to the different form of the anion exchanger and to the higher content of chitosan in the mixed layer than in the impregnated one.

Phenols

Aqueous eluents. Table II shows the R_F values of 36 phenols on layers of micro-

TABLE I

R_F VALUES FOR NUCLEOSIDES, NUCLEOTIDES AND NUCLEIC ACID BASES ON LAYERS OF MICROCRYSTALLINE CELLULOSE (a) ALONE, (b) IMPREGNATED WITH CHITOSAN FORMATE AND (c) MIXED WITH POWDERED CHITOSAN

Compound	*Mobile phase*						pK_a *of enol form*
	NH_4Cl *(0.25 M)* + NH_3 *(0.25 M)*			H_2O–*methanol (1:1, v/v)*			
	a	*b*	*c*	*a*	*b*	*c*	
Xanthine	0.54	0.58	0.56	0.46	0.51	0.35	7.4
Xanthosine	0.74	0.72	0.71	0.82	0.33	0.04	5.75
Xanthosine monophosphate	0.92	0.90	0.96	0.81	0.00	0.00	—
Xanthosine diphosphate	0.93	0.90	0.96	0.85	0.00	0.00	—
Xanthosine triphosphate	0.93	0.83	0.96	0.82	0.00	0.00	—
Thymine	0.76	0.78	0.78	0.75	0.70	0.77	9.8
Thymidine	0.90	0.86	0.86	0.82	0.77	0.83	—
Thymidine monophosphate	0.97	0.93	0.96	0.85	0.04	0.06	—
Guanine	0.39	0.39	0.40	0.41	0.44	0.44	9.2
Guanosine	0.64	0.60	0.60	0.57	0.53	0.52	9.16
Guanosine monophosphate	0.85	0.84	0.91	0.82	0.00	0.00	—
Hypoxanthine	0.57	0.55	0.58	0.57	0.57	0.57	8.9
Inosine	0.78	0.75	0.77	0.64	0.65	0.63	8.75
Inosine monophosphate	0.93	0.90	0.96	0.85	0.00	0.00	—
Adenine	0.34	0.35	0.37	0.50	0.56	0.57	9.8
Adenosine	0.53	0.53	0.52	0.52	0.56	0.59	—
Adenosine monophosphate	0.79	0.81	0.85	0.84	0.00	0.00	—
Adenosine diphosphate	0.79	0.83	0.87	0.85	0.00	0.00	—
Adenosine triphosphate	0.85	0.76	0.93	0.85	0.00	0.00	—
Cytosine	0.75	0.73	0.75	0.65	0.67	0.73	12.2
Cytidine	0.82	0.82	0.80	0.70	0.65	0.72	—
Uracil	0.76	0.74	0.75	0.73	0.73	0.73	9.5
Uridine	0.90	0.84	0.88	0.78	0.72	0.73	9.17

crystalline cellulose and on cellulose–powdered chitosan layers, with 0.1 *M* ammonium acetate or 0.1 *M* sodium bicarbonate as mobile phase. The results indicate that chitosan exhibits a higher retention power and a better selectivity towards the phenols.

On both layers, development with 0.1 *M* ammonium acetate leads to diffuse spots for many compounds, (including phenol), so that their detection is impossible. As the pH of the mobile phase is increased, the quality of the spots improves, and only phenol and *m*-cresol cannot be detected. On development with sodium bicarbonate, however, the number of polyhydric phenols giving rise to elongated spots increases, probably because of the easier oxidizability of such compounds as the pH on the layer is increased. As regards the chromatographic behaviour of the phenols on chitosan layers with the two mobile phases, a general increase in R_F value is observed on changing from ammonium acetate to sodium bicarbonate, except for those phenols of less marked acid character ($pK_a \geqslant 8.7$), which exhibit similar R_F values with the two developing solvents. The chromatographic behaviour of the phenols on the cellulose–powdered chitosan layers seems to depend on the pH value of the mobile phase and on the acid–base character of the compounds. For the dichlorophenols, with ammonium acetate (pH = 7.1) as mobile phase, only 2,6-dichlorophenol ($pK_a = 6.79$) ex-

TABLE II

R_F VALUES FOR PHENOLS ON LAYERS OF MICROCRYSTALLINE CELLULOSE (a) ALONE AND (b) MIXED WITH POWDERED CHITOSAN

n.d. = Not determined; e.s. = elongated spot.

Compound	*Mobile phase*						pK_a*
	CH_3COONH_4 (0.1 M)		$NaHCO_3$ (0.1 M)	H_2O–methanol (v/v)			
				4:1		1:1	
	a	*b*	*b*	*a*	*b*	*b*	
Phenol	n.d.	n.d.	n.d.	0.83	0.78	0.93	9.99
Catechol	0.79	e.s.	0.62	0.82	e.s.	e.s.	9.85
Resorcinol	0.79	0.58	0.61	0.82	0.60	0.77	9.81
Hydroquinone	0.80	0.62	e.s.	0.81	0.62	0.79	10.35
Pyrogallol	e.s.	e.s.	e.s.	e.s.	e.s.	e.s.	9.01
Phloroglucinol	0.65	0.60	e.s.	0.75	e.s.	0.65	8.45
Gallic acid	0.74	n.d.	e.s.	0.87	0.01	0.03	4.41
Pyrocatechuic acid	0.80	0.32	0.66	0.88	0.01	0.03	—
m-Cresol	n.d.	n.d.	n.d.	n.d.	n.d.	0.90	10.09
p-Nitrophenol	0.64	0.36	0.61	0.75	0.14	0.42	7.16
o-Nitrophenol	n.d.	0.49	0.71	n.d.	n.d.	0.52	7.23
m-Nitrophenol	0.71	0.50	0.63	0.76	0.50	0.76	8.40
2,4-Dinitrophenol	0.62	0.25	0.53	0.93	0.02	0.04	4.09
2,6-Dinitrophenol	0.75	0.34	0.64	0.94	0.02	0.03	3.71
2,5-Dinitrophenol	0.64	0.30	0.58	0.90	0.02	0.03	5.22
Picric acid	0.68	0.21	0.50	0.90	0.01	0.02	0.60
5-Aminosalicylic acid	0.79	0.49	0.72	0.93	0.03	0.04	2.74
4-Aminosalicylic acid	0.79	0.31	0.65	0.92	0.03	0.03	1.7
2-Amino-5-nitrophenol	0.43	0.28	0.36	0.52	0.31	0.61	—
2-Amino-4-nitrophenol	0.52	0.28	0.49	0.62	0.11	0.37	—
4-Amino-2-nitrophenol	0.60	0.46	0.61	0.66	0.43	0.68	—
2-Amino-4,6-dinitrophenol	0.49	0.14	0.34	0.88	0.00	0.01	—
o-Chlorophenol	n.d.	n.d.	0.63	n.d.	n.d.	0.83	8.48
p-Chlorophenol	0.72	0.55	0.52	0.79	0.62	0.83	9.38
m-Chlorophenol	0.70	0.55	0.55	0.76	0.61	0.83	9.02
p-Bromophenol	0.65	0.48	0.47	0.77	0.56	0.81	8.87
o-Bromophenol	n.d.	n.d.	0.60	n.d.	n.d.	0.81	8.42
2,5-Dichlorophenol	n.d.	0.35	0.58	n.d.	0.28	0.67	7.35
3,4-Dichlorophenol	0.57	0.34	0.45	0.69	0.45	0.79	8.39
2,4-Dichlorophenol	n.d.	n.d.	0.56	0.69	0.37	0.77	7.75
2,3-Dichlorophenol	0.58	0.36	0.57	0.70	0.31	0.71	7.45
2,6-Dichlorophenol	n.d.	0.40	0.65	n.d.	0.14	0.49	6.79
3,5-Dichlorophenol	0.58	0.34	0.50	0.69	0.39	0.77	7.93
1-Naphthol	0.50	0.26	0.27	0.62	0.35	0.76	9.34
2-Naphthol	0.46	0.25	0.25	0.58	0.37	0.78	9.51
Naphthalene-1,5-diol	0.35	0.16	0.16	0.51	0.20	0.58	—

* See ref. 6.

hibits an R_F value higher than those of the other isomers, whereas with sodium bicarbonate (pH = 8.5) the R_F values of the isomers increase with increasing pK_a value in accordance with the differing degrees of deprotonation of these compounds[6].

As regards the mechanism governing the retention of the phenols, and keep-

ing in mind that chitosan is a weak base[1] (and certainly the free-base form predominates at the pH of sodium bicarbonate solution), it follows that interactions between the primary amino group of chitosan and the phenolic hydroxyl groups are the parameters determining the retention.

Aqueous-organic eluents. Table II also shows the R_F values of phenols on layers of microcrystalline cellulose with water–methanol (4:1, v/v) as mobile phase and on layers of cellulose–powdered chitosan with similar mixtures for development.

As has already been pointed out for nucleic acid constituents, the use of aqueous-organic eluents increases the retention power of the chitosan layers. On microcrystalline cellulose, the phenols exhibit high R_F values, independent of their pK_a values and of substituents in the ring. On chitosan layers, however, the phenols are strongly retained and their chromatographic behaviour is correlated with both their pK_a values and the type and number of substituents in the ring. As regards the influence of the substituents in the ring on the chromatographic behaviour of the phenols, it should be noted that polyhydric phenols are retained more than phenol itself, despite their pK_a values, which in some instances (resorcinol and hydroquinone) are similar to, and in others (phloroglucinol) lower than, that of phenol. These compounds can give rise to hydrogen bonds with two or more of the functional groups of chitosan and therefore may be expected to be retained more than phenol.

The introduction into the ring of substituents that markedly increase the acid character of a phenol (*e.g.*, nitro, chloro or bromo groups), produces a decrease in R_F value.

Increase in the methanol concentration in the mobile phase leads to a general increase in R_F values and the production of more compact spots for polyhydric

TABLE III

SEPARATIONS ON LAYERS OF MICROCRYSTALLINE CELLULOSE MIXED WITH POWDERED CHITOSAN

Migration distance = 12.5 cm.

Mixture	*Mobile phase*	R_F
4-Amino-2-nitrophenol	water–methanol (4:1, v/v)	0.41
2-Amino-5-nitrophenol		0.30
2-Amino-4-nitrophenol		0.11
2-Amino-4,6-dinitrophenol		0.00
m-Nitrophenol	water–methanol (1:1, v/v)	0.72
o-Nitrophenol		0.50
p-Nitrophenol		0.41
5-Aminosalicylic acid	CH_3COONH_4 (0.1 *M*)	0.47
4-Aminosalicylic acid		0.30
o-Chlorophenol	$NaHCO_3$ (0.1 *M*)	0.60
p-Chlorophenol		0.50
o-Bromophenol	$NaHCO_3$ (0.1 *M*)	0.58
p-Bromophenol		0.46
3,4-Dichlorophenol	water–methanol (4:1, v/v)	0.44
2,4-Dichlorophenol		0.36
2,5-Dichlorophenol		0.28
2,6-Dichlorophenol		0.14
1- or 2-Naphthol	water–methanol (4:1, v/v)	0.34–0.36
Naphthalene-1,5-diol		0.19

phenols; indeed, some compounds impossible to detect with aqueous salt solutions as mobile phase can be detected in this system.

Comparison of results on chitosan layers with those obtained on cellulose-based anion exchangers (PEI- and DEAE-cellulose)[7] shows that, generally, chitosan exhibits a higher retention power and a better selectivity towards the phenols than do the exchangers cited. The R_F values on chitosan layers with water–methanol (4:1) as mobile phase are appreciably lower than those on PEI-cellulose with water as developer[7]. Further, on chitosan, mono-, di- and trihydric phenols and the three nitrophenols can be separated (see Table II); such separations cannot be achieved on PEI-cellulose, DEAE-cellulose or microcrystalline cellulose.

ANALYTICAL APPLICATIONS

Many separations of phenols can be achieved on the basis of their R_F values with aqueous and alcoholic–aqueous eluents; some are reported in Table III. Also, separations of nitro and polynitrophenols, and chloro and dichlorophenols, are possible.

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CHROM. 10,130

DANSYL HYDRAZINE AS A FLUORIMETRIC REAGENT FOR THIN-LAYER CHROMATOGRAPHIC ANALYSIS OF REDUCING SUGARS

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SUMMARY

Reducing sugars, particularly aldoses, react readily with dansyl hydrazine. The fluorescent hydrazones produced can be separated by thin-layer chromatography and determined quantitatively by spectrofluorimetry after elution from the chromatograms.

INTRODUCTION

Dansyl hydrazine [(1-naphthalenesulfonyl-5(dimethylamino)-hydrazide] has been suggested as a fluorimetric reagent for the analysis of ketosteroids[1,2]. It has been applied as a staining reagent for periodate oxidized glycoproteins separated by acrylamide gel electrophoresis[3,4]. In the present communication we report the use of dansyl hydrazine for the fluorimetric analysis of reducing sugars employing thin-layer chromatographic (TLC) procedures. The method described here for sugars is similar in principle to the many, commonly employed procedures available for the analysis of amino acids and other amines which use dansyl chloride as the fluorimetric reagent[5–20].

MATERIALS AND METHODS

Materials

D-[^{14}C(U)]Glucose was obtained from New England Nuclear (Boston, Mass., U.S.A.) and diluted to give a stock solution of 10 m*M* glucose with a specific activity of 0.2 μCi/μmole. Dansyl hydrazine was purchased from Pierce (Rockford, Ill., U.S.A.). Other chemicals used were obtained from Fisher (King of Prussia, Pa., U.S.A.) and Sigma (St. Louis, Mo., U.S.A.). TLC was carried out on Eastman-Kodak No. 13179 silica gel sheets and on Anasil G plates (Analabs, North Haven, Conn., U.S.A.). Fluorescence was measured in a spectro-fluorimeter[21] equipped with a 300-W xenon lamp and using cuvettes with a 1.0-cm light path. Radioactivity was measured with 87% efficiency for counting ^{14}C using the Aquasol liquid scintillation cocktail (New England Nuclear).

Reagents

Reagents used were: 4% (w/v) trichloroacetic acid, 1% dansyl hydrazine in ethanol, 1% (v/v) acetic acid in ethanol, 10 m*M* D-glucose (or other reducing sugar) stock solution.

Procedure

A 100-μl sample containing 0.04–2 μmoles reducing sugar is mixed with 100 μl trichloroacetic and 200 μl dansyl hydrazine solutions. The mixture is heated for 10 min at 80°, then cooled to room temperature. The same procedure can also be performed using smaller samples (2–10 μl) of test solution, keeping the same proportions of reagents as described above. A control tube containing no sugar is used as a reference system to aid in the detection of dansyl hydrazine and its degradation products.

Samples of the reaction mixture (2–10 μl, containing 2–100 nmoles sugar) are applied to starting points on silica gel plates and then developed by standard TLC procedures using solvents as described in Table I. After development and drying at room temperature, the plates are illuminated with a long-wave ultraviolet lamp. Sugar hydrazones appear as intensely fluorescent bright yellow spots. Dansyl hydrazine itself appears as a yellow fluorescent spot which is usually well separated from the

TABLE I

MOBILITY OF SOME SUGAR DANSYL HYDRAZONES ON TLC PLATES

Numbers indicate R_F values obtained on Anasil-G plates. Mobilities on Eastman-Kodak silica gel sheets differed only slightly from those reported here. Solvent systems: I, Benzene–pyridine–acetic acid (16:4:1); II, toluene–triethylamine–acetic acid (13:5:2); III, chloroform–ethylacetate–1% boric acid in methanol (3:5:2); IV, chloroform–*tert.*-butanol–acetic acid (5:4:1); V, benzene–chloroform–1% sulfamic acid in dimethylformamide (5:3:2).

Compound	R_F				
	I	*II*	*III*	*IV*	*V*
Maltose	0.01	0.11	0.15	0.08	0.20
Melibiose	0.01	0.09	0.10	0.06	0.12
D-Glucose	0.17	0.21	0.42	0.36	0.42
D-Galactose	0.13	0.19	0.27	0.29	0.38
D-Mannose	0.15	0.20	0.36	0.35	0.40
2-Deoxy-D-glucose	0.23	0.26	0.53	0.49	0.61
D-Fructose	0.23	0.23	0.46	0.50	0.54
L-Sorbose	0.25	0.25	0.50	0.60	0.47
D-Xylose	0.35	0.27	0.62	0.54	0.59
L-Arabinose	0.30	0.25	0.52	0.47	0.54
D-Ribose	0.41	0.32	0.59	0.52	0.66
L-Rhamnose	0.40	0.35	0.63	0.64	0.62
D-Fucose	0.42	0.36	0.58	0.56	0.65
2-Amino-2-deoxy-D-glucose	0	0.28	0.45	0.20	0
2-N-Acetylamido-2-deoxy-D-glucose	0.07	0.16	0.41	0.30	0.24
N-Acetylneuraminic acid	0	0.14	0	0.10	0.19
D-Glucuronic acid	0	0.14	0	0.04	0.23
DL-Glyceraldehyde	0.52	0.44	0.67	0.77	0.68
Dansyl hydrazine	0.86	0.56	0.94	0.96	0.98
Dansyl-OH	0.10	0.37, 0.51	0.26, 0.22	0.35, 0.18	0.25

hydrazones. A small amount of degradation products of dansyl hydrazine, mostly dansyl sulfonic acid (dansyl-OH), appear as green-blue fluorescent spots[5], easily distinguishable from the dansylhydrazones.

Elution and quantitative analysis

The yellow fluorescent spots are scraped off the plate (or cut out when Eastman-Kodak sheets are used) into a standard conical centrifuge tube. The hydrazones are then eluted by 10 min shaking with 2 ml of 1% acetic acid in ethanol. Particles are removed by centrifugation in a desk-top clinical centrifuge. The supernatant is measured in the spectrofluorimeter using the wavelength of 360 nm for exitation and 510 nm for emissions. If a radioactive sugar is used, a sample (0.1–1.0 ml) of this supernatant is counted by scintillation spectrometry.

RESULTS AND DISCUSSION

Optimal conditions for the formation of glucose dansyl hydrazone were worked out so as to formulate the analytical procedure described above. The optimum pH range for the reaction was found to be between 2 and 3. The rate of hydrazone formation at less acidic solutions was appreciably slower. Acetic acid (0.1 *M*) could substitute trichloroacetic acid in the reaction mixture. Hydrazone formation was found to be complete within less than 10 min when the system was heated up to 80°. The sugar dansyl hydrazones were relatively stable in the acidic solution and could be chromatographically and fluorimetrically analyzed with very little loss of fluorescence intensity also after 48 h if kept in the dark. The lowest limit of visual detection of the fluorescent spot on the TLC plate was found to be in the range of 1–2 nmoles glucose dansyl hydrazone. This is similar to the level of sensitivity described for the detection of dansyl amino acids on TLC plates[5,6,8]. The lowest level for the quantitative fluorimetric determination of the glucose dansyl hydrazone in solution after elution of the spot from the chromatographic plate was between 2 and 4 nmoles. This level of detection can most probably be lowered significantly through the use of a more sensitive instrumentation suitable for the fluorimetric analysis of smaller volumes than those used in the present study.

The results presented in Fig. 1 indicate that the procedure can be conveniently employed for the quantitative analysis of glucose. The use of [^{14}C]glucose in this experiment provided an internal control which indicated that about 90% of the glucose present was converted to its hydrazone under the assay condition described.

Many other reducing sugars yielded dansyl hydrazones with discrete mobilities which could be detected by TLC (Table I). The rate and yield of hydrazones formed with free aldoses was similar to that observed for D-glucose. However, 2-amino-2-deoxy sugars as well as ketoses (*e.g.*, D-fructose and L-sorbose) reacted very poorly with dansyl hydrazine and yielded low levels of hydrazones under the conditions described. Prolonged heating seemed to increase somewhat the yield of these hydrazones. Also, it was noted that if hydrazone formation was conducted at pH 4–6, ketoses and 2-amino-2-deoxy aldoses did not interact significantly with dansyl hydrazine even if heated to 100°, whereas glucose and other aldoses reacted readily under these conditions. A complete and comparative kinetic analysis of the interaction of ketoses, aminosugars and aldoses with dansyl hydroazine has not yet been performed.

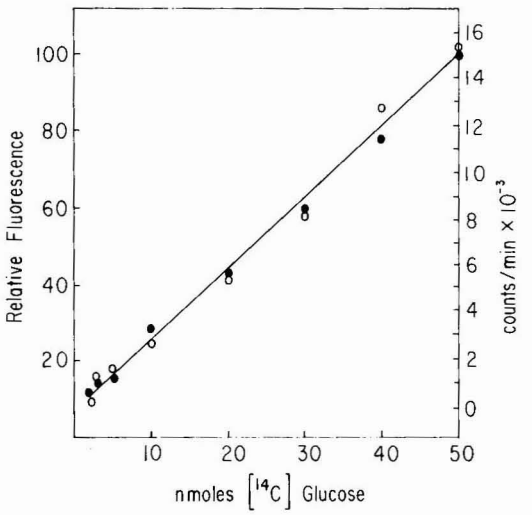

Fig. 1. Relationship between fluorescence intensity and glucose dansyl hydrazone concentration. Samples (10 μl) of reaction mixtures containing the indicated quantities of [^{14}C]glucose ($3 \cdot 10^5$ counts/min·μmole) were analyzed by TLC and subsequent elution as described in the text.

The arsenal of chromatographic, spectrophotometric and enzymatic procedures available to the analyst for the assay of reducing sugars is very large, but only a very limited number of reliable fluorimetric methods are available for this purpose. The method described here does not intend to substitute for any of the popularly employed procedures for the micro determination of reducing sugars in solution. However, it should be considered to be a useful addition which may occasionally provide a simple and helpful method for the detection of submicromolar quantities of sugars, primarily aldoses, in a mixture. Such occasions often exist during the analysis of acid hydrolysates of complex carbohydrates isolated in very small quantities from various biological preparations.

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GAS CHROMATOGRAPHIC INVESTIGATIONS OF THE SYSTEM TRICOBALT TETROXIDE–HYDROGEN

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SUMMARY

The system Co_3O_4–H_2 has been investigated by a gas chromatographic method in the temperature range 325–623 °K. Three intervals, in which the Co_3O_4–H_2 interaction differed, were found, namely, 323–448 °K, in which irreversible adsorption of H_2 on the Co_3O_4 surface predominated, 448–503 (553) °K, in which the reduction of Co_3O_4 began, and 503 (553)–623 °K, in which the reduction of Co_3O_4 continued.

INTRODUCTION

Cobalt oxides are used as catalysts for many contact reactions, and Co_3O_4 is known to be the most active catalyst in reactions involving the deep oxygenation of hydrocarbons[1-3] and of such inorganic compounds as ammonia[4,5] or carbon monoxide[6]. Other important catalytic reactions, carried out with this catalyst are dehydrogenation[7] and isomerisation[8] reactions and the decomposition of hydrogen peroxide[9].

Interactions in the system Co_3O_4–H_2, which are important in studies of the properties of oxygen adsorbed on the catalyst surface as well as oxygen bound in the crystal lattice, have hitherto been investigated mainly by static[10,11] or thermogravimetric[12] methods. A gas chromatograph method has been used only for determination of the irreversible adsorption of H_2 at temperatures from 78 to 293 °K[13].

Here we report gas chromatographic investigations of the system Co_3O_4–H_2 in temperature range 323 to 623 °K.

EXPERIMENTAL

Preparation of catalysts

Cobalt hydroxide, precipitated by aqueous ammonia from a solution of cobalt nitrate, was used as the starting material for all the oxides investigated. The series of catalysts was obtained by heating this cobalt hydroxide at 573, 673, 773, 873 or 973 °K in the presence of air; at these temperatures, decomposition of cobalt hydroxide was complete.

The specific surface areas of each oxide obtained was determined by the BET

method for the adsorption of krypton at 78 °K; for sintering temperatures of 573, 673, 773, 873 and 973 °K, the respective specific surface areas were 22.6, 15.5, 8.1, 5.7 and 2.0 m^2/g.

Studies showed that all the oxides had the structure of spinel, and derivatograms indicated that, in the temperature range under study, the oxides did not undergo any structural changes.

Methods

In this work, a gas chromatograph (ISCO 571 A) equipped with a thermal-conductivity detector was used, and the carrier gas was argon containing less than 20 ppm of oxygen. Oxygen was removed from the carrier gas by copper–alumina and manganese oxide–alumina catalysts[14,15], and moisture was removed by molecular sieve 5A. Electrolytic hydrogen was purified from traces of oxygen by means of a palladium–silica catalyst and dried on molecular sieve 5A.

Tablets prepared from powders of the oxides under test were crushed, and the size fraction 0.14–0.4 mm was used; 6–10 g of the sample were placed in a stainless-steel tube. Before the experiment, the oxide was standarised by heating at 573 °K for 2 h in a stream of the carrier gas.

Hydrogen was introduced into the column by means of a valve of volume 1 ml, and experiments were performed at temperatures in the range 323–623 °K. At higher temperatures in this range, additional chromatographic analysis of the reduction products was carried out with the use of a column of Porapak Q.

RESULTS AND DISCUSSION

Based on the difference in interaction between Co_3O_4 and H_2, the range of temperature investigated can be divided into three intervals, for which the chromatograms are shown in Fig. 1.

The interval 323–448 °K

In this interval, the first portions of H_2 are irreversibly adsorbed, as shown by the lack of peaks on the chromatogram. After a certain amount of H_2 has been adsorbed, further portions are practically not adsorbed (Fig. 1a). Thus, in this temperature range, the predominant process is irreversible adsorption of H_2. The amount of H_2 adsorbed increases with the temperature (Figs. 2 and 3).

A similar phenomenon, but in the temperature range 72–293 °K, was observed by Shigehara and Ozaki[13], who, by static and chromatographic methods, calculated that the amount of H_2 irreversibly adsorbed at 293 °K was 3 ml/100 m^2. In our work, the value measured for Co_3O_4 (sintered at 573 °K; surface area 22.6 m^2/g) changed from 6.4 to 10.8 ml/100 m^2 with increase in temperature from 323 to 448 °K. The amount of H_2 sorbed on oxides sintered at various temperatures (different as far as their specific surface area is concerned) decreases per mass unit of the oxide with increase in the sintering temperature; however, it remained constant per unit of surface area (Fig. 4).

From this, it follows that the process being studied occurs mainly on the surface. It can be used, therefore, for determination of the specific surface area of Co_3O_4

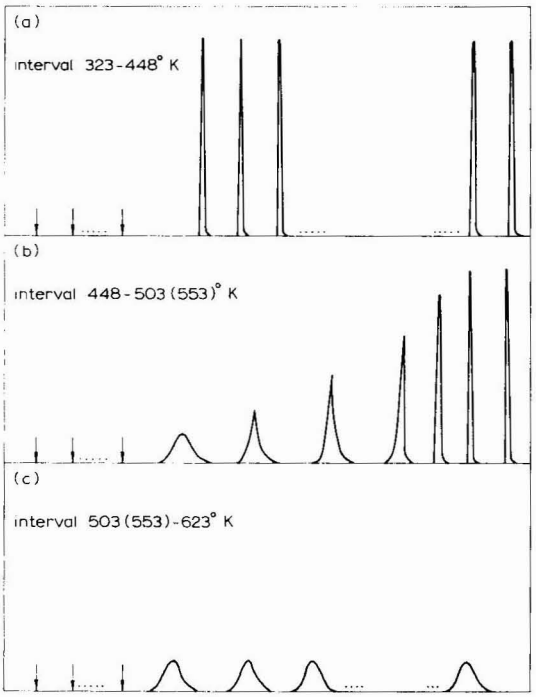

Fig. 1. Chromatograms for the system Co_3O_4–H_2.

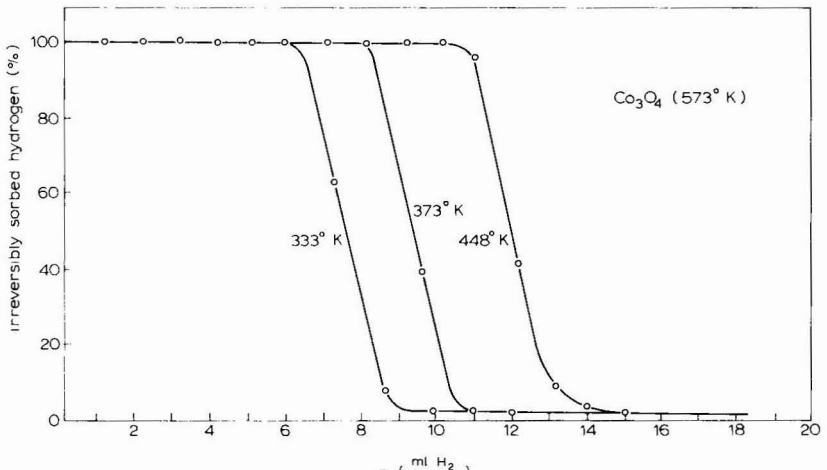

Fig. 2. Amounts (a) of H_2 irreversibly sorbed during introduction of successive volumes of H_2.

by the pulsed-chromatographic method, with H_2 as adsorbate. The probable mechanism of irreversible sorption of H_2 on the surface of the oxide is as follows:

$$-\text{Co}-\text{O}- + \text{H}_2 \rightarrow -\overset{\text{H}\quad\text{H}}{\underset{|\quad|}{\text{Co}}}-\text{O}-$$

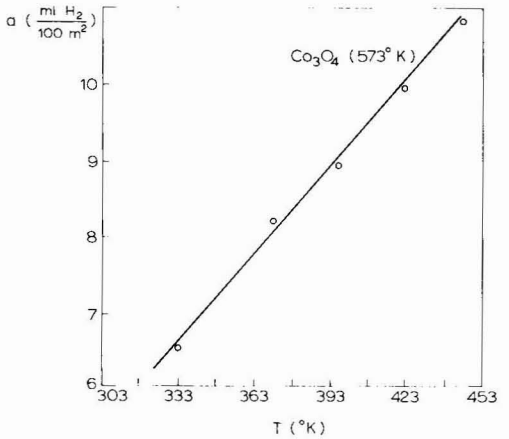

Fig. 3. Dependence of amount (a) of irreversibly sorbed H_2 on temperature.

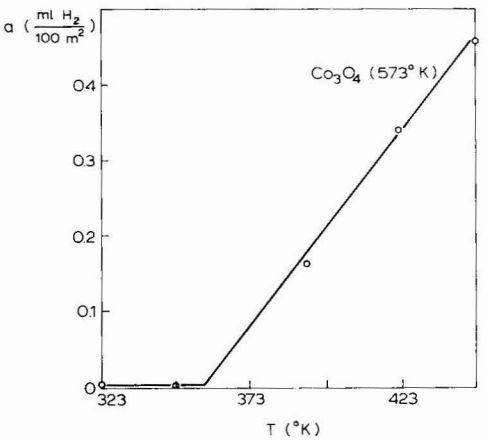

Fig. 4. Dependence of amount (a) of irreversibly sorbed H_2 on temperature after heating of the Co_3O_4 for 1 h at a given temperature.

In order to establish the irreversible character of the sorption in the temperature range under discussion, the test oxide was heated for 1 h at the measurement temperature in the stream of the carrier gas (after the maximum of the H_2 peak had been reached); after this period, H_2 was once more introduced. There was no reproduction of the active adsorption centers at temperatures of 323 and 358 °K, and for temperatures from 373 to 448 °K there was only slight reproduction (2–4% of the H_2 was sorbed, relative to the amount originally adsorbed); this is shown in Figs. 5 and 6.

It is probable that this is due to slight desorption of H_2 or migration of oxygen to the surface of the Co_3O_4 with simultaneous migration of H_2 into the catalyst.

The interval 448–503 (553) °K

In this interval, as in the preceding one, the first portions of H_2 are irreversibly sorbed; after a certain amount of H_2 has been sorbed, a diffuse peak for water appears on the chromatogram (Fig. 1b).

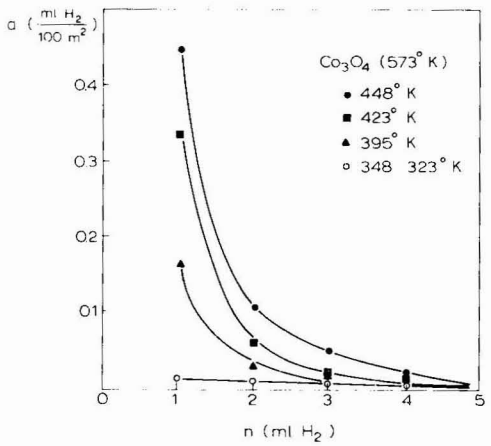

Fig. 5. Dependence of amount (a) of irreversibly sorbed H_2 on succesive volumes (n) of H_2 introduced hydrogen after heating of Co_3O_4 for 1 h at a given temperature.

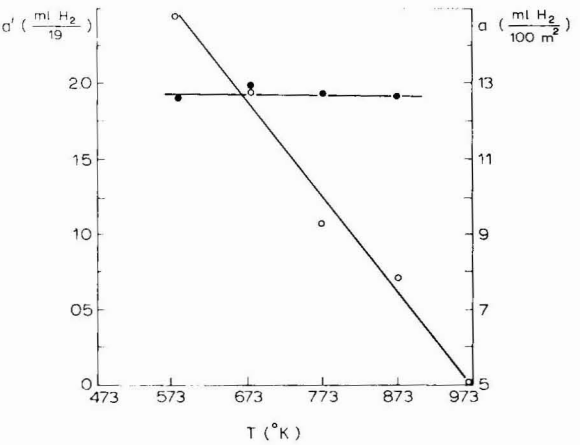

Fig. 6. Dependence of amount of irreversibly sorbed H_2 on sintering temperature (T) of Co_3O_4 per unit of surface (a–●) and mass (a'–○) of Co_3O_4.

With further portions of H_2, the peaks become more and more symmetrical, until eventually each portion gives a H_2 peak of constant height.

Thus, in this temperature range, reduction of oxide begins. The characteristic feature of this interval is the small amount of H_2 used in the reduction of the oxide. It is difficult precisely to assign the temperature at which reduction begins, but it increases with increase in the sintering temperature of the oxide (and thus with the decrease in specific surface area).

It can be assumed that reduction begins at the expense of the most mobile oxygen atoms on the surface of the oxide. This assumption is supported by the fact that for the oxide with a small specific surface area (sintered at 973 °K), reduction begins at a considerably higher temperature (about 553 °K).

The interval 503 (553)–623 °K

In this interval, after a definite amount of H_2 has been sorbed, further portions of H_2 appear on the chromatogram as diffuse peaks for water. Thus, all the H_2 introduced is used for reduction of the Co_3O_4 (Fig. 1c), which is the predominant reaction. The characteristic feature of this interval is the rapid reproduction of the active centres on the surface of the oxide. This can be explained by the diffusion of oxygen from the crystal lattice to the surface of the oxide, where it is liberated because the equilibrium of the reaction

$$2Co^{3+} + O^{2-} \rightleftharpoons 2Co^{2+} + O$$

is displaced to the right.

In this reaction, electron exchange is followed by a change in valency of the ions, which, in turn, is connected with the change in volume. This increase in temperature is followed by the formation of Co^{2+} ions, which have a radius greater than that for Co^{3+} ions, and the formation of oxygen atoms, with a radius significantly smaller than that of O^{2-} ions. The liberated oxygen atoms diffuse towards the surface of the oxide.

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Journal of Chromatography, 139 (1977) 355–363

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THIN-LAYER CHROMATOGRAPHIC BEHAVIOUR OF METALS ON DEAE-CELLULOSE IN OXALIC ACID AND MIXED OXALIC ACID–HYDROCHLORIC ACID MEDIA

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SUMMARY

The adsorption behaviour of 46 metals on layers of DEAE-cellulose has been examined in aqueous oxalic acid media. R_F values are given as a function of oxalic acid concentration over the range 0.010–0.30 *M* and are compared with those obtained in a similar manner with Avicel SF. R_F data are also presented for 48 metals in oxalic acid–hydrochloric acid mixtures, where the oxalic acid concentration was kept constant at either 0.030 or 0.25 *M* and that of hydrochloric acid was varied from 0.010 to 3.0 *M*. Pronounced ion exchange takes place in oxalic acid media for many metals, but a limited number of metals are adsorbed on DEAE-cellulose from the mixed acid media. The versatility of the chromatographic system investigated is demonstrated by many multi-component separations conducted without marked tailing.

INTRODUCTION

There have been few systematic studies on the behaviour and separation of metals on anion-exchange resins in oxalic acid, oxalate and oxalic acid–mineral acid mixtures. De Corte *et al.*[1] determined the distribution coefficients of 12 elements on Dowex 1 in oxalic acid medium (0.001–0.98 *M*) and developed a scheme for the chromatographic separations of As(III), Mn(II), Co(II), Zn(II) and Cu(II); Na(I), Mn(II) and Zn(II); and Ce(III) and Lu(III). Strelow *et al.*[2] measured the anion-exchange distribution coefficients with Bio-Rad AG-1 for 36 elements in oxalic acid–hydrochloric acid mixtures and presented elution curves for multi-component systems. Scattered information on the ion-exchange separation of metals in oxalic acid, oxalate and oxalic acid–mineral acid mixtures is also available[3–12].

This work was undertaken in order to investigate the adsorption characteristics of metals on the anion exchanger DEAE-cellulose (henceforth abbreviated to DEAE) in aqueous oxalic acid and oxalic acid–hydrochloric acid mixtures by thin-layer chromatography. The chromatographic systems investigated permit many useful separations of analytical interest to be conducted effectively on DEAE.

EXPERIMENTAL

Stock solutions of metals

Metal stock solutions (0.1 *M*) were prepared as described previously[13]. The solutions of platinum metals (3 *M* hydrochloric acid) except for Pt(IV) were aged for 2 years after their preparation. The stock solution of Pt(IV) was allowed to stand for 2 months before use. The aged stock solutions of Ru(III) and Rh(III) were chromatographed on a DEAE(Cl^-) layer with 3 *M* hydrochloric acid, each yielding two spots: R_F 0.52 and 0.76 for Ru(III) and R_F 0.00 and 0.78 for Rh(III).

Preparation of thin-layer plates

DEAE (Serva, Heidelberg, G.F.R., for TLC) was used as adsorbent. For comparison purposes the microcrystalline cellulose Avicel SF (F.M.C., Marcus Fook, Pa., U.S.A.) was also used as adsorbent.

DEAE (oxalate form) plates. About 13 g of DEAE were washed with 500-ml portions of 0.002 *M* sodium hydroxide solution by centrifugation until the supernatant liquid was free from chloride ions. DEAE was then washed once with deionized water. The DEAE was mixed with 200 ml of 0.2 *M* oxalic acid and stirred for 5 min, and finally washed with deionized water by centrifugation until the pH of the supernatant liquid was 3. The DEAE (oxalate form) thus prepared was slurried with 28 ml of deionized water and spread in 250-μm thick layers on five 20 $\times$ 20 cm glass plates. The plates were dried in air for 1 h and then at 40° for about 3 h, and were stored in a desiccator over saturated potassium bromide solution.

DEAE (chloride form) plates. About 13 g of DEAE were slurried with 200 ml of 1 *M* ammonium chloride solution, adjusted to pH 1 with hydrochloric acid, and stirred mechanically for 5 min. The DEAE then was washed with 200 ml of 0.1 *M* hydrochloric acid, and finally with deionized water until the pH of the supernatant liquid was 3; after each wash the aqueous phase was removed by centrifugation. DEAE in the chloride form was slurried with 28 ml of deionized water and spread in 250-μm thick layers on five 20 $\times$ 20 cm glass plates. The plates were dried and stored as above.

Avicel SF plates. After being washed with deionized water by centrifugation, about 18 g of Avicel SF was slurried with 30 ml of deionized water and spread in 250-μm thick layers on five 20 $\times$ 20 cm glass plates. The plates were dried and stored as for the DEAE plates.

Development

The metals (0.5 μl of stock solution) were applied to the layers and developed in rectangular glass tanks with ground-glass lids after equilibration for 1 h. The solvent front was allowed to rise 15 cm from the start. The solvents used were aqueous oxalic acid (0.010, 0.030, 0.10 and 0.30 *M*) and mixed solutions (0.030 or 0.25 *M* in oxalic acid and 0.010–3.0 *M* in hydrochloric acid). The DEAE (oxalate form) layers were used in the oxalic acid system and the DEAE (chloride form) layers in the mixed oxalic acid–hydrochloric acid system.

The metals were detected as described previously[14]. The R_F values reported are the averages of duplicate or triplicate determinations.

RESULTS AND DISCUSSION

In the oxalic acid and oxalic acid–hydrochloric acid media the acid fronts generally do not move to the liquid front. In Table I(A) are summarized the locations of the oxalic acid fronts on DEAE and Avisel SF in oxalic acid media. In addition to the oxalic acid front a hydrochloric acid front appeared in dilute mixed acid media and is indicated for each mixture in Table I(B).

TABLE I

OXALIC ACID AND HYDROCHLORIC ACID FRONTS ON DEAE AND AVICEL SF

(A) Oxalic acid (HOx) media

Solvent system	*Location of HOx front (R_F)*	
	DEAE	*Avicel SF*
0.01 *M* HOx	0.25	0.63
0.03 *M* HOx	0.40 (0.48)*	0.79
0.10 *M* HOx	0.63 (0.64)*	0.81
0.30 *M* HOx	0.78 (0.80)*	0.83

(B) Oxalic acid (HOx)–HCl media

Solvent system	*Location of fronts on DEAE (R_F)*	
	HOx	*HCl*
0.03 *M* HOx–0.01 *M* HCl	0.38	0.89
–0.1 *M* HCl	0.67	0.95
–0.5 *M* HCl	0.80	1.00
–1.0 *M* HCl	0.81	1.00
–3.0 *M* HCl	0.90	1.00
0.25 *M* HOx–0.01 *M* HCl	0.78	1.00
–0.1 *M* HCl	0.80	1.00
–0.5 *M* HCl	0.83	1.00
–1.0 *M* HCl	0.83	1.00
–3.0 *M* HCl	0.90	1.00

* Values in parentheses indicate average R_F values of the fronts which appeared when acidic sample solutions (mostly 0.5 μl of 3 *M* HCl) had been applied.

In Fig. 1, R_F values of 46 metals on DEAE (oxalate) are given as a function of oxalic acid concentration. R_F values on Avicel SF are also illustrated for comparison. It can be seen that most metals are not retained on Avicel SF to an appreciable extent from oxalic acid solutions, with some exceptions such as Te(IV), which is adsorbed to a lesser extent from oxalic acid solutions of lower concentration. On the other hand, pronounced adsorption of many metals takes place on DEAE from oxalic acid solutions. Over the tested concentration range, very strong retention ($R_F = 0$) was noted for Ti(IV), Cu (II), Mo(VI), Pd(II), W(VI), Ir(IV), Pt(IV), Au(III) and Bi(III). For other metals strong adsorption is exhibited, except for Cr(III), As(III) and Tl(I) at oxalate concentrations in the range 0.01–0.3 *M*. There are distinct differences in adsorbability between DEAE and Avicel SF for the metals including Be(II), Al(III), Sc(III), Ti(IV), V(IV), Mn(II), Fe(III), Co(II), Ni(II), Cu(II), Zn(II), Ga(III), Ge(IV), Se(IV), Y(III), Zr(IV), Nb(V), Mo(VI), [Ru(III)], [Rh(III)], [Pd(II)], Cd(II), In(III), Sn(IV), Sb(III), La(III), Sm(III), Yb(III), Hf(IV), W(VI), Re(VII), Ir(IV), Pt(IV), Au(III), Hg(II), [Pb(II)], [Bi(III)], [Th(IV)] and U(VI) (for the metals in square brackets tailing takes place on either adsorbent, but general differences in absorbability are apparent).

These metals were located on the DEAE layer either along or below the oxalate

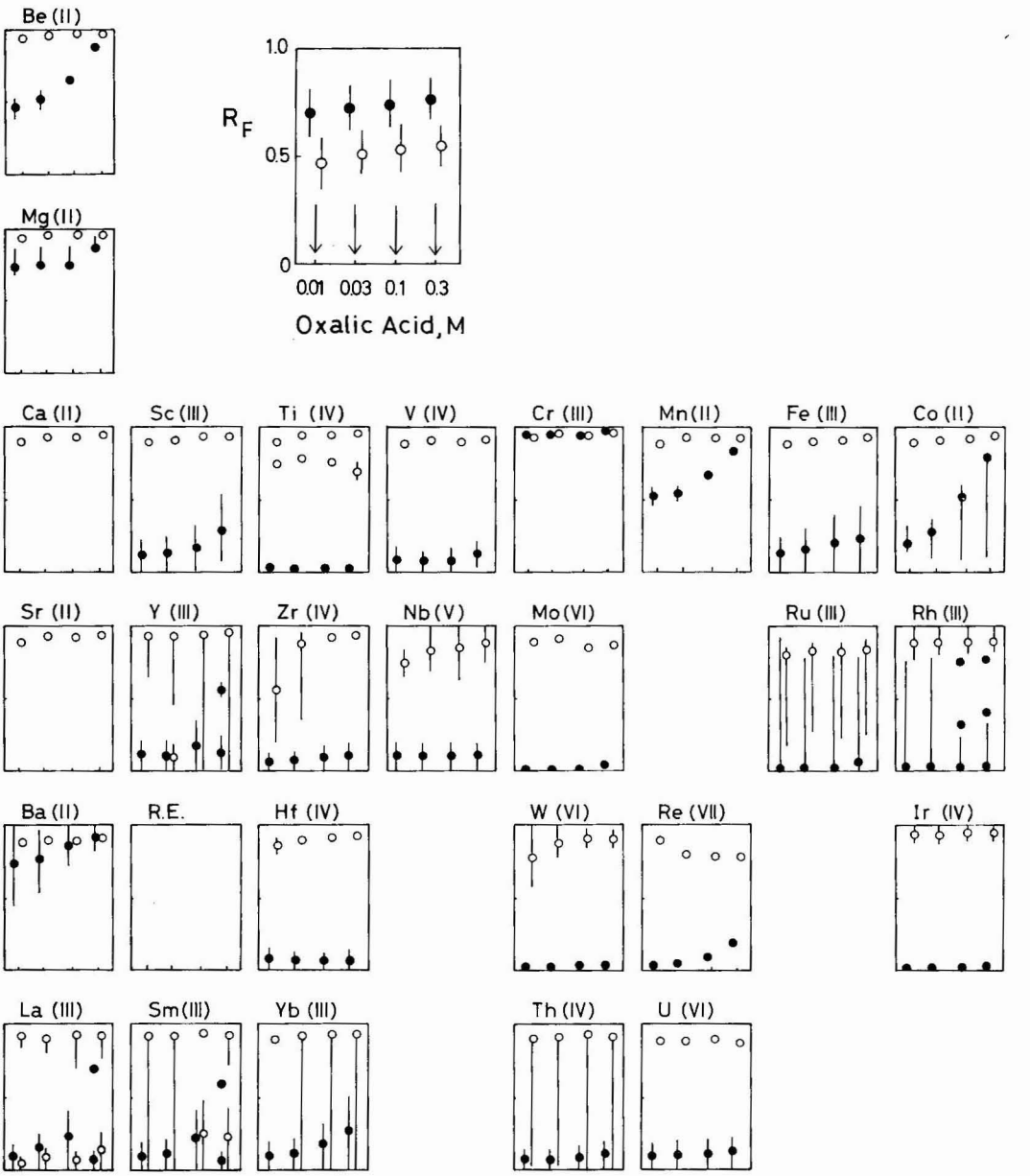
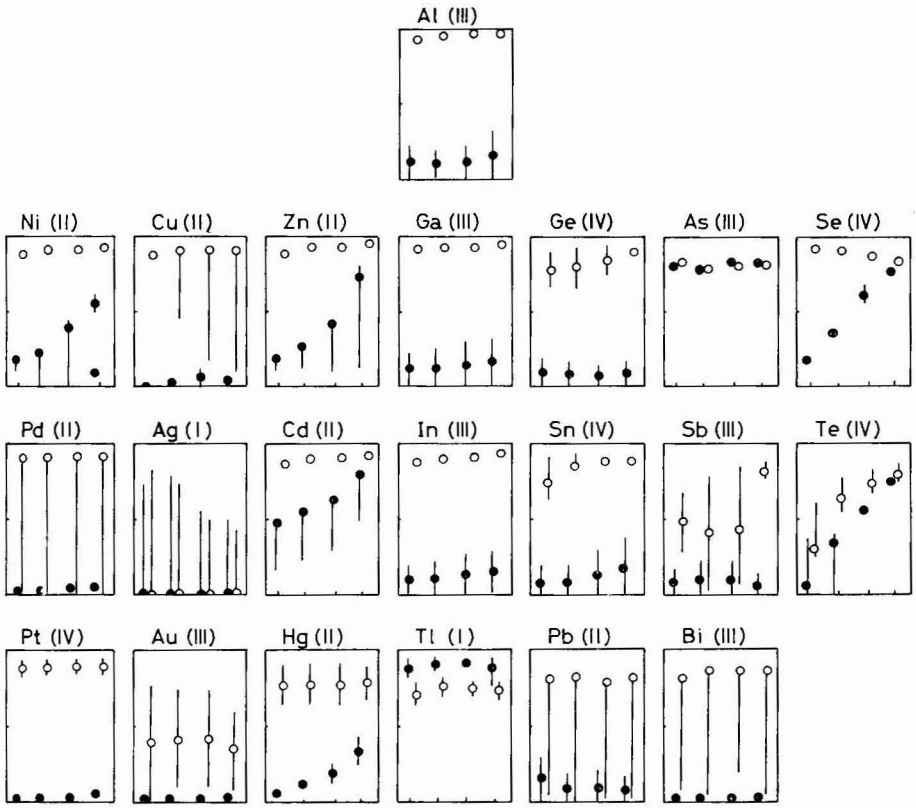


Fig. 1. R_F values of metals on DEAE (●) and Avicel SF (○) in oxalic acid solutions. For convenience, R_F values on Avicel SF are shifted arbitrarily to the right on the abscissa.

front over the oxalic acid concentration range tested; Be(II), Mn(II), Cd(II) and perhaps Se(IV) moved just along the oxalate front.

The different behaviours of the metals towards DEAE and Avicel SF can be understood by assuming the formation and subsequent anion-exchange adsorption



of oxalato complexes of these metals in oxalate media. Regarding the retention of the precious metals, their retention can probably be ascribed to ion exchange of their chloro complexes, because of their application to the DEAE layer from hydrochloric acid solution[13–15] and of their low oxalato complex formation tendencies.

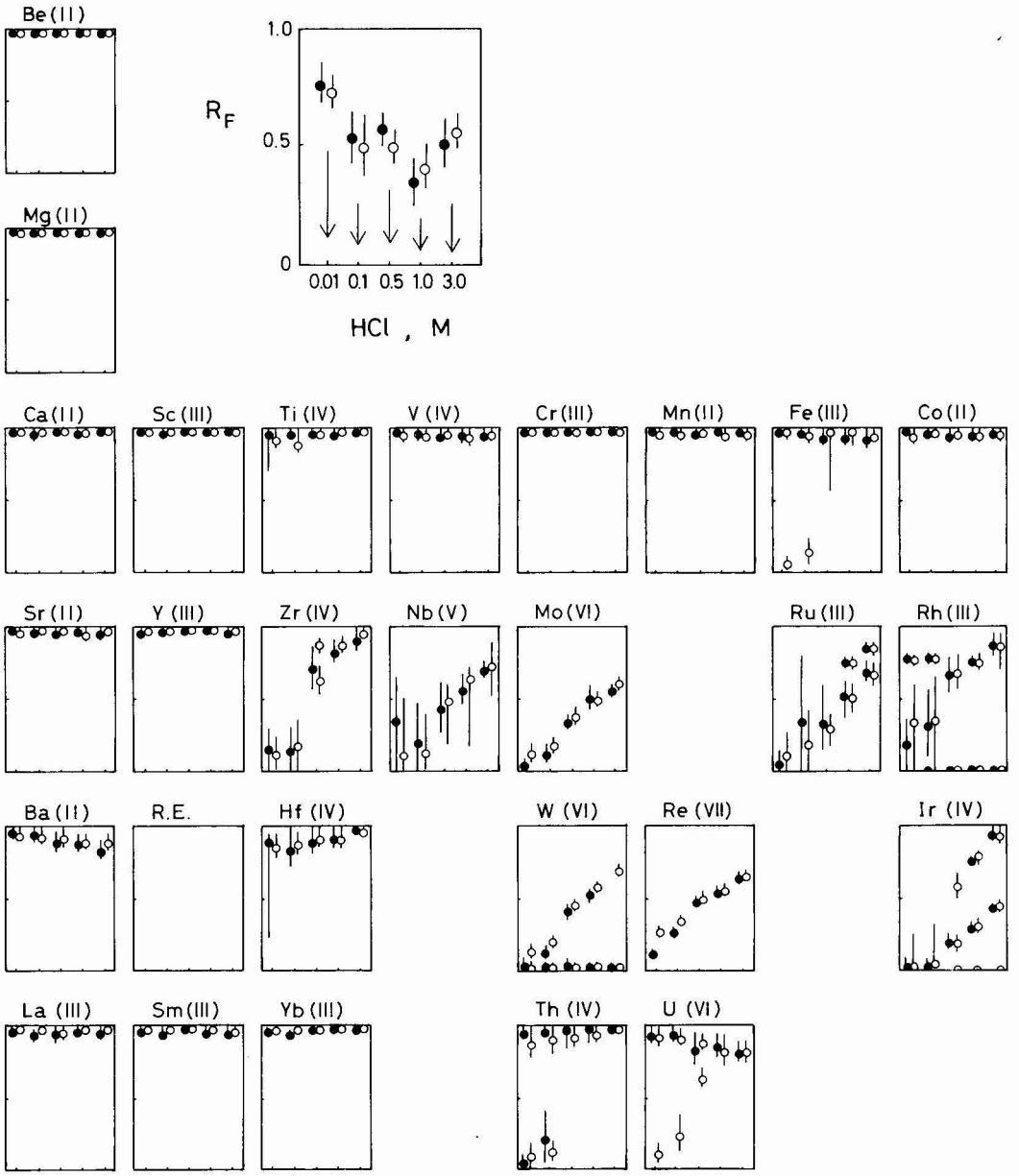
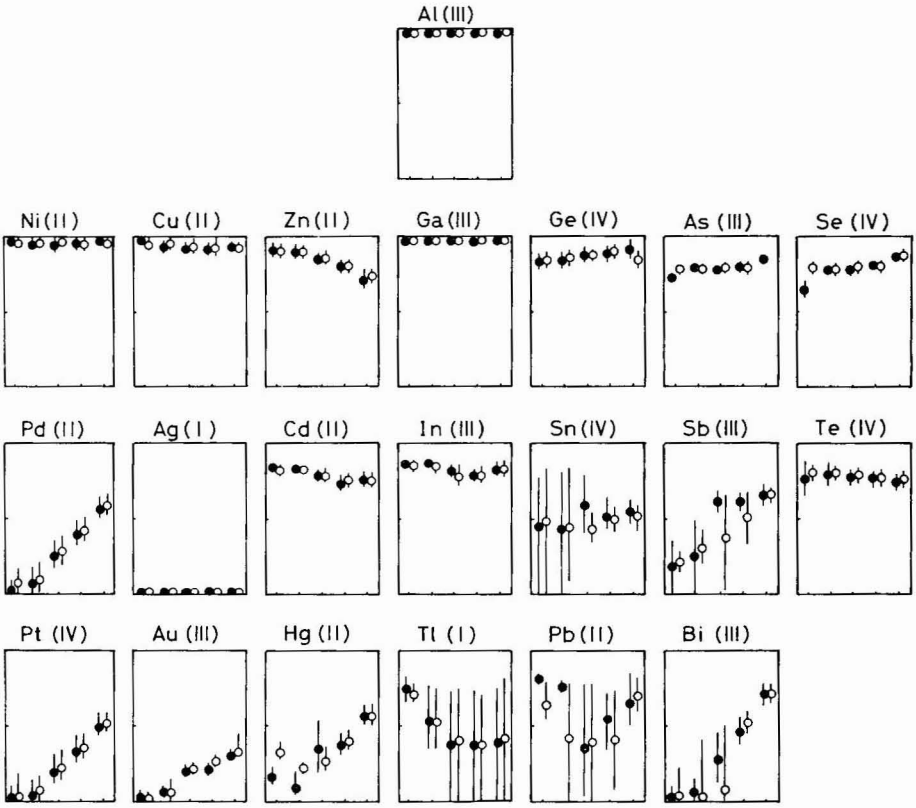


Fig. 2. R_F values of metals on DEAE in oxalic acid–HCl mixtures. The concentration of oxalic acid was kept constant at either 0.030 *M* (●) or 0.25 (○) *M*.

It is worth noting the difference in the adsorption sequences of the metals toward DEAE and a strongly basic anion-exchange resin in oxalic acid media. Fortunately, distribution coefficients for twelve metals on Dowex 1 are now available[1] for oxalic acid solutions of concentration 0.001–0.98 *M* (saturated solution).



The adsorption sequence of metals from 0.01 *M* oxalic acid solution is Mo(VI)[$>10^5$] >In(III), Lu(III), Sc(III) [$>10^4$] >Cu(II) [*ca.* 10^4] >Ce(III) [*ca.* 5000] >Zn(II) [*ca.* 3500] >Hg(II) [2000] >Co(II) [400] >Mn(II) [*ca.* 20] >As(III) [1.7], where the values in square brackets are the distribution coefficients, mostly obtained by inter-

polation from the original data. In our 0.01 *M* oxalic acid–DEAE system, the sequence is Mo(VI) >Cu(II), Hg(II) >Sc(III), In(III), REE >Zn(II) >Co(II) >Mn(II) >As(III) (REE represents rare-earth elements). Metals which are adsorbed weakly on Dowex 1 are not retained on DEAE to any significant extent. However, the selectivity sequences of metals on the two exchangers do not always coincide.

In Fig. 2 are illustrated the R_F values of 48 metals on DEAE(Cl^-) in mixed oxalic acid–hydrochloric acid media. The concentration of oxalic acid is kept constant at either 0.030 or 0.25 *M* and the concentration of hydrochloric acid is varied over the range 0.010–3.0 *M*. The addition of hydrochloric acid resulted in a rapid decrease in adsorption for nearly all of the metals tested, regardless of the concentration of oxalic acid. Even the presence of 0.01 *M* hydrochloric acid renders many metals virtually non-adsorbable on DEAE; thus Sc(III), Ti(IV), V(IV), Fe(III), Co(II), Ni(II), Cu(II), Zn(II), Ga(III), Ge(IV), Se(IV), Y(III), Cd(II), In(III), Te(IV), REE, U(VI), etc., exhibit R_F values of unity or near unity in spite of the presence of oxalic acid (0.030 and 0.25 *M*). Some metals are still retained on DEAE from hydrochloric acid of lower concentrations, as is found, for example for Zr (IV), Mo(VI), Pd(II), Sb(III), W(VI), Re(VII), Ir(IV), Pt(IV), Au(III), Hg(II) and Bi(III). These metals move well below the oxalic acid front. For some of these metals Lederer and Ossicini[16] have reported similar trends in R_F values on DEAE-paper as a function of hydrochloric acid concentration, although the comparison is possible only for a limited range of hydrochloric acid concentrations. The similar behaviour of metals on DEAE in both hydrochloric acid and mixed hydrochloric acid–oxalic acid does not exclude the role of oxalato and chloro-oxalato complexes on the retention mechanism. However, it is true that the apparent role of oxalic acid is not significant in mixed oxalic acid–hydrochloric acid media.

Strelow *et al.*[2] determined anion-exchange distribution coefficients (K_d) with AG 1-X8, a strongly basic anion-exchange resin, for 36 elements in oxalic acid–hydrochloric acid mixtures. The adsorption behaviour of metals in AG 1 appears to be almost independent of those on DEAE in the mixed acid media. Some examples can be mentioned. Sn(IV) is retained on AG 1 the most strongly of the 36 elements studied from the mixtures, with $K_d > 10^4$ for 0.05 *M* oxalic acid + 0.01–2.0 *M* hydrochloric acid and also $K_d > 10^4$ for 0.25 *M* oxalic acid + 0.01–1.0 *M* hydrochloric acid mixtures. In our system the corresponding R_F values are near 0.5, although tailing is marked at lower concentrations of hydrochloric acid. The R_F values of Ti(IV) on DEAE actually remain unchanged (*ca.* 1.0), but the corresponding K_d on AG 1 decreases rapidly with increasing concentration of hydrochloric acid: $>10^4$ for 0.01 *M* HCl, 7800 for 0.1 *M* HCl, 1450 for 0.5 *M* HCl, 213 for 1 *M* HCl and 1.3 for 3 *M* HCl (0.25 *M* oxalic acid present), Mo(VI) has K_d values on AG 1 very similar to those of Ti(IV), but the R_F values vary from 0 to 0.6 (see Fig. 2). It is likely that oxalato complexes dominate and control the R_F values on DEAE in oxalic acid media (Fig. 1), but chloride ions compete with the complexes for ion-exchange sites and release them easily even at lower concentrations of hydrochloric acid in the mixed acid acid media. DEAE strongly favours chloride ions.

A knowledge of the R_F values for metal ions chromatographed on DEAE in oxalic acid and the mixed acid media will permit many separations of analytical interest to be conducted easily. We accomplished separations involving La(III)–Ba(II), Be(II)–Mg(II), Ni(II)–Mn(II), Co(II)–Mn(II), Hg(II)–Cd(II), V(IV)–Cr(III),

Ti(IV)–Cr(III), Sb(III)–As(III), Bi(III)–As(III), Pb(II)–Tl(I), etc., in oxalic acid media and Hg(II)–Zn(II), Hg(II)–Cd(II), Zr(IV)–Ti(IV), Zr(IV)–V(IV), Ir(IV)–Co(II), In(III)–Ga(III), In(III)–Al(III), W(VI)–Mo(VI), U(VI)–Mn(II), Fe(III)–In(III)–Se(IV) Hg(II)–Au(III), Mn(II)–Cd(II)–Hg(II)–Au(III), Cu(II)–Zn(II)–Sn(IV)–Au(III), Ni (II)–Cd(II)–Hg(II)–Au(III), Mn(II)–U(VI)–Sn(IV)–Au(III), Ni(II)–Pd(II)–Au(III), Cu (II)–Pt(IV)–Au(III), etc., in oxalic acid–hydrochloric acid mixtures, confirming the validity of the R_F values shown in Figs. 1 and 2.

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Journal of Chromatography, 139 (1977) 364–369

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Note

Pyrolysis gas chromatographic–mass spectrometric study of medicinal sulphonamides

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The identification of polymers[1], biological macromolecules[2] and microorganisms[3] by analytical pyrolysis gas chromatography (GC)[4] or pyrolysis mass spectrometry (MS)[5] is well documented. The application of these techniques to the study of lower molecular weight compounds[6,7] and in particular to antibacterial substances[8–10] has been limited. Our interest in the analysis of these compounds, especially in formulated products or in biological fluids, has led us to study the application of analytical pyrolysis in this field and here we report some of our results concerned with the identification of medicinal sulphonamides. Various methods have been developed for the identification of these compounds and chromatographic procedures include paper and thin-layer[11] chromatography, and high-pressure liquid[12] and ion-pair partition chromatography[13]. Although sulphonamides are generally too polar for direct GC and decompose during elution[14,15], several procedures are now available for derivatisation prior to analysis. These include methylation[16], permethylation and perethylation[17] for GC–MS studies and the use of perfluoracyl and pentafluorobenzyl compounds for electron capture detection of simple[18] or medicinal[19] sulphonamides. Methylated acetal derivatives have also recently been used for this purpose[20]. Degradation techniques are limited to hydrolysis and GC of the liberated amines[21] and to preparative pyrolysis and thin-layer chromatography for pyrimidine sulphonamides[22]. The TAS[23] (thermomicro and transfer-application-substance) procedure has also been applied to some sulphonamides[24].

EXPERIMENTAL

Preparation of samples

The samples (50–100 μg) were dissolved in 10–20 μl of a suitable solvent (usually methanol) and applied to a rotating wire by a microsyringe[25]. The solvent was evaporated using a hairdrier and the coated wires were stored under vacuum.

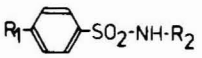
Apparatus and conditions

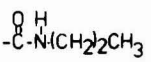
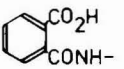
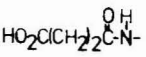
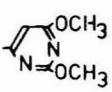
A Pye Curie point pyrolyser was used at a temperature of 770° maintained for 5 sec. A new wire and quartz tube were used for each sample. Chromatography was carried out on a Pye GCV gas chromatograph with 1.5 m × 3 mm I.D. dual glass columns packed with 2% KOH + 8% Carbowax 20M on Chromosorb W AW

DMCS, 100–120 mesh (Phase Separations, Queensferry, Great Britain). The temperature was programmed from 100° to 240° at 2°/min with a final hold of 10 min. The injection port was held at 275° and the detector oven heated to 350°. The air pressure was maintained at 0.5 kg/cm^2, the hydrogen at 1.3 kg/cm^2 and the nitrogen flow-rate was 50 ml/min. The data was collected using an Infotronics CRS 304-30 integrator. The identification of the pyrolysis fragments was achieved by means of a GC-linked VG Micromass 12B mass spectrometer operated with a trap current of 100 μA, an accelerating voltage of 4 kV and an ionisation energy of 22 eV.

TABLE I

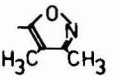
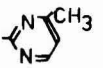
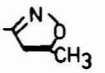
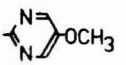
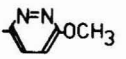
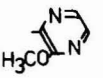
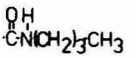
CHEMICAL STRUCTURE OF SULPHONAMIDES



No.	*Sulphonamide*	R_1	R_2	*Proprietary product*
1	Benzene sulphonamide	H	H	
2	Benzene sulphanilide	H		
3	Chlorpropamide	Cl		Diabinese (Pfizer)
4	*p*-Ethyl benzene sulphanilide	CH_3CH_2		
5	Phthalylsulphathiazole			Thalazole (May & Baker)
6	Succinylsulphathiazole			Sulphasuximide (MSD)
7	Sulphacetamide	H_2N		Eye drops
8	Sulphanilamide	H_2N	H	
9	Sulphadiazine	H_2N		Sulphatriad (May & Baker)
10	Sulphadimethoxine	H_2N		Madribon (Roche)
11	Sulphadimidine	H_2N		Sulphamezathine (ICI)

(Continued on p. 366)

TABLE I *(continued)*

No.	*Sulphonamide*	R_1	R_2	*Proprietary product*
12	Sulphafurazole	H_2N		Gantrisin (Roche)
13	Sulphaguanidine	H_2N		
14	Sulphamerazine	H_2N		Sulphatriad (May & Baker)
15	Sulphamethoxazole	H_2N		Gantanol, Bactrim (Roche) Septrin (Wellcome)
16	Sulphamethoxydiazine	H_2N		Durenate (Bayer)
17	Sulphamethoxypyridazine	H_2N		Midicel (Parke Davis) Lederkyn (Lederle)
18	Sulphametopyrazine	H_2N		Dalysep (Syntex) Kelfazine (Montedison)
19	Sulphaphenazole	H_2N		Orisulf (Ciba)
20	Sulphapyridine	H_2N		M & B 693 (May & Baker)
21	Sulphathiazole	H_2N		Sulphatriad (May & Baker)
22	Tolbutamide	CH_3		Rastinon (Hoechst)
23	*p*-Toluene sulphonamide	CH_3	-H	

RESULTS AND DISCUSSION

The sulphonamides used in this investigation are recorded in Table I together with the proprietary and ethical names for the various formulated products. The pyrolysis of those sulphonamides recorded in Table II was found to be characterised by fission about the labile sulphonamido group and yielded a simple pyrogram in each case. This consisted of a short retention time gas composed solely of sulphur dioxide followed by two intense peaks due to aniline and a heterocyclic amine. These identifications were confirmed by retention time comparisons and by MS. Aniline was found to be common to all fragmentations and served as an internal reference. The heterocyclic amine was produced by cleavage at the sulphonamido group and characterised the sulphonamide under test. This is illustrated for the pyrimidine sulphonamides in

TABLE II

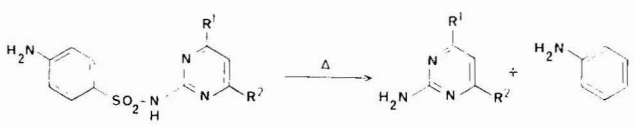
RETENTION INDICES (ANILINE = 1.00) OF SULPHONAMIDES WITH SIMPLE FRAGMENTATION

Sulphonamide	*Retention index*
Sulphapyridine	1.26
Sulphadiazine	1.30
Sulphamerazine	1.33
Sulphadimidine	1.47
Sulphamethoxydiazine	1.85
Sulphaphenazole	3.16

Table II, and in Fig. 1. This data demonstrates the variation in retention time which enables the identification of a specific sulphonamide to be achieved.

MS fragmentation of sulphonamides[26,27] is characterised by the presence of diarylamines derived via the extrusion of sulphur dioxide from the molecular ion. Simple sulphonamides (2 and 23 in Table I) have been found to undergo analogous secondary reactions and small amounts of biphenyl, carbazole and diphenylamine were detected on pyrolysis of benzene sulphonamide, in addition to the major components benzene and aniline. The results of methoxy-substituted sulphonamides again produced characteristic pyrograms. In this series however, only sulphamethoxydiazine underwent the simple decomposition noted previously to yield sulphur dioxide, aniline and 2-amino-5-methoxy-pyrimidine. The remaining sulphonamides (Table III) yielded secondary products resulting from *trans*-methylation reactions involving aniline and the methoxyheterocyclic. Thus sulphametopyrazine and sulphamethoxypyridazine yielded N-methylaniline and sulphadimethoxine, which has two methoxy substituents, in addition yielded N,N-dimethylaniline. This activity may be accounted for by the known tendency for the methoxyl groups positioned α to a π-deficient ring nitrogen atom to undergo thermally-initiated free-radical intermolecular rearrangements resulting in methyl migration[28,29].

In sulphacetamide an acetyl group replaces a heterocyclic substituent. This change increases the incidence of secondary reactions and pyrolysis of the hydrated sodium salt yields several products, among these acetanilide is an intense peak and is unique to sulphacetamide and so may be used as the diagnostic fragmentation product. Smaller amounts of carbon dioxide, sulphur dioxide, acetonitrile, acetic acid, benzene and acetophenone as well as aniline were detected.



	R^1	R^2
SULPHADIAZINE	H	H
SULPHAMERAZINE	CH_3	H
SULPHADIMIDINE	CH_3	CH_3

Fig. 1. Pyrolytic composition of pyrimidine sulphonamides.

TABLE III

RELATIVE AREAS OF AMINES OBTAINED FROM METHOXYSULPHONAMIDES SHOWING TRANS-METHYLATION

Sulphonamide	*Relative areas*			*Retention index R_2NH_2 (aniline = 1.00)*
	Me_2NPh	*MeNHPh*	*R_2NH_2*	
Sulphametopyrazine	—	0.07	0.23	2.48
Sulphamethoxypyridazine	—	0.18	—	—
Sulphadimethoxine	0.63	0.94	2.75	0.24

Formulated sulphonamides may also be studied by this method as it has been found that the excipients in tablets, *e.g.* magnesium stearate, lactose and starch, do not interfere either with the fragmentation pathways or with the overall appearance of the pyrogram. The technique is particularly useful for formulated mixtures. Fig. 2 records data obtained from Sulphatriad which contains a mixture of three sulphonamides and illustrates the clear identification of the components.

The technique has also been applied to the quantitative analysis of these drugs and also to the detection of sulphonamides and metabolites in urine. These results will be communicated at a later date[30].

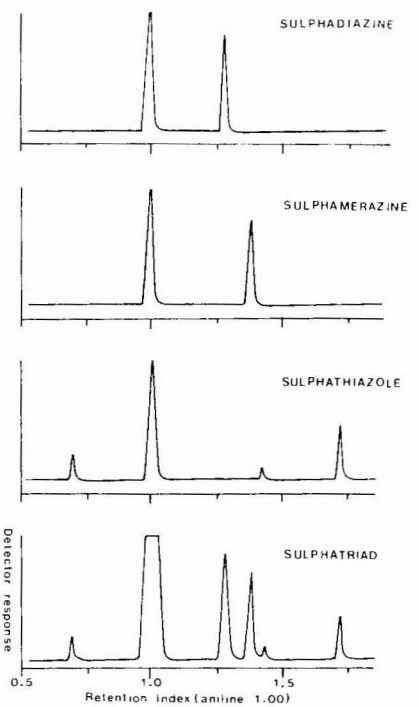


Fig. 2. Pyrogram of formulated mixture.

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Note

High-performance liquid chromatography of adenine and hypoxanthine arabinosides

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Adenine arabinoside (ara-A)* is a purine nucleoside first synthesized in the early 1960s[1,2] and later isolated from culture filtrates of *Streptomyces antibioticus*[3]. It was reported to exhibit marked inhibitory activity against DNA viruses in cell culture[4–7]. Its antiviral activity has been extensively evaluated in a variety of animal systems[8] and utilized for therapeutic purposes in man[9]. The determination of ara-A and its principle metabolite, hypoxanthine arabinoside (ara-Hx) in biological fluids has been reported by high-performance liquid chromatography (HPLC) on cation- and anion-exchange resins[10–13]. In our earlier work, good separations of ara-A, ara-Hx, Hyp, Ino and internal marker (8-amino ara-A) were obtained with Aminex A-28 columns using 0.2 *M* sodium acetate at pH 7.4 as the mobile phase. However, when present, Ado was not separated from ara-A in this system. The separation of this epimeric pair required the use of an additional column of a strong cation-exchange resin such as Aminex A-6 or A-7.

The chromatographic separation of saccharides[14–16] and nucleosides[17,18] has been accomplished through borate complexing. In an effort to eliminate the use of a two-column system for the efficient separation of ara-A from its metabolites, the use of borate buffers was investigated in the Aminex A-28 system.

EXPERIMENTAL

Chemicals and reagents

Sodium borate decahydrate and acetic acid (reagent grade) were obtained from J. T. Baker (Phillipsburg, N.J., U.S.A.). Sodium acetate (anhydrous analytical reagent) was obtained from Mallinckrodt (St. Louis, Mo., U.S.A.). Ado, Ino, Hyp and Ado deaminase were obtained from Calbiochem (San Diego, Calif., U.S.A.). Ara-A, ara-Hx and 8-amino ara-A were obtained from Parke, Davis & Co. (Ann Arbor, Mich., U.S.A.). Stock solutions of 0.04 *M* sodium borate and 2 *M* sodium acetate were used to prepare mobile phase buffer solutions by appropriate dilution in distilled water. The pH was adjusted by the addition of 5% (0.83 *M*) acetic acid.

* Abbreviations used: ara-A = 9-β-D-arabinofuranosyladenine, arabinosyladenine, vidarabine; ara-Hx = 9-β-D-arabinofuranosylhypoxanthine, arabinosylhypoxanthine; 8-amino ara-A = 8-aminoarabinosyladenine; ara-AMP = arabinosyladenine 5′-monophosphate; Ado = adenosine; Ino = inosine; Hyp = hypoxanthine.

Equipment

A Series 4200 liquid chromatograph (Varian Assoc., Walnut Creek, Calif., U.S.A.) with a 254-nm fixed-wavelength UV detector and an A-25 recorder was used throughout this study. A Model 153 fixed-wavelength UV detector (Altex Scientific, Berkeley, Calif., U.S.A.) was used to provide dual column capability. Stainless-steel columns (15 cm × 0.37 I.D.) were slurry-packed with Aminex A-28 resin (Bio-Rad Labs., Richmond, Calif., U.S.A.) in distilled water. The Aminex A-28 resin was pre-equilibrated for 24 h in 2 *M* sodium acetate solution. The temperature was maintained at 60° to facilitate diffusion and to decrease the viscosity of the solvent. The flow-rate of the mobile phase was adjusted to 0.5 ml/min, which required a pressure of 700–1000 p.s.i. Samples were injected on-column by means of a syringe through a stop-flow injector.

Methods

Plasma samples were deproteinized by centrifugation through CF25 or CF50A Centriflo membrane cones (Amicon Corp., Lexington, Mass., U.S.A.) at 100 *g* for 60 min. Urine samples were filtered through No. 497 paper (Schleicher and Schüll, Keene, N.H., U.S.A.) and diluted with distilled water as required. The internal marker, 8-amino ara-A, was added to all samples provided that interference peaks were not present. Peaks were quantitated manually using peak-height measurements. Ara-A and ara-Hx concentrations in biological samples were determined from calibration graphs prepared by linear regression analysis of standards at four or five different concentrations.

RESULTS AND DISCUSSION

Anion-exchange techniques were selected for the separation of ara-A and ara-Hx in the presence of Ado, Ino and Hyp for several reasons. Firstly, except for an overlap of Ado and ara-A, all other components are separated in a single analytical system. Secondly, borate complexing provides an additional parameter for the effective separation of ribosyl and arabinosyl nucleosides. As borate complexing is most pronounced with sugars that contain *cis*-hydroxyl groups in a furanoid structure[19], the borate-diol of ribose shows a high affinity for strongly basic anion exchangers.

With 0.005 *M* sodium borate solution as the mobile phase, the effect of pH on the retention of ara-A, 8-amino ara-A, ara-Hx, Ado and Ino on Aminex A-28 is shown in Table I. Optimal separation of these components occurred at pH 6.3. Ado, ara-A and 8-amino ara-A have no ionic properties that can be manipulated in order to change their retentions in anion-exchange systems except at strongly alkaline pH. The apparent exception of Ado is attributed to the formation and exchange of the borate-complex anion. The retention of ara-Hx and Ino is based upon true anion exchange. In addition, the formation of a borate complex with Ino resulted in total resin retention in the pH range tested.

The effect of the molarity of the mobile phase on the retention of components at a constant pH of 6.3 is shown in Table II. Maximum separation of components was achieved at a borate concentration of 0.01 *M*. As expected for true anion exchange, the retention of ara-Hx was inversely proportional to borate concentration, while ara-A and 8-amino ara-A were not greatly affected. In contrast, the retention of Ado was

TABLE I

INFLUENCE OF pH ON THE RETENTION TIME OF NUCLEOSIDES ON AMINEX A-28

Mobile phase 0.005 *M* sodium tetraborate solution.

pH	*Retention time (min)*				
	ara-A	*8-amino ara-A*	*Ado*	*ara-Hx*	*Ino*
9.0	11.0	20.0	N.E.*	N.E.	N.E.
7.3	9.5	—	N.E.	N.E.	N.E.
7.0	7.0	—	28.0	N.E.	N.E.
6.3	6.0	8.0	10.0	17.5	N.E.
6.0	6.0	8.0	8.5	11.5	—
5.0	6.0	—	7.0	5.0	—

* N.E. = Not eluted from column.

directly proportional to borate concentration, as would be expected from increased borate-complex anion formation.

The chromatographic separation of nucleosides and Hyp on Aminex A-28 using a mobile phase consisting of 0.01 *M* sodium borate at pH 6.3 is shown in Fig. 1A. Although the separations were reproducible from day to day and the columns appeared to be stable, a gradual increase in the retention times of Ado, ara-Hx and Hyp was found with columns used over periods of weeks or months. The chromatographic profile on the same column after a 3-month period is shown in Fig. 1B. As freshly prepared columns operated satisfactorily, the gradual replacement of acetate with borate ion on the resin appeared to be a possibility. The addition of sodium acetate to the mobile phase restored the retention times of the above components to their initial values. Subsequently, a mobile phase consisting of 3 parts of 0.01 *M* sodium borate and 1 part of 0.01 *M* sodium acetate at pH 6.4 was employed.

The response of the UV detector to known amounts of ara-A and ara-Hx was linear throughout the range (0–20 μg/ml), as shown in Fig. 2. The reproducibility of the peak heights of ara-A and ara-Hx was established in a trial in which 10 replicate assays were run at a concentration of each component of 20 μg/ml. The coefficient of variation was 2.1% for ara-A and 7.4% for ara-Hx. Standard graphs prepared by the addition of known amounts of ara-A and ara-Hx to deproteinized plasma, urine or water showed no significant differences in recoveries.

The sensitivity of detection was enhanced by the low noise level of the chromatographic system and the relatively sharp chromatographic peaks. This permitted

TABLE II

INFLUENCE OF MOLARITY ON THE RETENTION TIMES OF NUCLEOSIDES ON AMINEX A-28

Mobile phase sodium tetraborate solution at pH 6.3.

Molarity	*Retention time (min)*				
	ara-A	*8-amino ara-A*	*Ado*	*ara-Hx*	*Hx*
0.005	6.0	8.0	10.0	17.5	—
0.01	6.5	9.0	12.5	16.5	19.0
0.02	6.8	9.5	15.2	13.0	—

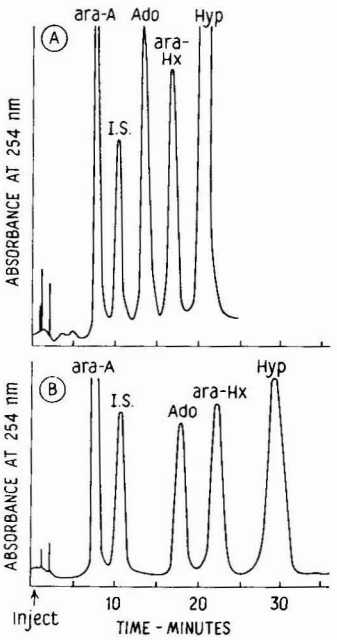
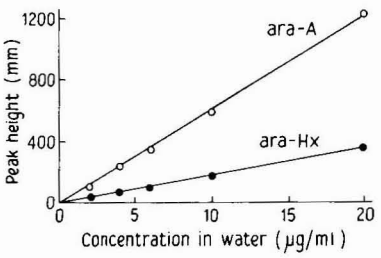


Fig. 1. HPLC of a nucleoside mixture on an Aminex A-28 column. (A) Initial separation; (B) separation after 3 months.

operation of the UV detectors at maximal sensitivity. Under these conditions, the lower limits of detection were about 2 ng for ara-A and 5 ng for ara-Hx. A typical chromatogram of human plasma taken 30 min after the start of an i.v. infusion of ara-A (10 mg/kg·day over a 12-h infusion period) is shown in Fig. 3. A 20-μl volume of internal marker (20 μg/ml in water) and 20 μl of 0.1 *M* sodium acetate buffer at pH 6.3 were added to 160 μl of deproteinized plasma and a 10-μl sample of this mixture



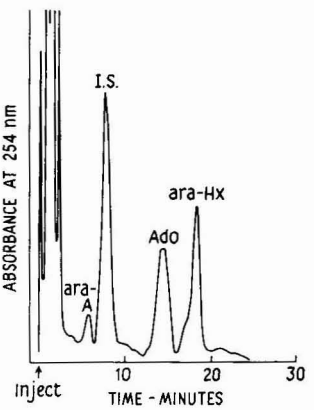


Fig. 2. Response curves for ara-A and ara-Hx.

Fig. 3. HPLC of deproteinized human plasma 30 min after start of i.v. infusion of ara-A (10 mg/kg·day).

was injected on the column. Ado and a small amount of ara-A were found in addition to the major peak representing ara-Hx.

Presumptive identification of ara-A and ara-Hx in biological samples was made by comparing the retention times of the peaks with those of known standards. Identification of ara-A was confirmed by use of an enzymic peak-shift technique[20]. A study in which human eyes were treated with a 3% ophthalmic solution of ara-AMP required the assay of the aqueous humor. A 20-μl sample was assayed directly without deproteinization because of limitations on the sample size; this produced an elevated base-line. A 5-μl volume of internal marker (10 μg/ml in water) and 5 μl of Ado deaminase (42 I.U./ml in water) were added to 20 μl of sample and the mixture was incubated at room temperature for 15 min. The results shown in Fig. 4 indicate the complete disappearance of the ara-A peak and a corresponding rise in ara-Hx produced by the deamination of ara-A. This technique is not applicable when co-vidarabine[21] or other inhibitors of Ado deaminase are added to freshly collected blood samples.

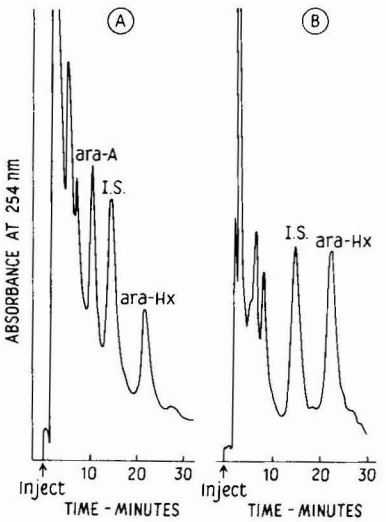


Fig. 4. HPLC of human aqueous humor following topical application of 3% ara-AMP ophthalmic solution. (A) Untreated sample; (B) after treatment with Ado deaminase.

CONCLUSION

By means of HPLC in a borate buffer system, ara-A and ara-Hx are separated from ribosyl nucleosides and determined quantitatively in blood and urine.

ACKNOWLEDGEMENT

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Note

Thin-layer chromatographic measurement of low activities of tritiated substances mixed with non-radioactive quenching plant pigments

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Tritium (^3H) is frequently used as a tracer in radiochromatography of samples of biological origin, although difficulties arise in its determination owing to the low electron energies (E_{max} = 18.5 keV) involved. For radiocarbon (^{14}C), the electron energies are larger by a factor of *ca.* 10, and the problems of detection are greatly reduced. The standard method of film detection of low activities of ^{14}C in thin-layer chromatography (TLC) is predominantly an autoradiographic process entailing the direct interaction of the ^{14}C β-particles with the emulsion[1], and is several thousand-fold less sensitive for ^3H. To enhance the speed and the sensitivity of ^3H film detection, scintillators must be incorporated into the TLC plate, or applied to the plate after chromatographic development, which in turn produces an image in the photographic emulsion (fluorography)[1–3]; a sensitivity as low as 1 nCi ^3H per cm^2 per day can be obtained, but it varies strongly with the experimental conditions[1]. The TLC spots are scraped off and suspended in a scintillation solution, and the ^3H radioactivity is measured using a liquid scintillation spectrometer[4–6]. These methods involve the detection of luminescence induced by β-particles, and can thus be hampered by quenching. During TLC analysis of samples from plant extracts, spots of coloured plant pigments, especially the chlorophylls, can partly or completely quench the radioactivity of the spots of the tritiated compounds under study (the pesticides and their metabolites, for example). We have developed a sensitive and reliable method for detection of tritiated substances on TLC plates when their spots are mixed with those of such pigments. The TLC of the tritiated fungicide triforine [*NN'*-bis-(1-formamido-2,2,2-trichloroethyl)piperazine, uniformly ^3H-labelled in the piperazine ring] is given as an example.

MATERIALS AND METHODS

^3H-Triforine (105 μCi/mg) was received from Cela Merck (Ingelheim am Rhein, G.F.R.), and stored as a powder until use. TLC was carried out with activated (105°, 24 h) DC-Plastikfolien Kieselgel 60 F_{254} (Merck, Darmstadt, G.F.R.) divided into strips (2 × 20 cm/0.25 mm). ^3H Measurements were made at 8° in polyethylene counting vials (Packard) with a liquid scintillation counter (Packard, Model Tri-Carb 2425) in scintillant I (60 g naphthalene, 4 g PPO, 200 mg POPOP, 100 ml methanol,

20 ml ethylene glycol, dioxane to 1 l) or II (7 g PPO, 0.6 g POPOP, toluene to 1 l), prepared with scintillation grade products from Packard (Downers Grove, Ill., U.S.A.). The other chemicals were of analytical grade from Merck.

Standard solutions of ^{3}H-triforine in methanol or benzene were prepared, and counted (100 μl) in scintillant II. Absolute activities and counting efficiencies were determined by using internal standards of ^{3}H-toluene (Packard), the sample giving a homogeneous solution with the scintillant. A mixture of plant pigments was obtained by the extraction of barley leaves with chloroform and concentrated in a rotatory vacuum evaporator[7]. The total chlorophyll content of the concentrate was measured by visible absorption spectrophotometry ($\lambda = 652$ nm) of an aliquot dissolved in 80% aqueous acetone[8,9]. Aliquots of the concentrate were added to solutions of ^{3}H-triforine, giving standard solutions of mixtures of ^{3}H-triforine and chlorophylls (and other plant pigments), which were spotted (20 μl) on the TLC strip. This was developed for 16 cm with ethyl acetate, the R_F of triforine being 0.67. The dried chromatogram was cut into 12 equal segments, the radioactivities of which were measured separately, and then summed. The distribution of the radioactivity along the chromatogram was thus determined. Several methods were tried for the determination of the radioactivity on the TLC strip.

Method 1. The TLC segment was put directly into the counting vial containing scintillant II (10 ml).

Method 2. The TLC segment was scraped off. The powder was transferred quantitatively into the counting vial containing scintillant II (10 ml), and the whole was shaken for 1 min.

Method 3. The TLC segment was scraped off. The powder was transferred into the counting vial containing 0.2 ml Cl_2–water (*ca.* 5 g Cl_2 per l water) and shaken for 2 h after which scintillant I (10 ml) was added.

Method 4. The TLC segment was scraped off. The powder was transferred into the counting vial containing 0.1 ml Br_2–water (saturated solution) and shaken for 2 h, after which scintillant I (10 ml) was added.

Method 5. The TLC segment was put into the counting vial containing a 10 g% solution (1.2 ml) of benzoyl peroxide in toluene. After irradiation for 2 h under intense light, scintillant II (10 ml) was added.

Method 6. The TLC segment was scraped off. The powder was treated in a sealed tube (105°, 17 h) with propan-2-ol (0.3 ml) and 30 vol% hydrogen peroxide (0.3 ml). The contents of the cooled sealed tube were transferred quantitatively into scintillant I (10 ml).

The absolute activity (as well as the amount of chlorophylls) spotted on the TLC strip was always accurately known. By comparing this with the sum of the measured activities of the segments, obtained after chromatography and application of one of the methods outlined above, we measured the total efficiency of the method. If the solution studied was spotted on a TLC segment which was not chromatographically developed, the radioactivity measurement efficiency was similar to that observed after chromatography. All six methods gave similar results. All samples were counted at least four times with 10,000 counts collected in the counting channel, and the counting was repeated several hours later to check the absence of disturbing luminescence after an adequate time of dark adaptation. The background count rates varied from 10 to 18 cpm.

RESULTS AND DISCUSSION

The radioactivity of a solution of pure ^{3}H-triforine in toluene was measured in scintillant II. The counting efficiency was 44 $\pm$ 0.5%, and independent of the activity of the sample between $2 \cdot 10^3$ and $2 \cdot 10^5$ dpm. When a solution of pure ^{3}H-triforine was spotted on a TLC strip, and the radioactivity of the developed chromatogram was measured, the efficiency of counting was the same with both methods 1 and 2 (Fig. 1).

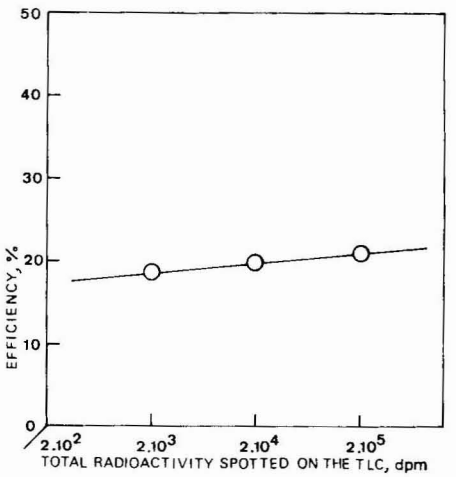

Fig. 1. Efficiency of pure ^{3}H-triforine radioactivity measurement after TLC and use of methods 1 and 2.

Standard solutions of mixtures of ^{3}H-triforine and chlorophylls (and other coloured plant pigments) were spotted on TLC strips, which were developed with ethyl acetate. The spot of triforine was mixed with those of plant pigments. The counting efficiency was low and similar with methods 1 and 2 (no bleaching) (Fig. 2). Similar results were obtained when there was no chromatographic development. There was no reliable relationship between efficiency and channels ratio. The efficiency was so poor that low activity (less than $2 \cdot 10^4$ dpm) of ^{3}H-triforine could not be usefully distinguished on developed TLC strips. Moreover, the counting solutions were green and luminescent so that, sometimes, as much 4 h elapsed before counting was possible.

When the same assays with mixtures of ^{3}H-triforine and plant pigments were performed using the bleaching methods 3 and 4 poor results were obtained, the counting efficiencies being less than 10%. Somewhat better results were obtained with the bleaching method 5 (Fig. 3); however, it was inefficient as the amounts of chlorophylls spotted on the TLC strip were usually *ca.* 30 μg. Similar efficiencies were obtained when there was no chromatographic development.

TLC assays with mixtures of ^{3}H-triforine (spotted activities between $2 \cdot 10^3$ and $2 \cdot 10^5$ dpm) and chlorophylls (spotted amounts of 0–150 μg) were performed using the bleaching method 6. The counting efficiencies after chromatographic development (or without development) were constant (22 $\pm$ 0.5%), and correspond to the counting

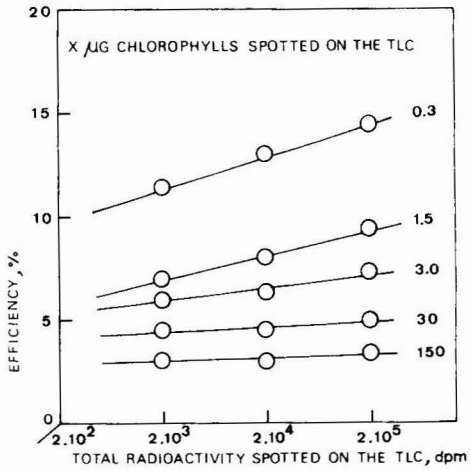

Fig. 2. Solutions of mixtures of ^{3}H-triforine and chlorophylls were spotted on TLC strips and, after chromatographic development, the efficiencies of radioactivity measurement by methods 1 and 2 (no bleaching) were recorded.

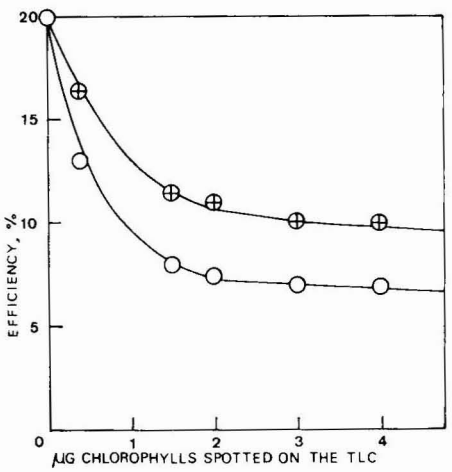

Fig. 3. Solutions of mixtures of ^{3}H-triforine and chlorophylls were spotted on TLC strips, the tritiated spotted activity being constant at $2 \cdot 10^4$ dpm. After chromatographic development, the efficiencies of radioactivity measurement by methods 1, 2 (no bleaching; similar results for both: ○), and 5 (⊕) were recorded.

efficiency of ^{3}H-toluene in scintillant I (10 ml) to which the pure bleaching solvents were added (0.3 ml of 30 vol% hydrogen peroxide, and 0.3 ml isopropanol). Counting could be performed immediately, as there was no interfering luminescence. Independently of the spotted amount of chlorophylls, ^{3}H-triforine activities as low as $2 \cdot 10^3$ dpm gave very clear spots after TLC development. This method is thus useful for the TLC measurement of low tritiated activities mixed with quenching coloured plant pigments. It also enables the measurement of the distribution of the radioactivity along the chromatogram, as the counting efficiency is constant and inde-

pendent of the presence of coloured spots mixed with the radioactive one. The limiting factor, although not concerned with the measurement method, was the amount of spotted chlorophylls. Indeed, above 50 μg chlorophylls, the TLC was oversaturated. This problem could be solved by using preparative TLC.

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Note

Détermination rapide de polyamines et de quelques mono- et diamines dans des extraits végétaux

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(Reçu le 18 avril 1977)

La chromatographie automatique sur colonne d'échangeur d'ions est une des techniques de choix pour l'analyse de mélanges de mono-, di- et polyamines. Bien qu'un grand nombre de méthodes aient été publiées (par ex. bibl. 1–4) elles concernent souvent l'analyse de matériel d'origine animale. Très peu d'entre elles ont été consacrées à la determination des amines contenues dans du materiel d'origine végétale[5]. D'autre part ces méthodes donnent de bons résultats, lorsqu'il s'agit d'échantillons purifiés, par contre on obtient des chromatogrammes difficiles à interpreter si l'on analyse des extraits bruts. Quand on cherche à détecter la présence de ces composés aminés, pouvant se trouver en petite quantité, il vaut mieux éviter la purification préalable de l'échantillon, car ceci peut contribuer à la perte de quelques constituants mineurs. De plus, dans les cas où l'on doit effectuer un grand nombre d'analyses, cette purification préalable représente une perte considerable de temps.

Dans cette note, nous rapportons une nouvelle méthode permettant l'analyse directe de mono-, di- et polyamines d'origine végétale, dont la durée estdedeux heures, ce qui représente un progrès par rapport aux techniques décrites antérieurement, qui nécessitaient huit heures d'analyse[5]. L'emploi de l'*o*-phtalaldehyde pour le dosage par fluorimétrie augmente la sensibilité de la méthode permettant la détection de ces composés à des concentrations de 10^{-12} *M*.

La Fig. 1 montre le chromatogramme d'un mélange standard préparé avec de produits commerciaux et les Figs. 2 et 3 les chromatogrammes obtenus avec des extraits bruts des plantes de soja (*Phaseolus mungo*) et de petits pois (*Pisum sativum*).

MATÉRIEL ET MÉTHODES

Nous avons utilisé un analyseur automatique Liquimat-Labotron muni d'un fluorimètre Labotron FFM-31 (Soc. Kontron, Boulogne, France) possédant une microcellule de circulation de 50 μl de capacité. La colonne (0.4 × 9 cm) de résine Durrum DC 6A est thermostatée à 66°. La composition des deux tampons servant à l'élution est comme suit; premier tampon (pH 5.65): citrate de sodium·2 H_2O 0.2 *N*, NaCl 1.0 *N*; deuxième tampon (pH 5.65): citrate de sodium·2 H_2O 0.2 *N*, NaCl 2.6 *N*. Les deux tampons ont été filtrés sur une membrane Millipore (0.22 μm). Les valeurs

* Boursiers du gouvernement français.

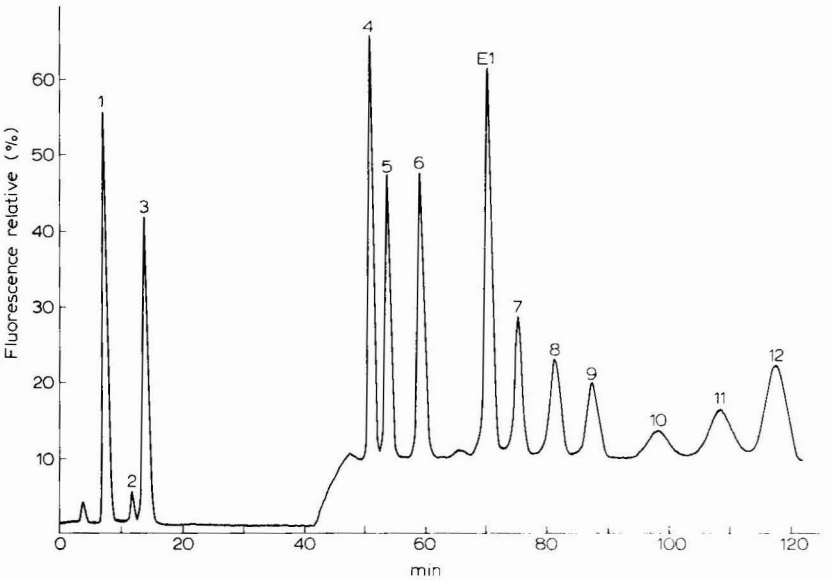

Fig. 1. Chromatogramme d'un mélange standard contenant 500 pmoles de chaque amine sauf pour l'étalon interne (E.I.) qui contient 375 pmoles. Voir Tableau I pour leur identité et comportement chromatographique.

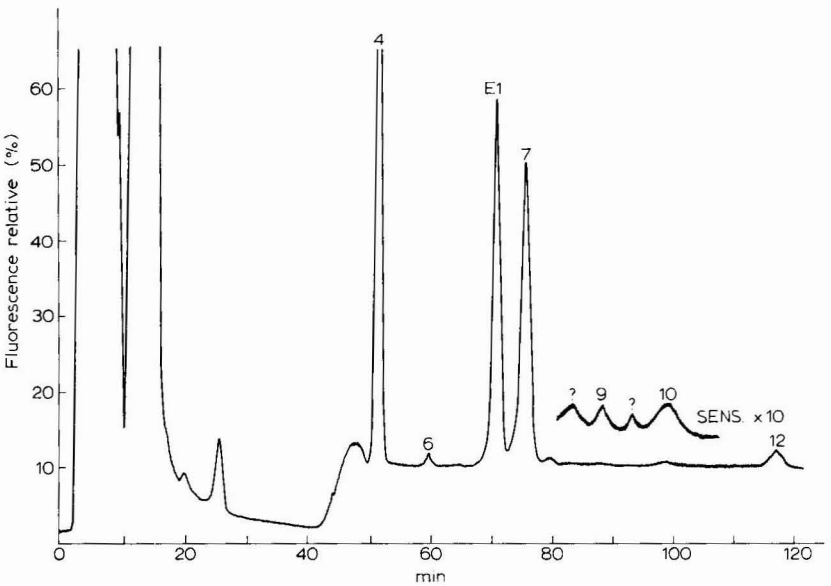

Fig. 2. Chromatogramme d'un extrait de soja (mis à germé pendant dix jours). Voir Tableau I pour l'identité des produits.

pH sont ajustés avec HCl après avoir ajouté 5% d'éthanol à chaque tampon. La détection est effectuée à l'aide d'une solution d'*o*-phtalaldehyde (Fluka, Buchs, Suisse)[6,7]. L'enregistreur, W + W 600-Tarkan (Soc. Kontron) est reglé sur 100 mV pour 100% de fluorescence relative ce qui laisse la possibilité d'augmenter encore de

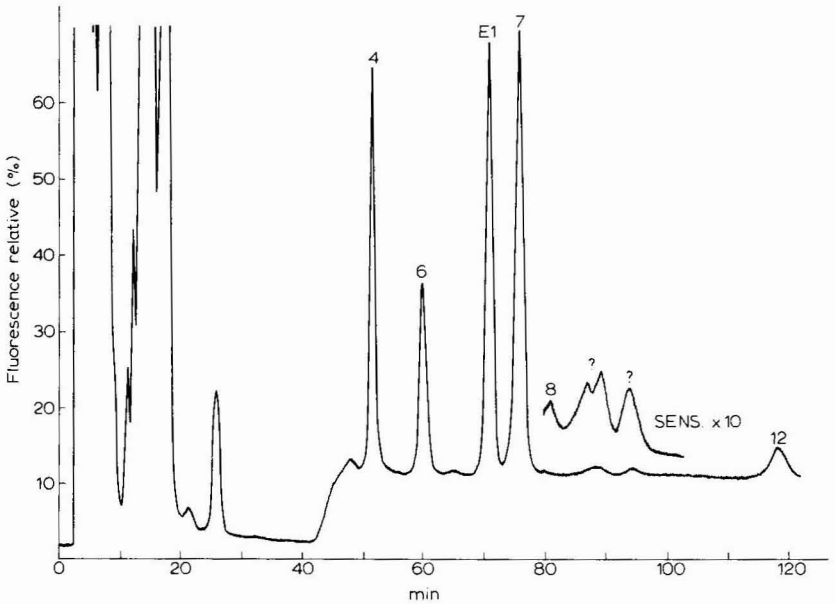

Fig. 3. Chromatogramme d'un extrait de petit pois (mis à germé pendant deux jours). Voir Tableau I pour l'identité des produits.

10 fois la sensibilité de l'enregistreur. Ceci nous a été utile pour la détection de quelques composés mineurs des extraits analysés (voir Figs. 2 et 3).

La quantification est faite avec un integrateur ICAP 10 (LTT Conflans Saint Honorine, France) par la méthode de l'étalon interne.

Préparation des échantillons

La solution standard (10 μM) contenant les produits aminés (voir Tableau I) a été préparé dans un tampon citrate de sodium 0.2 N (pH 2.2). La 4-aza heptaméthylène 1,7-diamine (Merck) a été employée comme étalon interne (7.5 μM).

L'extraction des polyamines à partir du soja ou des petits pois a été realisée sur des graines mises à germer pendant des périodes allant de 1 à 10 jours. Les plantes ainsi obtenues ont été broyées dans un mortier et extraites deux fois avec une solution de TCA à 5% contenant HCl 0.05 N. Après centrifugation les surnageants sont reunis et une partie aliquote de cet extrait est directement analysée sans purification préalable.

Les volumes analisés, solution standard ou extraits de végétaux, étaient de 25 à 100 μl.

Méthode

Un débit de 22.5 ml/h est utilisé pour les tampons d'élution et un débit de 25 ml/h pour la solution d'*o*-phtalaldehyde. Lors de la chromatographie, les temps de passage sont: pour le premier tampon de 35 min et pour le deuxième tampon de 83 min; 10 min de passage de NaOH 0.2 N suffisent pour le recyclage de la colonne et 20 min du premier tampon pour la réequilibrer.

TABLEAU I

RÉSULTATS DE LA MÉTHODE

Surfaces des pics: pour 500 pmoles de produit. Coefficients de réponse:réference, étalon interne (E.I.).

No.	*Composé*	*Temps de rétention (min)*	*Surface des pics*	*Coefficient de réponse*
1	Lysine	7.6 ± 0.1	104877	1879
2	Ammoniaque	12.1 ± 0.1	25747	7657
3	Arginine	14.2 ± 0.1	122319	1611
4	Putrescine	51.1 ± 0.1	130135	1515
5	Histamine	53.8 ± 0.3	96753	2037
6	Cadaverine	59.6 ± 0.1	90712	2173
E.I.	4-Aza heptaméthylènediamine	70.4 ± 0.1	147873 (375 pmoles)	
7	Spermidine	75.2 ± 0.1	71378	2762
8	Hexaméthylènediamine	81.7 ± 0.1	80221	2457
9	Agmatine	87.7 ± 0.1	75012	2628
10	Tyramine	99.1 ± 0.1	87078	2264
11	Phényléthylamine	109.3 ± 0.1	109557	1799
12	Spermine	116.5 ± 0.2	125046	1576

RÉSULTATS

La Fig. 1 montre le chromatogramme obtenu lors de la séparation d'un mélange standard contenant 500 pmoles de chaque composé. Le premier tampon sert à éluer tous les acides aminés et à éliminer toute autre substance moins basique que les polyamines. Le deuxième tampon élue par la suite les polyamines et amines apparentées. L'éthanol qu'on ajoute aux tampons est necessaire pour obtenir une bonne séparation. Sur le Tableau I figurent les temps de rétention, les surfaces des pics et les coefficients de réponse obtenus.

A titre d'exemple les Figs. 2 et 3 montrent les chromatogrammes correspondant aux polyamines des plantes de soja et de petits pois, dont les résultats sont en accord avec les données bibliographiques[8,9].

La reproductibilité de la méthode chromatographique est comparable à celle de l'analyse automatique d'acides aminés. Le fait de chromatographier des échantillons bruts non tamponés, comme c'est le cas pour les extraits végétaux analysés n'a aucune influence sur la séparation de polyamines et il n'est pas observé de déplacement anormal des pics. Ceci a été vérifié en chromatographiant un mélange d'extrait brut avec une solution standard de polyamines. La méthode permet, grace à l'utilisation de la quantification fluorimétrique, la détection des composés aminés présents à des concentrations de l'ordre de picomole.

Nous employons cette méthode pour (1) la détection rapide de mono-, di- et polyamines dans du matériel végétal; (2) le dosage simultané des décarboxylases de quelques acides aminés tels que la tyrosine, la phénylalanine, l'ornithine, la lysine, l'histidine, l'arginine. Une étude concernant les variations des concentrations des polyamines par rapport à la croissance végétale est actuellement en cours.

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Note

The use of Amberlite XAD-2 resin for the quantitative recovery of fenitrothion from water —a preservation technique

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Amberlite marcoreticular resins are hard, insoluble beads of porous polymer, ranging in physical properties from being essentially non-polar to very polar. They have been studied in terms of their usefulness for the recovery of various organic contaminants from environmental water.

For instance, Burham *et al.*[1] have used Amberlite XAD-2 for the quantitative recovery in the ppb range of methyl isobutylketone, ethyl butyrate, benzene, naphthalene, benzoic acid, 2,4-dimethylphenol, *p*-nitrophenol, 2-methylphenol, aniline and *o*-cresol. They have been used for the extraction and recovery of chlorinated insecticides and polychlorinated biphenyls from water[2,3]. This has led to the development of a multi-residue technique for the extraction of organochlorine pesticides and polychlorinated biphenyl from natural waters[4]. They have also been used to determine pesticides such as Atrazine, DDE and Dieldrin from various Iowa waters[5].

Recently Amberlite XAD-4 has been applied to the analysis of phosphorous-containing hydrolytic products of organophosphorous insecticides in water[6]. This has shown the potential of using Amberlite resins for the recovery of organophosphorous pesticides.

This study demonstrates the use of Amberlite XAD-2 resin for the recovery of fenitrothion, an organophosphorous insecticide, from aqueous environmental samples. More interesting is the fact that the insecticide is stable on the column for extended time periods making the procedure suitable as a preservation technique.

EXPERIMENTAL

Analytical grade fluorescamine was purchased from Fisher Scientific (Montréal, Canada) and a 0.025% (w/v) solution was prepared in acetone. A solution of stannous chloride was prepared by dissolving 0.5 g in 5 ml of concentrated hydrochloric acid and diluting to 120 ml with a solution of 50 ml of water plus 65 ml of acetone. This solution was always freshly prepared. Fenitrothion (Folithion) was obtained from Chemagro (Kansas City, Mo., U.S.A.). A stock solution was prepared 1 μg/μl in ethanol and dilutions were made in *n*-hexane.

Layers (250 μm thick) of silica gel H (20 × 20 cm) were prepared using a

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mixture of 30 g of silica gel H (Brinkmann, Rexdale, Canada) and 80 ml of 0.1 *M* KH_2PO_4. The plates were left to dry in air and were not activated before use.

Amberlite XAD-2 resin was supplied by BDH (Toronto, Canada). Prior to use it was washed successively with 50 ml of ether, 25 ml of methanol and 1 l of distilled water. The glass column was 50 cm × 2.2 cm I.D. and had a PTFE stopcock.

Apparatus

A Turner Fluorometer Model III (G. K. Turner Assoc., Palo Alto, Calif., U.S.A.) equipped with a Camag thin-layer chromatography (TLC) scanner was used for all quantitative fluorometric measurements. Excitation filter no. 7-60 (360 nm) and secondary filter No. 2-A (> 415 nm) both available from Corning (New York, N.Y., U.S.A.) were utilized.

A Perkin-Elmer 3920 gas–liquid chromatograph (Montréal, Canada) equipped with a flame photometric detector (FPD) was also used. The column contained 3% OV-101 on Chromosorb W. The temperature was 190°.

Methods

Preparation of the column. A glass-wool plug is first inserted in the column which is then filled with distilled water. The stopcock is opened while the resin (in a water slurry) is added until the desired length is obtained. Another glass-wool plug is put on top of the column.

Flow-rate. The flow-rate is easily measured by observing the time it takes for a water sample to pass through a 10-cm length of column. A vacuum may be used to accelerate the flow.

Recovery of fenitrothion and/or degradation products from water. A 1000-ml water sample containing 50 ppb of fenitrothion is allowed to percolate down the column. The level of water in the column may be kept above that of the resin but the water may also be removed or the column allowed to drain *e.g.*, for field purposes, as a preservation technique.

The column is then eluted with an appropriate organic solvent *e.g.*, ethyl ether or ethyl acetate. The eluting solvent is then either evaporated to a smaller volume or diluted to a known volume for analysis.

Analysis. For TLC and *in situ* fluorometric analysis the eluted sample is concentrated to 1 ml or less and a 10-μl aliquot is spotted on a TLC plate. The latter is developed in hexane–acetone (4:1) along with appropriate standards.

The nitro group is reduced as follows: the plate is sprayed to saturation with stannous chloride, allowed to stand 5 min and dried in a stream of cold air. The excess acid is neutralized by spraying lightly with aqueous (2 *M*) sodium carbonate. Fluorescence is obtained by spraying with fluorescamine.

For gas chromatographic (GC) analysis the eluted sample is concentrated to 50 ml in a volumetric flask (*i.e.*, for a concentration of at least 50 ppb) and a 5-μl aliquot is injected in the chromatograph. For samples less concentrated than 50 ppb, the solvent can be evaporated as desired.

RESULTS AND DISCUSSION

The conventional technique of recovering fenitrothion from natural waters is

TABLE I

RECOVERY OF FENITROTHION FROM DISTILLED WATER USING XAD-2

Method, TLC and *in situ* fluorometry; eluting solvent, diethyl ether (3 × 30 ml); column length, 12 cm.

Experiment No.	*Flow-rate (ml/min)*	*Recovery (%)*
1	147	99
2	142	82
3	152	88
4	142	97
5	147	96
6	144	104
Average	146	94
Standard deviation		7.9

by solvent extraction using chloroform, as an example. Since fenitrothion is very unstable in water approx. 25 ml of chloroform are added to the bottle at the sampling site and the sample is rushed to the laboratory for quick analysis. Analyses are usually carried out by GC using an FPD[7] or by *in situ* fluorometry after TLC[8]. Both analytical procedures are used in this study for comparison.

In a first experiment the recovery of fenitrothion from water at the 50-ppb level using an XAD-2 column instead of solvent extraction, has been studied (Table I). The average percent recovery is good considering the inherent error with the *in situ* fluorometric technique of analysis. The flow-rate is given in ml/min, and is obtained by adjusting the flow such that it takes 14–16 sec for the water to cross a 10-cm length of the column. Since $V = \pi r^2 l$, where the length l is equal to 10 cm and the column radius r 1.1 cm, the volume V is 38 ml.

The flow-rate, adjusted to 1 min, is shown in Table I. A small vacuum is necessary to attain this flow-rate and it takes approx. 7 min to pass a 1-l sample of water through an XAD-2 column.

The *in situ* fluorometric results for fenitrothion are confirmed by GC (Table II) even though a different solvent is used for elution; ethyl acetate is preferred to diethyl ether as a solvent for GC. In this experiment the volume of eluting solvent as well as the column length are optimized. Three portions of 30 ml of ethyl acetate seem

TABLE II

RECOVERY OF FENITROTHION FROM NATURAL WATER USING XAD-2

Method, GC with FPD; percent recoveries are the averages of three separate analytical determinations.

Volume of eluting solvent (ethyl acetate)	*Column length (cm)*	*Recovery (%)*
3 × 30	12	95
3 × 30	12	91
3 × 30	12	90
3 × 30	10	92
3 × 30	8	93
3 × 30	6	66
3 × 20	10	56
2 × 45	10	77

TABLE III

PRESERVATION OF FENITROTHION ON A 12-cm XAD-2 COLUMN

I, TLC and *in situ* fluorometry; solvent, diethyl ether; recoveries are averages of 3 TLC developments. II, GC with FPD; solvent, ethyl acetate; recoveries are averages of 3 injections.

I		*II*	
Time (h)	*Recovery (%)*	*Time (h)*	*Recovery (%)*
0	92	0	95
24	106	—	—
48	108	48	97
72	94	504	92
96	96	840	92
192	105		
240	98		

most appropriate and at a concentration of 50 ppb a 10-cm column can be used for 1 l of water. Recovery starts to decrease when the XAD-2 resin length is less than 10 cm.

Experiments have been carried out to determine whether fenitrothion is stable in the column or not. Some results by *in situ* fluorometry are given in Table III, and they show that after 10 days degradation is not important. These results are confirmed by GC whereas no degradation is visible over a 5-week period.

CONCLUSIONS

The use of Amberlite XAD-2 resin to recover fenitrothion from environmental water is a worthwhile venture. The procedure is adequate for fenitrothion but remains to be adapted to its degradation products. The most important aspect, however, is that the compound is stable in the column and as such the method becomes a preservation technique. In practice the water sample containing fenitrothion can be processed in the field and the column can be eluted and its content analysed some time afterwards. During all this time fenitrothion remains unchanged in the column and its concentration is representative of the time the water was sampled. This is a great improvement over the current technique of adding chloroform to the sample bottle after collection to preserve the sample only for a few days.

Another advantage is that the columns can be regenerated and re-used many times. Some columns have been used 20 times to recover fenitrothion from water without showing any signs of deterioration.

Several conditions, however, should be optimized. These are: the size of the glass column and consequently the volume of resin in the column; the volume of water that can be processed and the volume and type of solvent to use; the type of resin. Work is currently in progress to optimize these conditions for fenitrothion and its degradation products in water.

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Note

Usefulness of N-ethylmaleimide in the identification of ^{75}Se-labeled selenocysteine

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The discovery of several catalytically active selenoproteins[1–5] necessitates methods for identifying the form of selenium in these proteins. Identification of selenol compounds is particularly difficult because they are unstable[6]. They tend to decompose to elemental Se, oxidize to diselenides, and to react with sulfhydryl compounds; as a result, problems arise in their chromatographic identification[7]. Therefore, reagents that bind the selenol group to provide stable derivatives would be advantageous.

Carboxymethylation has recently been described for the identification of selenocysteine[8], and in the selenium-containing subunit of glycine reductase from *Clostridium sticklandii*, this selenoamino acid has been identified by formation of Se-carboxymethyl, Se-carboxyethyl, and Se-aminoethyl derivatives[5]. The sulfhydryl reagent, N-ethylmaleimide (NEM), has proved useful in the study of thiols[9]; it combines with the sulfhydryl group of cysteine yielding a derivative with distinctive chromatographic properties[10,11]. The similarity between sulfhydryl and selenol groups prompted us to investigate the ability of NEM to provide a similar derivative of selenocysteine.

MATERIALS AND METHODS

L-Cysteine · HCl was obtained from Eastman-Kodak (Rochester, N.Y.,U.S.A.); L-cystine from Nutritional Biochemicals (Cleveland, Ohio, U.S.A.); D,L-selenocystine and N-ethylmaleimide from Sigma (St. Louis, Mo., U.S.A.); N-ethylmaleimide (ethyl-1-^{14}C), 8.4 mCi/mmole, was obtained from New England Nuclear (Boston, Mass., U.S.A.). All reagents were analytical grade.

Conditions known to succeed with cystine were chosen to form the NEM adduct of selenocysteine. One milliliter of a D,L-selenocystine or L-cystine solution (6 μmoles/ml) was reduced by the addition of 1 ml of 0.6 *M* sodium borohydride prepared immediately before use. Reduction was carried out under nitrogen for 90 min. The reaction mixture was acidified to pH 5.0 with a solution containing 1.0 *M* HCl and 1 *M* KH_2PO_4 (ref. 12) and diluted to twice the initial volume with water. To an

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aliquot was added an equal volume of 6 m*M* NEM in 0.2 *M* phosphate buffer (pH 7.0); the mixture was allowed to react at room temperature for 1 h. L-cysteine·HCl was treated with NEM similarly. Aliquots were applied to Whatman No. 1 chromatography paper and developed in three different solvent systems[11,13]. Chromatograms were sprayed with 0.25% ninhydrin in acetone.

In experiments with ^{14}C-labeled NEM, 1 μCi of the radioactive NEM was added to the non-radioactive NEM. Ratemeter tracings of radioactive chromatograms were obtained with a Packard Model 7201 radiochromatogram scanner.

The reaction of NEM with cysteine or selenocysteine was monitored spectrophotometrically at 300 nm according to the method of Roberts and Rouser[14].

RESULTS AND DISCUSSION

Many selenium compounds exhibit the same paper chromatographic (PC) properties as their sulfur analogs[15]. It would be expected, therefore, that the R_F value of a selenocysteine–NEM adduct be identical to that of the cysteine–NEM adduct. The R_F values listed in Table I, obtained with three different solvent systems, show that the reaction between borohydride and selenocystine generated a product that reacted with NEM to give a ninhydrin positive spot with an R_F identical to that of the cysteine–NEM adduct. Radioactive NEM travelled close to the solvent front. The reaction mixture could be frozen for several weeks with no apparent decomposition of the NEM adduct.

TABLE I

PAPER CHROMATOGRAPHY OF CYSTEINE- AND SELENOCYSTEINE-[^{14}C]NEM ADDUCTS IN USING THREE SOLVENT SYSTEMS

Solvent systems: EBFW = ethanol–*tert.*-butanol–formic acid–water (60:20:5:15)[11]; BFW = *tert.*-butanol–formic acid–water (70:15:15)[11]; BAW = *n*-butanol–glacial acetic acid–water (60:15:25)[13].

Compound	R_F *values*		
	EBFW	*BFW*	*BAW*
Cysteine·HCl	0.56	0.44	0.24
Cystine	0.16	0.06	0.02
Selenocystine	0.17	0.06	0.03
Cysteic acid	0.20	0.08	0.03
Cysteine-[^{14}C]NEM	0.66	0.57	0.33
$NaBH_4$ reduced cystine-[^{14}C]NEM	0.67	0.56	0.33
$NaBH_4$ reduced selenocystine-[^{14}C]NEM	0.66	0.55	0.32
[^{14}C]NEM	0.92	0.95	0.94

Ratemeter tracings of radiochromatograms verified the formation of the selenocysteine–[^{14}C] NEM adduct. Fig. 1 shows the coincidence of radioactivity with the predominant ninhydrin positive spot. A second, radioactive spot of lesser intensity and with an R_F equal to that of cystine and selenocystine was also present. The NEM adduct travelled as a discrete spot with no evidence of streaking. The absence of radioactivity at the R_F of [^{14}C] NEM and with the presence of a single radioactive peak, associated with the single ninhydrin positive spot of the adduct in three different solvent systems, indicate a stoichiometric utilization of NEM.

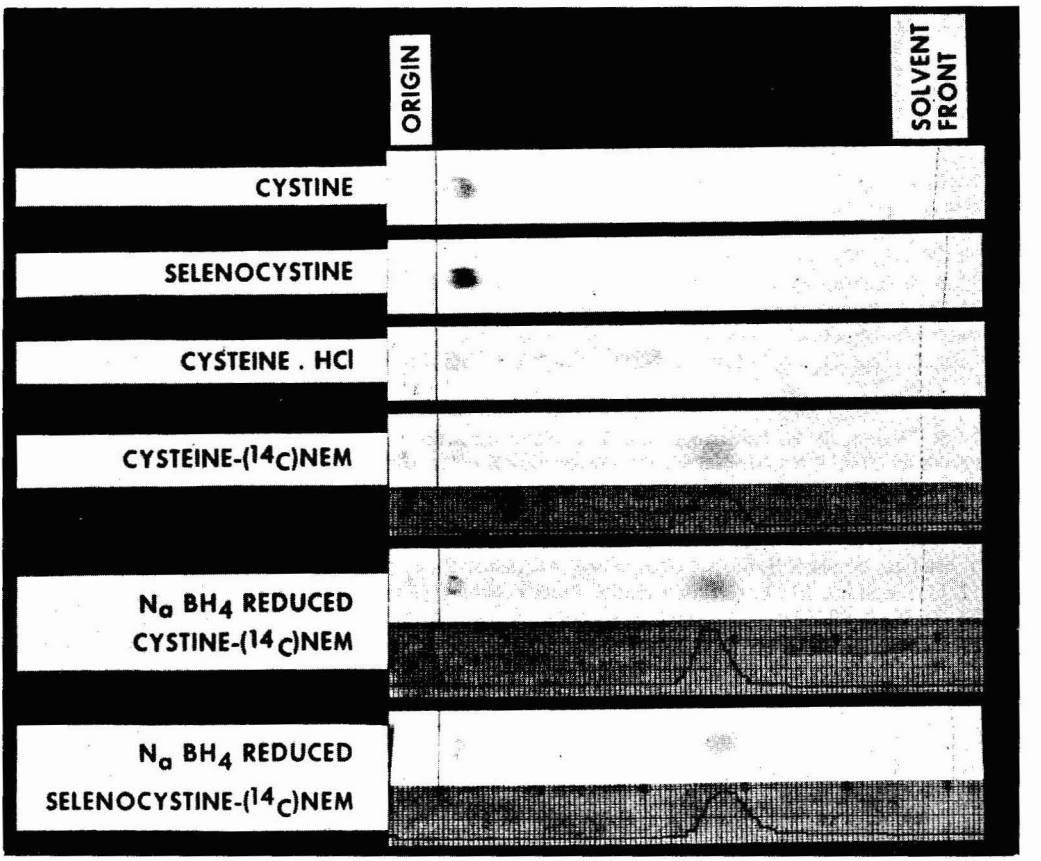

Fig. 1. Paper chromatograms of cysteine- and selenocysteine-[^{14}C]NEM adducts. Solvent: *tert.*-butanol–formic acid–water (70:15:15). R_F values are given in Table I.

The reaction of NEM with cysteine or selenocysteine was also monitored spectrophotometrically at 300 nm. However, quantitative determinations of selenol concentrations could not be obtained because the spectrophotometric data were variable (the cause of this variability is under investigation). Nevertheless, a decrease in absorbance was observed in all experiments, and is further evidence for the reaction between NEM and selenocysteine.

The binding of NEM to selenocysteine provides another approach to the identification of selenol compounds in cell extracts and proteins. A stabilized selenol group would be less likely to undergo oxidation during the extraction procedure; it is also likely that decomposition and streaking during chromatography would be eliminated. The NEM adduct should also be stable to enzymatic hydrolysis as has been found with the Se-carboxymethyl, Se-carboxyethyl, and Se-aminoethyl derivatives derived from digests of glycine reductase treated with the respective alkylating agents[5]. These advantages, we believe, will facilitate the identification of the selenium moiety in ^{75}Se-labeled selenoproteins.

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CHROM. 10,142

Note

Identification and determination of 1,3-diisobutyrate-2,2,4-trimethylpentane (Texanol isobutyrate) in polyvinyl chloride plastisol coating formulations

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Analytical problems may arise in the quality control of the manufacture of plastisol films with desired durability and resistance to staining by foot traffic due to the necessarily low levels of plasticisers in the final products and also from the differential loss of certain components of plasticer blends during processing. A mixture of Texanol isobutyrate (TXIB), butyl benzyl phthalate (BBP), and an epoxydised tall oil (ETO) have been used in the manufacture of a fused plastisol film from a paste polyvinyl chloride resin. The addition of TXIB was to attain a suitable plastisol spreading viscosity, the BBP was the primary plasticiser[1] and ETO a heat stabiliser to protect the plastisol from degradation during fusion. Because of the high fusion temperature, 200°, and the large exposed surface area to volume ratio (film thickness 0.016 in.) much of the volatile plasticiser components are lost, particularly TXIB, the residual amount of which is important in final product performance.

EXPERIMENTAL

Materials and formulations

The plasticisers used herein are specified in Table I. The solvent used, tetrahydrofuran, was of technical reagent grade (BDH, Poole, Great Britain). The dibutyl phthalate, internal standard, was supplied by Geigy (Manchester, Great Britain). The paste resin was Breon P 130/1[2] (BP, London, Great Britain).

Plastisol formulations were prepared in the usual manner by mixing components under vacuum, to remove air, in a paddle type blender. A typical formulation composition is shown in Table II.

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TABLE I

PLASTICISERS

No.	*Name*	*Abbreviation code*	*Source*
1	Texanol isobutyrate	TXIB	Eastman (Hemel Hempstead, Great Britain)
2	Butyl benzyl phthalate	BBP	Monsanto (Ruabon, Great Britain)
3	Epoxydised tall oil	ETO	Lankro (Manchester, Great Britain)

TABLE II

TYPICAL FORMULATION COMPOSITION BEFORE FUSION

Component	*Percentage by weight*
Paste polymer	71.7
TXIB	17.9
BBP	3.6
EPO	3.6
Other components	3.12

Apparatus

A Becker Model 417 gas chromatograph equipped with a flame ionisation detector was used isothermally. The optimised conditions for analysis, determined in preliminary studies were: column 1 m × 2.6 mm I.D. stainless steel; stationary phase, 10% SE-30 on Chromosorb W HP (80–100 mesh); oven temperature, 200°; injection block temperature, 250°; detector temperature, 275°; air flow-rate 300 ml/min; hydrogen flow-rate, 30 ml/min; carrier gas, nitrogen; nitrogen flow-rate, 30 ml/min; recorder, 10 mV; attenuation, × 8.

Preparation of sample solutions

TXIB calibration standards were 0.025–0.10% (w/v) in tetrahydrofuran containing 0.2% (w/v) dibutyl phthalate. BBP and EPO solutions were 0.1% (w/v) in tetrahydrofuran containing 0.2% (w/v) dibutyl phthalate.

The finished flooring products normally consist of several layers of different materials. The upper plastisol layer, the wear resistant layer, thus has to be carefully pared from the flooring base layer composition to avoid contamination. The separated upper layer (0.2 g) was cut into small pieces and dissolved in tetrahydrofuran (10 ml) containing 0.2% (w/v) dibutyl phthalate. Gentle warming facilitates the dissolution. 3.5-μl volumes of each sample solution, in turn, were injected directly on column and chromatograms recorded.

RESULTS

It was found that only TXIB, BBP, the internal standard and solvent eluted from the column and were resolved well under the conditions specified. ETO and the other components remained on column. The retention data are given in Table III.

The calibration graph for TXIB was linear over the range examined. Chromatograms of dissolved plastisol films showed only peaks for TXIB and that BBP was absent from the fused materials examined as shown in Fig. 1. Quantitative examination based on peak heights of 5 replicate injections from single samples gave

TABLE III

RETENTION TIMES

Component	*Time (min)*
Tetrahydrofuran	0.1
TXIB	0.83
Dibutylphthalate	4.13
BBP	5.1
ETO	retained on column

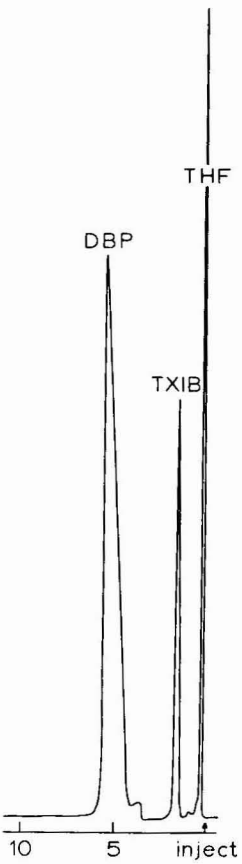

Fig. 1. Typical chromatogram of fused polyvinyl chloride plastisol coating. DBP = dibutyl phthalate; THF = tetrahydrofuran.

standard deviations of 0.11% TXIB at levels which were for each sample studied in the range 1.3–1.6% TXIB.

DISCUSSION

The differential loss of plasticisers during the fusion stage of the manufacturing process was confirmed by infrared (IR) examination of non-volatile portions of

extracts of fused plastisol films. Extraction (24 h) of fused coating[3] in ethanol (94 vol %) gave a non-volatile oily residue (105°) of 6.5% (w/w). The initial plasticiser plus oil content was 28.2% thus some 21.7% was lost on fusion.

IR examination of the extract showed it to be mainly ETO (b.p. 260° at 2 mm Hg). The peaks between 650 cm^{-1} and 800 cm^{-1}, characteristic of BBP (b.p. 370° at 768 mm Hg), were absent and those for TXIB were weak.

The proposed method is more rapid than solvent extraction and/or hydrolysis[4] procedures and allows ready quantitation of the residual volatile plasticiser, TXIB, and is thus suitable for routine quality control in the manufacture of plastisol based products.

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Journal of Chromatography, 139 (1977) 399

CHROM. 10,146

Book Review

Analysis of essential oils by gas chromatography and mass spectometry, by Y. Masada, Halsted (Wiley), New York, London, Sydney, Toronto, 1976, XV + 334 pp., price £ 28.90, US$ 49.70, ISBN 0-470-15019-X.

This book, which is a development of the author's earlier book on the "Analysis of essential oils by gas chromatography" published in 1968, is divided into two parts. The first part consists of reports on the gas–liquid chromatography of 64 essential oils and the mass spectrometry of 52 constituents of these oils. The work was undertaken in the author's laboratories using glass capillary columns coated with Carbowax 20M on an Hitachi-K53 gas chromatograph coupled to an Hitachi RMU-6E single focussing mass spectrometer employing an ionising voltage of 70 eV.

The value of the results as far as an analyst is concerned is like the proverbial curate's egg, good in some parts and bad in others and as is usual in such situations, where it is good it is very very good but where it is bad it is useless. The information and data provided in the monograph on oil of chamomile is an example of the latter. As the text states there are two oils of chamomile, one from Roman chamomile (*Anthemis nobilis*, now known as *Chamoemelum nobile*) and the other from German chamomile (*Matricaria chamomilla*), the main constituents of the former being the esters of angelic and isobutyric acid and that of the latter being chamazulene and α-bisabolol. However, the chromatogram contains none of these constituents and makes no attempt to explain the origin of the oil examined. Similar criticisms may be made of the monograph on oils of anise, coriander and nutmeg chiefly because there is no attempt to differentiate between oils from plant material of different biological sources (*e.g. Pimpinella anisum* or *Illicium verum*) or from different geographical sources. On the other hand the information given on oils of cardamonis, cinnamon, citranellol, hops, pepper, peppermint and orange is very comprehensive and is very useful for analytical purposes. Some information is given about a number of unusual essential oils, *e.g.* Ho Leaf oil, Kuromoji oil and Vetiver oil, all of which are obtained from plants which grow in the Far East.

In Part II there are some very brief notes, in Japanese, on essential oils, gas–liquid chromatography and mass spectrometry but even if the text had been in English it would hardly have added to the value of the book. Any analyst who uses these techniques in the analysis of essential oils must already have far more knowledge about them than these notes provide.

Nevertheless, it is a reference book that essential oil analysts will find useful, particularly as it possesses a good bibliography.

London (Great Britain) E. J. SHELLARD

Author Index

Errata

J. Chromatogr., 136 (1977) 147–153

Page 150, Table II, $\frac{1}{2}(RI)$ values of substitution patterns "2, 3, 4, 5" and "2, 3, 4, 5, 6", for columns 1–13 should read:

2,3,4,5	1312	1304	1349	1533	1354	1475	1559	1571	1631	1679	1410	1770	1423
2,3,4,5,6	1380	1369	1392	1620	1417	1549	1620	1576	1648	1702	1480	1773	1439

J. Chromatogr., 136 (1977) 289–300

Page 294, Table I, 3rd column, last line "1.95730" should read "0.95730".

Vol. 139, No. 2

journal of
chromatography news section

APPARATUS

N-1034

TUBING CLIP

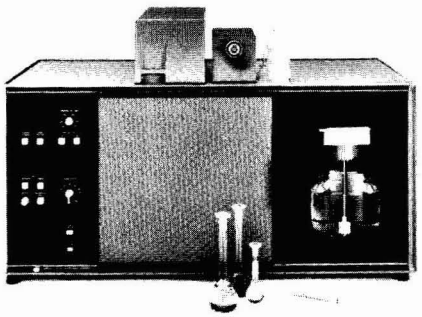
The tubing clip recently introduced by Hamilton Bonaduz can be fixed on beakers, flasks, test tubes, instrument boards, etc., up to a wall thickness of 4 mm. The tubing is guided, held and clamped, thus minimising cracking, kinking and leaking.

For further information concerning any of the news items, apply to the publisher, using the reply cards provided, quoting the reference number printed at the beginning of the item.

N-1041

NEW HIGH-PERFORMANCE LIQUID CHROMATOGRAPH

Hewlett-Packard announce a new, more compact, high-performance liquid chromatograph which performs with an accuracy and precision similar to that of the HP 1080 series. All components including pump, injector, column, detector and solvent reservoir, are contained in the mainframe of the instrument. The solvent

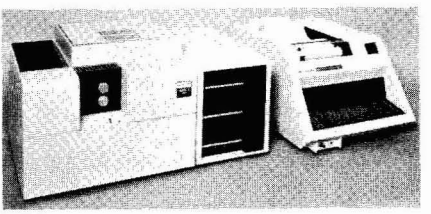


flow is set by a push-button-controlled stepping motor which adjusts the strokes of the single-head diaphragm pump. The valve loop injector (allowing sample introduction at full column pressure without flow interruption) has a standard 20-μl loop, but loops of 10, 50 and 100 μl are also available. The single-wavelength UV detector with high signal-to-noise ratio operates at 254 nm. The instrument, with analogue-signal output, can be linked directly to conventional integrators and strip-chart recorders or interfaced to a computer via an A to D converter.

N-1036

NEW LINE OF GAS CHROMATOGRAPHS

Perkin-Elmer has announced a new line of four complementary gas chromatographs, a lab data system and accessories – the Sigma Series – which provides a selection of interchangeable components. The system comprises: Sigma 1 (photograph), a system combining one or more gas chromatographs with integral control and data handling; Sigma 2, a multi-detector, microprocessor-controlled gas chromatograph;

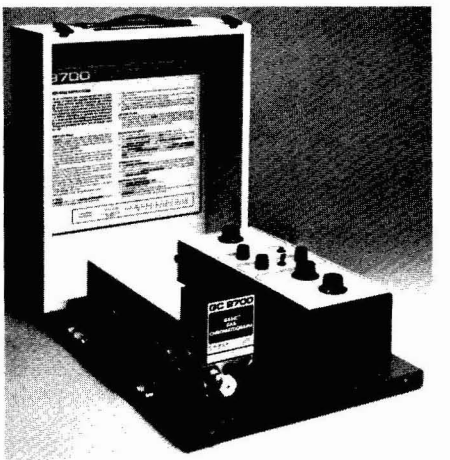


Sigma 3, designed for routine, temperature-programmed analysis, a microprocessor-controlled gas chromatograph; Sigma 4, an isothermal gas chromatograph, especially suited for routine applications and for instructional purposes. Sigma 10 is a stand-alone lab data system version of the data reduction, printer/plotter capability that is standard with Sigma 1. Components and accessories include detectors, pneumatic units, columns and column oven, injectors and sampling systems.

N-1039

BASIC™ GAS CHROMATOGRAPHS FROM CARLE

The new models of Basic gas chromatographs from Carle Instruments are factory-dedicated instruments able to perform one of 15 different applications. Models are available with thermal conductivity (GC 8700) or flame ionization (GC 9700, photograph) detectors, and feature built-in valving when appropriate, using Carle mini volume valves, placed in a heated compartment at the front of the instrument.



MEETING

THE 28TH PITTSBURGH CONFERENCE ON ANALYTICAL CHEMISTRY AND APPLIED SPECTROSCOPY

The 28th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy was held from February 28 till March 4 this year. For many years, this conference, organised by the Society for Analytical Chemists of Pittsburgh and the Spectroscopy Society of Pittsburgh, has been held in the Cleveland Convention Center in Cleveland, Ohio, U.S.A.

The spacious Mall Exhibit Area of the Cleveland Convention Center traditionally houses the exhibition of modern laboratory equipment. This instrument show is gaining in importance every year and has become the major spot in the world in the last few years for introducing new or modified apparatus, especially that designed for use in analytical chemistry. The exhibition is dominated by the instruments employed in spectroscopy and in chromatography.

At this year's "Cleveburgh Show", the Perkin-Elmer Corporation from Norwalk, Conn., occupied even more space than at last year's exhibition. The company showed no fewer than 23 new products. Spectacular and dominating the vast booth area of Perkin-Elmer, was the totally new

SIGMA series of gas chromatographs. This series is designed in the U.S.A. by a team of scientists and technicians from the factories in the U.S.A., Great Britain and the G.F.R. The series consists of four gas chromatographs and a new data processing system called SIGMA-10.

The SIGMA-1 gas chromatograph consists of one or two analytical units controlled by a microprocessor system. All injector and detector options of the series can be used with this SIGMA-1. The analytical parameters are set using a keyboard. The microprocessor system of the SIGMA-1 provides data reduction for up to four detectors simultaneously. The SIGMA-2 is a dual-channel, microprocessor-controlled, temperature-programmable gas chromatograph. The analytical parameters are set using a built-in keyboard or are entered through the available card reader accessory. Especially designed for the routine temperature-programmed analyses is the third member of the family, the SIGMA-3, which is also controlled by a microprocessor. Designed for simple routine analyses is the SIGMA-4, which is compactly built and for use in isothermal work with most of the available detectors.

The new SIGMA-10 data system can handle data from the gas chromatographs of the series or from other gas or liquid chromatography instruments. Annotated chromatograms can be obtained for any of the four active channels connected to the SIGMA-10 and multiple data files and methods can be stored.

As an extension of their liquid chromatography programme, Perkin-Elmer introduced three new solvent delivery systems based on a reciprocating pump. This series consists of the modular-built instruments Series 2/1, Series 2/2 and Series 3. The last, and most versatile, instrument is controlled by a microprocessor. The new instruments are compatible with all commercially available liquid chromatography detectors. Also for liquid chromatographic work, Perkin-Elmer introduced the new LC-65T detector/oven. This LC-65T features detection in the far UV region, specific detection at whatever wavelength is required for the compound of interest and linear detector response over the working range.

Mention of the slightly modified fluorescence spectrometers of the Series 1000 and 204 may serve to close this imcomplete survey of the Perkin-Elmer chromatography news from the Pittsburgh Conference 1977.

Waters Associates Inc. from Milford, Mass., exposed their new automated sample injector Model 710 WISP (Waters' Intelligent Sample Processor). This sample injector is the first part of a new Waters liquid chromatography system. Later this year, the company will introduce the corresponding data/control terminal. The WISP and the data/control terminal represent a continuation of Waters' modular-design philosophy. This new generation of Waters' instrumentation will, of course, be microprocessor-based.

Also microprocessor-based is the Model 429 gas chromatograph, the newest product of Packard-Becker BV, the Dutch branch of the Packard Instrument Company, Inc., Downers Grove, Ill. The new instrument is an extension of the gas chromatography line started with the routine Model 427, shown at the 1976 Pittsburgh Conference. The microprocessor in the 429 instrument controls the analytical parameters which can be set using the instrument's simple keyboard. With this new gas chromatograph, Packard-Becker introduced their new detectors especially designed for the 42X series.

Philips Electronic Instruments, Inc., Mahwah, N.J., showed the Pye-Unicam LC-3 liquid chromatograph. The instrument is already quite well-known in Europe, but it was new for the U.S.A. market.

Hewlett-Packard's Avondale Division exhibited the German-designed 1082/4 liquid chromatographs for the first time at the Pittsburgh Conference. The automatic sampler for this line of instruments was especially new. A further fresh note was HP's 5985, a gas chromatography–mass spectometry combination, based on the analyser system of the little 5992 and extended with a chemical ionisation possibility and a more powerful data system.

Varian Associates, Palo Alto, Calif., showed the capillary version of the Model 3700 gas chromatograph, the totally automated version 3711 and a number of extensions in the 8500 liquid chromatography programme, including the new fluorescence detector Fluorichrom. From Germany came the Varian MAT 44 gas chromatography–mass spectrometry combination, an instrument

with digital electronics, fully microprocessor-controlled and equipped with a CRT display. The instrument parameters are set using a keyboard unit connected to the instrument by a flexible cable. The Varian MAT 44 was shown with the new SS200 data system, which has also been developed in the G.F.R.

The trends in chromatography instrumentation were clearly visible in the exhibition area of the Pittsburgh Conference 1977. In the first place, there was a dominating trend of using microprocessors and microcomputers as an advanced piece of intelligence in the instruments. Their purpose is to control the actions of certain essential parts of the instrument and to stand guard for failures in the operation. Secondly, there was the still-growing interest in the application of capillary columns in gas chromatography. Not only did the manufacturers show capillary-friendly apparatus and splitless systems, but also the scientific world expressed strong interest. At the Conference itself several papers were presented on this and related subjects. Finally, there was growing interest in auto-sampling systems, not only for the purpose of handling vast numbers of samples, but more for the purpose of gaining reproducibility.

NEW BOOKS

Lipid chromatographic analysis, Vol. 3, by G.V. Marinetti, Marcel Dekker, New York, Basel, 2nd (revised) ed., 1976, ix + 289 pp., price SFr. 115.00, ISBN 0-8247-6357-2.

Principles of organic chemistry, by T.A. Geissman, Freeman, Reading, 4th ed., 1977, 1035 pp., ISBN 0-7167-0177-4.

Physical biochemistry - Applications to biochemistry and molecular biology, by D. Freifelder, Freeman, Reading, 1976, 507 pp., price £ 12.80, US$ 20.30 (hard cover), £ 8.00, US$ 12.00 (soft cover), ISBN 0-7167-0559-1.

Experimental biochemistry, edited by J.M. Clark, Jr. and R.L. Switzer, Freeman, Reading, 2nd ed., 1977, in press.

Chemistry of marine sediments, by T.F. Yen, Ann Arbor Sci. Publ., Ann Arbor, Mich., 1977, 266 pp., price US$ 24.95, ISBN 0-250-40103-7.

Identification and analysis of organic pollutants in water, by L.H. Keith, Ann Arbor Sci. Publ., Ann Arbor, Mich., 1976, 707 pp., price US$ 27.50, ISBN 0-250-40131-2.

Carbonate chemistry of aquatic systems, Vol. II, by R.E. Loewenthal and G.v.R. Marais, Ann Arbor Sci. Publ., Ann Arbor, Mich., 1977, price US$ 24.50, ISBN 0-250-40150-9.

Colorimetric chemical analytical methods, by L.C. Thomas and G.J. Chamberlin, Wiley, London, New York, Sydney, Toronto, and Tintometer, Salisbury (G.B.), Dortmund, 8th ed. 1974 (published 1977), XLI + 626 pp., price £ 20.00, US$ 34.40, ISBN 0-471-99525-8.

Vibrational spectra and stucture: A series of advances, Vol. 6, edited by J.R. Durig, Elsevier, Amsterdam, Oxford, New York, 1977, XIV + 398 pp., price Dfl. 146.00, US$ 59.60, ISBN 0-444-41588-2.

Rodd's Chemistry of carbon compounds, Vol. IV, Heterocyclic compounds, Part E, edited by S. Coffey, Elsevier, Amsterdam, Oxford, New York, 2nd ed., 1977, XVIII + 494 pp., price Dfl. 195.00, US$ 79.75 (subscription price Dfl. 169.00, US$ 68.95), ISBN 0-444-41363-4.

Assay of drugs and other trace compounds in biological fluids, (Methodological Developments in Biochemistry, Vol. 5), edited by E. Reid, North-Holland, Amsterdam, New York, 1976, X + 254 pp., price Dfl. 65.00, US$ 26.75, ISBN 0-7204-0584-X.

The analysis of rocket propellants, by H.E. Malone, Academic Press, New York, London, 1976, x + 148 pp., price US $ 14.25, £ 5.50, ISBN 0-12-466750-3.

Pesticide chemistry in the 20th century (ACS Symp. Series No. 37), edited by J.R. Plimmer, American Chemical Society, Washington, D.C., 1977, ix + 310 pp., price US $ 20.00, ISBN 0-8412-0364-4.

GENERAL INFORMATION

(A leaflet *Instructions to Authors* can be obtained by application to the publisher.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (full-length papers), short communications and notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as full-length papers, but should preferably not exceed four printed pages. For reviews, see page 2 of cover under Submission of Papers.

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Summary. Full-length papers and review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes are published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. One original and two photocopies are required. Attention should be given to any lettering (which should be kept to a minimum) and to spacing on axes of graphs in order to ensure that numbers etc. remain legible after reduction. Axes of a graph should be clearly labelled. The figures should preferably be of such a size that the same degree of reduction can be applied to all of them. Photographs should have good contrast and intensity. Sharp, glossy photographs are required to obtain good halftones. References to the illustrations should be included in appropriate places in the text using arabic numerals. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet*. If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the authors' expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

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1 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
3 H. C. S. Wood and R. Wrigglesworth, in S. Coffey (Editor), *Rodd's Chemistry of Carbon Compounds, Vol. IV, Heterocyclic Compounds*, Part B, Elsevier, Amsterdam, Oxford, New York, 2nd ed., 1977, Ch. 11, p. 201.
4 E. C. Horning, J.-P. Thenot and M. G. Horning, in A. P. De Leenheer and R. R. Roncucci (Editors), *Proc. 1st Int. Symp. Quantitative Mass Spectrometry in Life Sciences, Ghent, June 16–18, 1976*, Elsevier, Amsterdam, Oxford, New York, 1977, p. 1.

Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts*. Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication". The *Journal of Chromatography; Journal of Chromatography, Biomedical Applications* and *Chromatographic Reviews* should be cited as *J. Chromatogr.*

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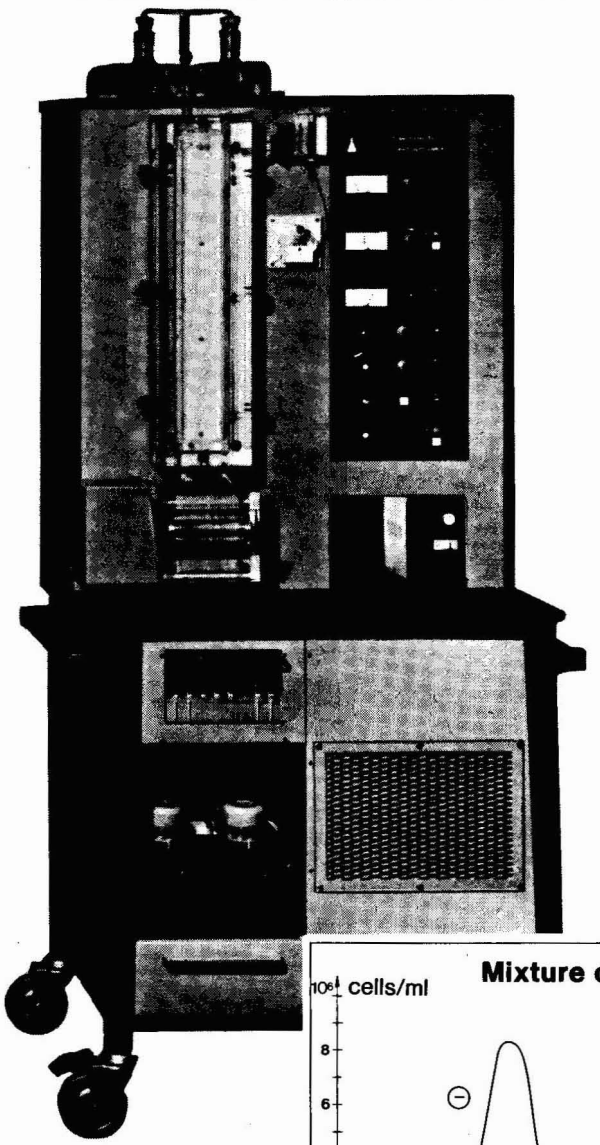

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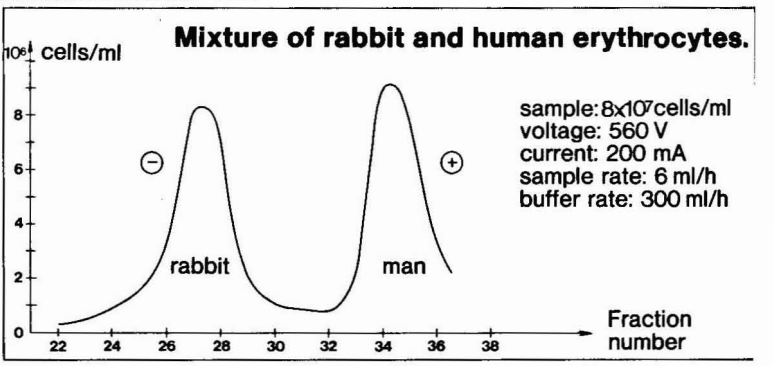

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