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ORTNIGHTLY

VOL. 95 NO. 2 AUGUST 14, 1974

THIS ISSUE COMPLETES VOL. 95

URNAL OF CHROMATOGRAPHY

TERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

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CONTENTS

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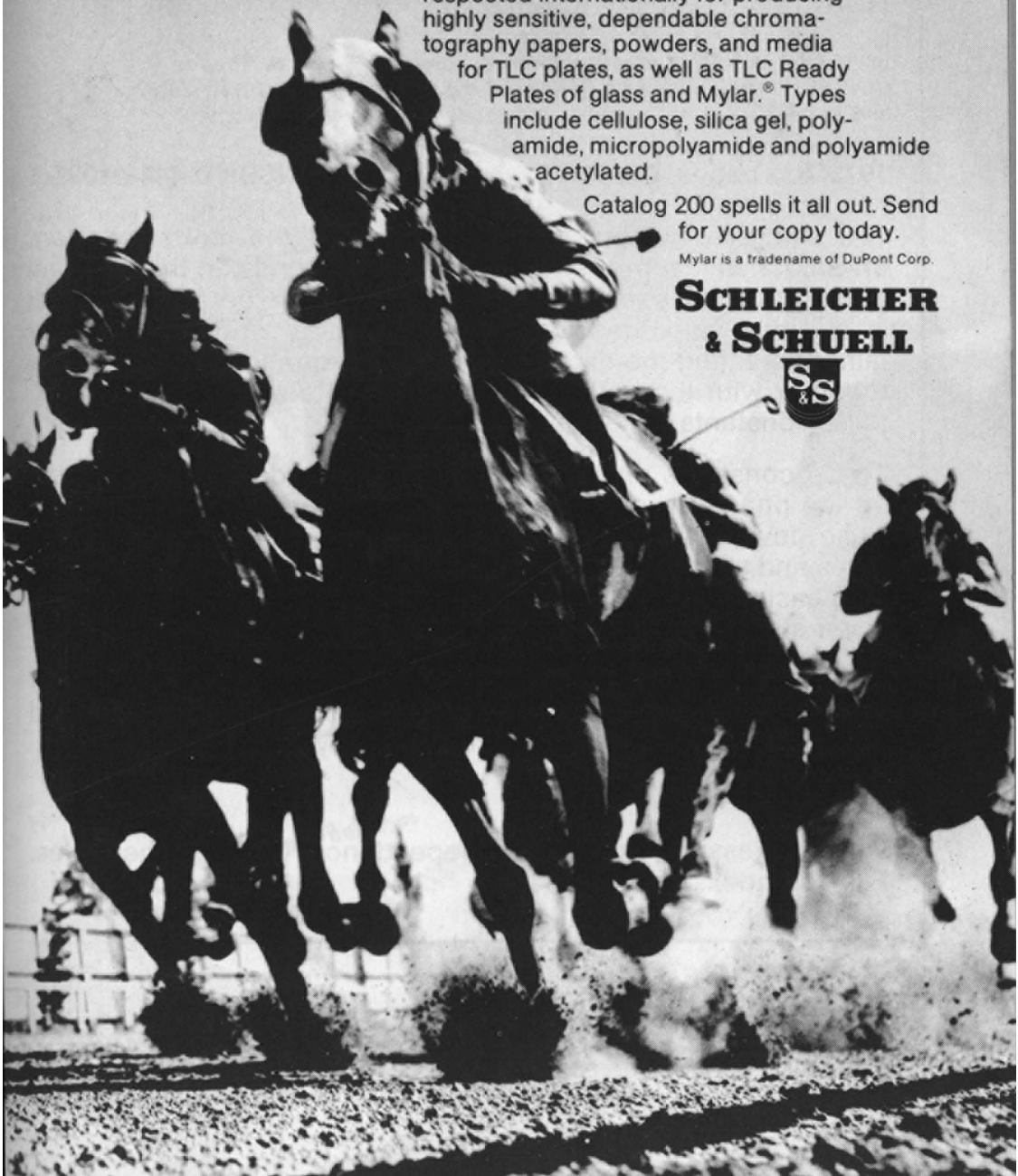

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The Vapour Pressures of Pure Substances

Selected Values of the Temperature Dependence of the Vapour Pressures of Some Pure Substances in the Normal and Low Pressure Region.

by TOMÁS BOUBLÍK, *Czechoslovak Academy of Sciences, Prague,*
VOJTECH FRIED, *Brooklyn College of the City University of New York,*
and ERUARD HÁLA, *Czechoslovak Academy of Sciences, Prague.*

1973. 632 pages. Dfl. 75.00 (about US$26.30). ISBN 0-444-41097-X

The saturated vapour pressure is one of the more important physicochemical properties of pure compounds. In this book an extensive set of experimental vapour pressure data is collected; in addition smoothed values of the vapour pressures, as obtained by fitting the data to the Antoine equation, are presented together with the calculated boiling points and sets of the Antoine constants for each compound.

These constants provide in themselves condensed information on the phase behaviour, very suitable for practical application, while deviations of the smoothed from the experimental pressures and standard deviations (also listed) indicate the accuracy of measurements and the reliability of the equation with the given set of constants.

The choice of systems presented in the tables was governed by the availability and quality of the experimental data, and partly by the practical importance of the system in question.

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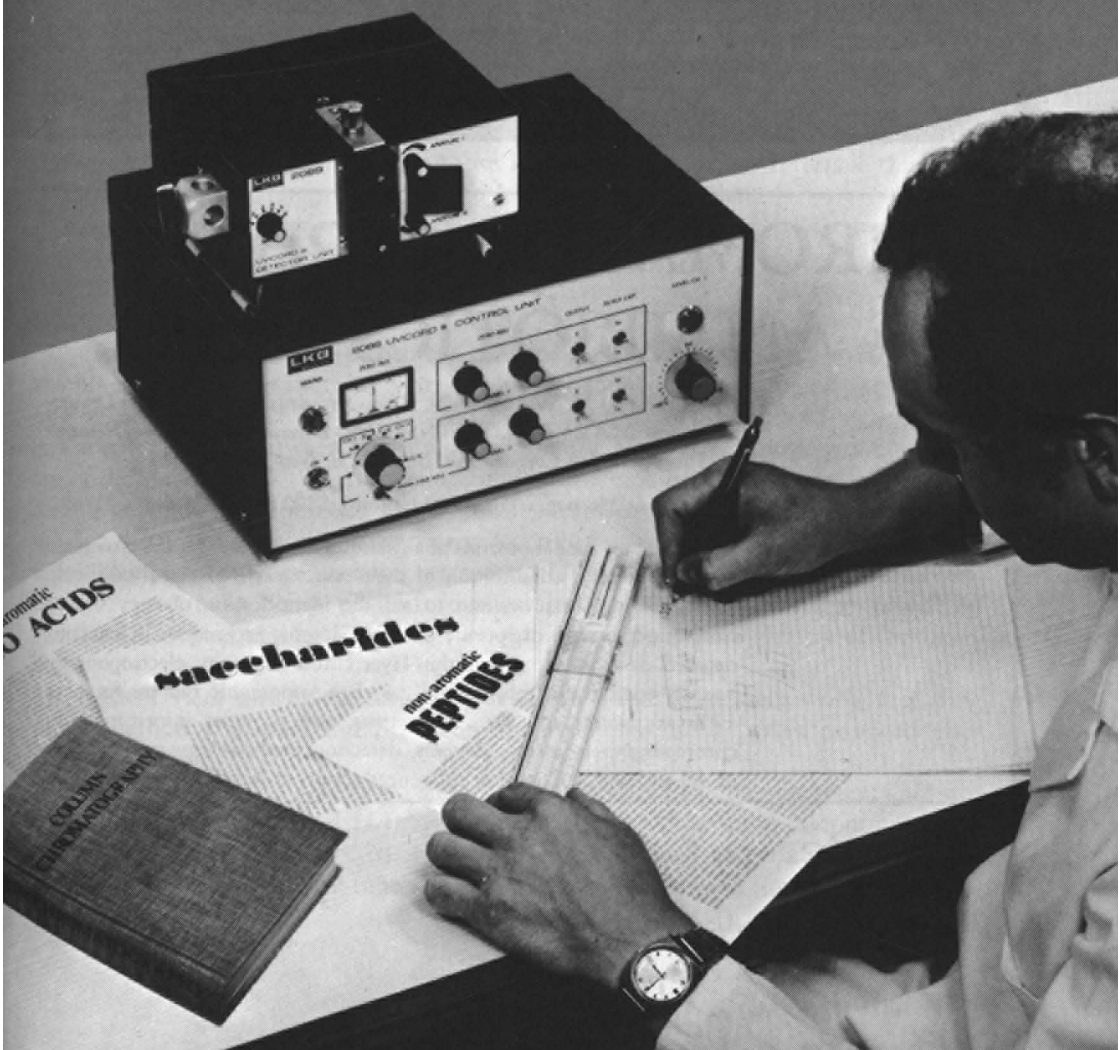

Journal of Chromatography, 95 (1974) 133–145

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ISOTACHOPHORESIS (DISPLACEMENT ELECTROPHORESIS, TRANSPHORESIS) THEORY

STRUCTURE OF THE IONIC SPECIES INTERFACE

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(Received January 2nd, 1974)

SUMMARY

An approximate solution is given for the equations governing the structure of the inter-species ionic interface in discrete sample isotachophoresis (displacement electrophoresis, transphoresis) in an ideal one-dimensional system, in the presence of a common counterion and in the absence of continuous mobility spectrum spacer ampholytes. It is shown that the approximation is reasonably good for values of the Kohlrausch-regulated terminator/leader concentration ratio greater than 0.5. The dependence of interface thickness on mobility and concentration ratios is discussed and results are compared to those in previous literature. Interface thickness is found to be inversely proportional to leader voltage gradient.

INTRODUCTION

Isotachophoresis[1] (displacement electrophoresis[2], transphoresis[3]) is an electrophoretic separation method characterized by equal velocities for all ions migrating in the system once equilibrium has been attained. The sample to be separated is placed in a tube between leader and terminator electrolyte solutions whose ions are of the same sign as the sample ions, and whose mobilities are respectively higher and lower than the mobilities of any of the sample ions. In the absence of co-moving continuous mobility spectrum spacer ampholytes[1], the sample ions separate into a number of contiguous compartments arranged in the order of their mobilities, each compartment being of uniform characteristic concentration governed by the Kohlrausch regulating function[4] (see eqn. 33) except in the vicinity of the interfaces.

The system was first described by Kendall[5], and subsequent work, including direct method transport number determinations, has been partly reviewed elsewhere[1,3,6–10].

Because of diffusion effects, the interfaces between the compartments are not sharp (although in most cases they are much sharper than ionic discontinuities in other forms of electrophoresis), but rather a continuous distribution of all ions exists throughout the entire tube. However, in discrete sample (as opposed to con-

tinuous frontal) separations, the bulk of the concentration change of the individual ion species occurs in a very short length of tube if the mobility differences are not excessively small. We refer to the distance over which the concentration changes from 99% to 1% as the interface thickness, Δ. This paper presents an approximate method for determining the structure of the concentration and potential gradient profiles and the interface thicknesses based on a one-dimensional analysis of the interface which would exist between leader and terminator in a sample-free model.

Such a one-dimensional system has no physical existence, but may approximate isotachophoretic behaviour under ideal conditions. It is, therefore, a first step in the theory from which the three-dimensional case may follow if the radial and longitudinal non-uniformities of real systems can be estimated. Such non-uniformities include the effects of radial and longitudinal temperature gradients and the various property variations which result from them, electro-osmosis, electro-convection, and gravity-dependent phenomena such as sedimentation and thermal convection. Further corrections are also required for partly ionized compounds and macromolecules.

A theoretical prediction of the thickness of isotachophoretic interfaces is of importance in evaluating the maximum resolution of the method for preparative applications, and in determining in what ways experimental parameters may be varied to achieve optimal performance.

A list of symbols is given at the end of the paper.

PREVIOUS WORK

Previous work concerning the determination of the interface thickness has been restricted for the most part to one-dimensional interfaces (*i.e.*, no variations in a radial direction, steady state conditions) between two essentially 100% dissociated electrolyte solutions having a common counterion. The effects of electro-osmosis and temperature gradients have been dealt with only in a qualitative manner. The basic differences between the various analyses to date lie in the additional assumptions made in each case to render the problem tractable.

Longsworth[11] obtained solutions for the concentration profiles of leader, terminator, and counterion for the case in which the mobilities of leader and counterion were equal and twice the mobility of the terminator. In his analysis he imposed the condition of electrical neutrality throughout the entire system. Although this is certainly true for points removed from the interface region, Gauss's law requires some net charge to exist (however small) in the interface region in order that an electric field gradient exists there.

Martin and Everaerts[2] consider the case where leader and terminator mobilities are approximately equal and assume that diffusion effects can be characterized by a single diffusion coefficient for all three ion species. Their results do not show a dependence of front thickness on counterion mobility. They and Routs[12] rewrite the Kohlrausch regulating function to account for partial ionization and compartmental pH differences, with attendant mobility and concentration differences.

Westhaver[13] considers the case of a very thick interface where there is a small mobility difference, the interface representing a form of frontal enrichment.

Konstantinov and Oshurkova[14] consider only the species continuity equations for leader and terminator and obtain solutions for the ratio of either leader or terminator concentration to the sum of the leader and terminator concentrations. A similar solution is obtained by Routs[15] for the ratio of leader concentration to terminator concentration.

Hall and Hinckley[16] obtain solutions valid at points removed from the vicinity of the interface and then estimate interface thicknesses for all three ion species from this. Hinckley[17] has predicted that the interface thickness should be inversely proportional to the leader voltage gradient on the basis that difussion depends on both mobility and concentration gradient, and the latter increases with reduction of interface thickness (*cf.* Routs[15]).

Brouwer and Postema[18] consider the unsteady problem but neglect diffusion, thus arriving at a steady state in which all components are separated into distinct compartments, each compartment containing only one component, and hence do not obtain estimates of interface thickness.

GOVERNING EQUATIONS FOR THE ONE-DIMENSIONAL INTERFACE

The governing equations for the one-dimensional interface in isotachophoresis are the species continuity equations for the leader, terminator, and common counterion, and Gauss's law. These may be written as

$$\frac{\partial n_1^*}{\partial t} + \nabla\cdot(\mu_1 n_1 \overset{*\to *}{E}) = D_1 \nabla^2 n_1^* \tag{1}$$

$$\frac{\partial n_2^*}{\partial t} + \nabla\cdot(\mu_2 n_2 \overset{*\to *}{E}) = D_2 \nabla^2 n_2^* \tag{2}$$

$$\frac{\partial n_3^*}{\partial t} - \nabla\cdot(\mu_3 n_3 \overset{*\to *}{E}) = D_3 \nabla^2 n_3^* \tag{3}$$

$$\nabla\cdot\overset{\to *}{E} = \frac{q}{\varepsilon^*}(n_1^* + n_2^* - n_3^*) \tag{4}$$

If variations in properties over the cross-section of the tube are neglected, then the above equations may be simplified to give

$$\frac{\partial n_1^*}{\partial t} + \mu_1 \frac{\partial (n_1^* E^*)}{\partial x^*} = D_1 \frac{\partial^2 n_1^*}{\partial x^{*2}} \tag{5}$$

$$\frac{\partial n_2^*}{\partial t} + \mu_2 \frac{\partial (n_2^* E^*)}{\partial x^*} = D_2 \frac{\partial^2 n_2^*}{\partial x^{*2}} \tag{6}$$

$$\frac{\partial n_3^*}{\partial t} - \mu_3 \frac{\partial (n_3^* E^*)}{\partial x^*} = D_3 \frac{\partial^2 n_3^*}{\partial x^{*2}} \tag{7}$$

$$\frac{\partial E^*}{\partial x^*} = \frac{q}{\varepsilon^*}(n_1^* + n_2^* - n_3^*) \tag{8}$$

Transforming the problem to a coordinate system moving with the interface by putting $\tilde{x}=x^*-Ut$, the following equations are obtained:

$$D_1 \frac{d^2 n_1^*}{d\tilde{x}^2} = \mu_1 \frac{d}{d\tilde{x}}(n_1^* E^*) - U \frac{dn_1^*}{d\tilde{x}} \tag{9}$$

$$D_2 \frac{d^2 n_2^*}{d\tilde{x}^2} = \mu_2 \frac{d}{d\tilde{x}}(n_2^* E^*) - U \frac{dn_2^*}{d\tilde{x}} \tag{10}$$

$$D_3 \frac{d^2 n_3^*}{d\tilde{x}^2} = -\mu_3 \frac{d}{d\tilde{x}}(n_3^* E^*) - U \frac{dn_3^*}{d\tilde{x}} \tag{11}$$

$$\frac{dE^*}{d\tilde{x}} = \frac{q}{\varepsilon^*}(n_1^* + n_2^* - n_3^*) \tag{12}$$

Eqns. 9, 10 and 11 may be integrated once to obtain

$$D_1 \frac{dn_1^*}{d\tilde{x}} = \mu_1 n_1^* E^* - U n_1^* + C_1 \tag{13}$$

$$D_2 \frac{dn_2^*}{d\tilde{x}} = \mu_2 n_2^* E^* - U n_2^* + C_2 \tag{14}$$

$$D_3 \frac{dn_3^*}{d\tilde{x}} = -\mu_3 n_3^* E^* - U n_3^* + C_3 \tag{15}$$

The boundary conditions are:

$$\begin{aligned} \tilde{x} \to -\infty \qquad & n_1^* \to N \\ & n_2^* \to 0 \\ & n_3^* \to N \\ & E^* \to E_A \end{aligned} \tag{16}$$

$$\begin{aligned} \tilde{x} \to +\infty \qquad & n_1^* \to 0 \\ & n_2^* \to M \\ & n_3^* \to M \\ & E^* \to E_B \end{aligned} \tag{17}$$

Applying boundary conditions 16, and noting that $\mu_1 E_A = \mu_2 E_B = U$, eqns. 13, 14 and 15 become

$$D_1 \frac{dn_1^*}{d\tilde{x}} = \mu_1 n_1^* E^* - U n_1^* \tag{18}$$

$$D_2 \frac{dn_2^*}{d\tilde{x}} = \mu_2 n_2^* E^* - U n_2^* \tag{19}$$

$$D_3 \frac{dn_3^*}{d\tilde{x}} = -\mu_3 n_3^* E^* - U n_3^* + E_A N(\mu_1 + \mu_3) \tag{20}$$

According to the Einstein relation $D=\mu kT/e$. For monovalent ions, this is equivalent to $D=\mu kT/q$. Using this, and introducing the dimensionless variables n, E and x, eqns. 18, 19, 20 and 12 become

$$\frac{dn_1}{dx} = n_1 E - n_1 \tag{21}$$

$$\frac{dn_2}{dx} = n_2 E - \frac{n_2}{b_2} \tag{22}$$

$$\frac{dn_3}{dx} = -n_3 E - \frac{n_3}{b_3} + \theta_2(1+1/b_3) \tag{23}$$

$$\varepsilon \frac{dE}{dx} = n_1 + n_2 - n_3 \tag{24}$$

In non-dimensional terms, the boundary conditions become

$x \to -\infty$

$$n_1 \to \theta_2 \tag{25a}$$

$$n_2 \to 0 \tag{25b}$$

$$n_3 \to \theta_2 \tag{25c}$$

$$E \to 1 \tag{25d}$$

$x \to +\infty$

$$n_1 \to 0 \tag{26a}$$

$$n_2 \to 1 \tag{26b}$$

$$n_3 \to 1 \tag{26c}$$

$$E \to \theta_1 \tag{26d}$$

Eliminating E from eqns. 21 and 22 gives

$$n_2 = n_1 C e^{\alpha x}$$

Choosing the origin at the point where $n_1=n_2$ gives $C=1$, thus

$$n_2 = n_1 e^{\alpha x} \tag{27}$$

From eqn. 21

$$E = 1 + 1/n_1 \frac{dn_1}{dx} \tag{28}$$

Thus

$$\frac{dE}{dx} = \frac{1}{n_1^2}\left[n_1 \frac{d^2 n_1}{dx^2} - \left(\frac{dn_1}{dx}\right)^2\right] \tag{29}$$

Substitution of eqns. 27 and 29 in eqn. 24 yields

$$n_3 = n_1(1+e^{\alpha x}) - \varepsilon \frac{n_1''}{n_1} + \varepsilon\left(\frac{n_1'}{n_1}\right)^2 \tag{30}$$

where

$$n_1' = \frac{dn_1}{dx}, \text{ etc.}$$

Thus

$$n_3' = n_1 \alpha e^{\alpha x} + n_1'(1+e^{\alpha x}) - \varepsilon\left(\frac{n_1 n_1''' - n_1' n_1''}{n_1^2}\right) + \varepsilon\left(\frac{2n_1^2 n_1' n_1'' - 2n_1 n_1'^3}{n_1^4}\right) \tag{31}$$

Substitution of eqns. 28, 30 and 31 in eqn. 23 gives

$$2n_1^3 n_1'(1+e^{\alpha x}) + n_1^4 \alpha e^{\alpha x} - \varepsilon n_1^2 n_1''' + \varepsilon n_1^2 n_1''(3-\beta) - \varepsilon n_1'^3 -$$

$$\varepsilon n_1 n_1' n_1'' + \beta\varepsilon n_1 n_1'^2 + \beta n_1^4(1+e^{\alpha x}) - \frac{\alpha\theta_2}{1-\theta_2} n_1^3 = 0 \tag{32}$$

The last term in eqn. 32 is obtained by using the relationship

$$\beta = (1+1/b_3) = \alpha/(1-\theta_2) \tag{33}$$

This may be obtained by applying boundary conditions 25 and 26 to eqn. 15 after it has been put in dimensionless form. Boundary condition 25 yields as before

$$\bar{C}_3 = \theta_2(1+1/b_3)$$

Boundary condition 26 gives

$$\bar{C}_3 = \theta_1 + 1/b_3$$

Equating the two expressions for $\bar{C}_3$ gives

$$\theta_2(1+1/b_3) = \theta_1 + 1/b_3$$

Noting that $b_2\theta_1 = 1$, eqn. 33 is obtained by algebraic manipulation. Eqn. 33 has been described by Kohlrausch[4] and Weber[19] and is known as the Kohlrausch regulating function.

Eqn. 32 may be rearranged to give

$$2n_1'(1+e^{\alpha x}) + n_1 \alpha e^{\alpha x} - \varepsilon \frac{n_1'''}{n_1} + \varepsilon \frac{n_1''}{n_1}(3-\beta) - \varepsilon\left(\frac{n_1'}{n_1}\right)^3 - \varepsilon \frac{n_1' n_1''}{n_1^2} +$$

$$+\beta\varepsilon\left(\frac{n_1'}{n_1}\right)^2 + \beta n_1(1+e^{\alpha x}) = \frac{\alpha\theta_2}{(1-\theta_2)} \tag{34}$$

SOLUTION OF THE PROBLEM

The problem consists of finding a solution for eqn. 34 that satisfies the boundary conditions 25 and 26. The nature of the problem and the boundary conditions indicate the general shape of the solution curve (it might be referred to as a distorted sigmoid). The results of the aforementioned references tend to confirm this statement. Hence we assume a solution of the form

$$n_1 = \frac{\theta_2}{(1+\psi(x)e^{\phi(x)})^{N_1}} \tag{35}$$

Hence

$$n_2 = \frac{\theta_2 e^{\alpha x}}{(1+\psi(x)e^{\phi(x)})^{N_1}}$$

It is seen that boundary condition 25b is satisfied if

$$\phi(x) \leqslant (\alpha/N_1)x$$

Boundary condition 26b is satisfied only if the following conditions are met:

(1) $\phi(x)=(\alpha/N_1)x$

(2) $\psi(x)=K$

(3) $\frac{K^{N_1}}{\theta_2} = 1$

Hence the assumed solution for n_1 becomes

$$n_1 = \frac{\theta_2}{(1+Ke^{\alpha x/N_1})^{N_1}} \tag{36}$$

where

$$K=\theta_2^{1/N_1} \tag{37}$$

It is easily seen that eqn. 35 also satisfies the other boundary conditions.

Substitution of eqn. 36 into eqn. 34 yields an equation of the form

$$f(x)=\beta\theta_2 \tag{38}$$

As an indication of how well our assumed solution satisfies eqn. 38, we define the error in the system to be

$$\text{Error } (\%) = \left| \frac{100[f(x)-\beta\theta_2]}{\beta\theta_2} \right|$$

In general, the error will be a function of x, and the exponent N_1 must be chosen in such a way as to minimize the maximum error for each set of experimental conditions considered.

RESULTS

A numerical search routine was employed to determine the optimum value of N_1 for each set of parameters considered, the calculations being performed on the CDC-6400 computer at the University of Arizona Computer Center. The optimum value of N_1 and the resulting maximum error were found to be highly dependent on θ_2 but essentially independent of α and ε. The variations of N_1 and the maximum error with θ_2 are shown in Figs. 1 and 2, respectively.

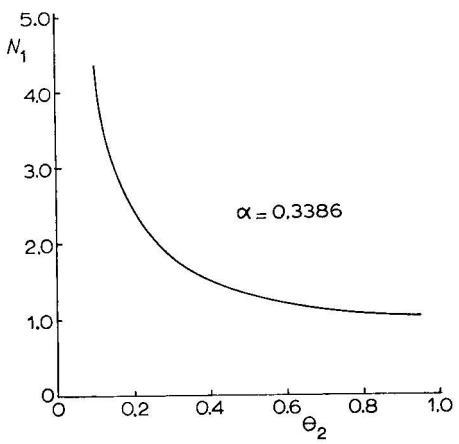

Fig. 1. Variation of N_1 with θ_2.

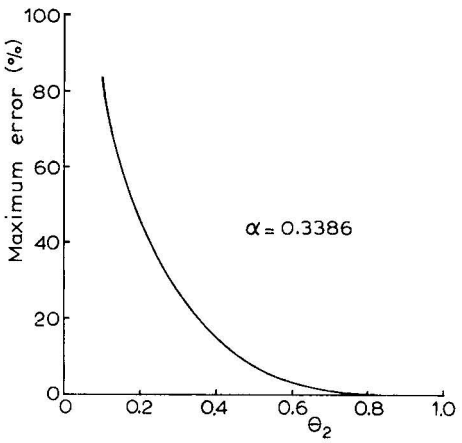

Fig. 2. Variation of maximum error with θ_2.

The values of interface thickness for leader and terminator ions were found to be equal and highly dependent on α and θ_2, but independent of ε. This same parameter dependency was found to be true for the counterion and electric field interface thicknesses. The variations of interface thickness with α and θ_2 are shown in Figs. 3–5.

DISCUSSION

As can be seen from Fig. 2, the assumed solution satisfies the governing equations with negligible error for $\theta_2 > 0.75$. The error increases rapidly for decreasing θ_2. At $\theta_2 = 0.5$ the maximum error is about 7.5%.

A comparison of Figs. 3 and 4 shows that δ_3 is less than δ_1 and δ_2. This result was also predicted by Hall and Hinckley[16].

A comparison of Figs. 3 and 5 shows that for large values of θ_2, δ_E is approximately equal to δ_1, but that for small values of θ_2, δ_E is considerably greater than δ_1. Since a common means of determining δ_1 experimentally is to measure δ_E, it would appear that at low values of θ_2 this method would give values of δ_1 which are larger than they should be. However, since it is for low values of θ_2 that the error in our calculations is very large, this may not actually be the case.

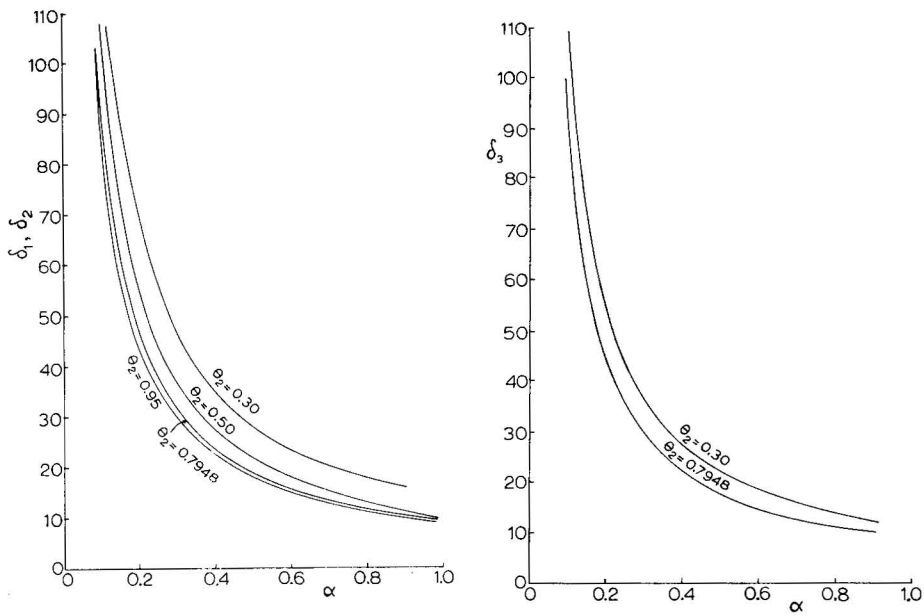

Fig. 3. Leader and terminator interface thickness.

Fig. 4. Counterion interface thickness.

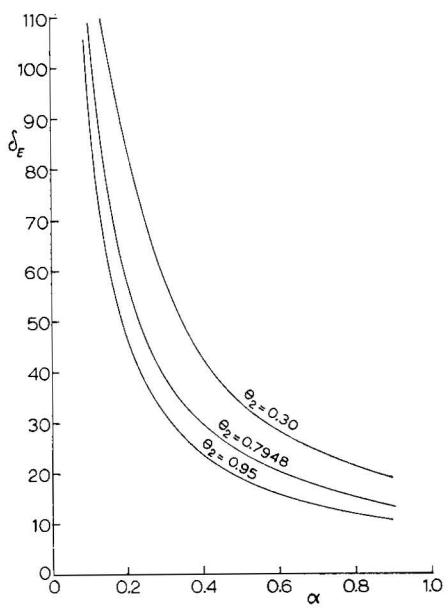

Fig. 5. Electric field interface thickness.

TABLE I

EXPERIMENTAL PARAMETERS

Parameter	*Experimental value*
n_1^*	Sodium
n_2^*	Potassium
n_3^*	Chloride
T	283 °K
μ_1	$35.98 \cdot 10^{-5}$ cm$^2 \cdot$ V$^{-1} \cdot$ sec^{-1}
μ_2	$54.4 \cdot 10^{-5}$ cm$^2 \cdot$ V$^{-1} \cdot$ sec^{-1}
μ_3	$55.25 \cdot 10^{-5}$ cm$^2 \cdot$ V$^{-1} \cdot$ sec^{-1}
E_A	250 V/cm
M	1 millimolar
ε^*	$7.4471 \cdot 10^{-10}$ F/m
α	0.3386
θ_2	0.79494
ε	$1.98 \cdot 10^{-4}$

As a numerical example for use in comparison with the results of previous analyses we consider the conditions given in Table I. For the parameters given, Fig. 3 gives $\delta_1 = 27.68$. This is a dimensionless interface thickness, and it may be put back into dimensional form by employing the definition of the dimensionless distance x given in the symbol table. Thus

$$\Delta = \frac{\delta_1 kT}{qE_A}.$$

For the conditions given this yields an interface thickness Δ of 0.027 mm.

Martin and Everaerts[2] predict an interface thickness of 1.2 mm from the results of their analysis. Applying the data of Table I to the formulations of Hall and Hinckley[16] and Konstantinov and Oshurkova[14] gives values of 0.0038 mm and 0.0115 mm, respectively, although the latter is based on a definition of interface thickness different to that used here.

Arlinger and Routs[20], using a UV detector, detected interface thicknesses of less than 1 mm, and Hinckley[17] has detected alkali metal interfaces of 0.2 mm or less with a d.c. electrometric detector although exact measurement is difficult because of the short distances involved.

The concentration and electric field profiles for the conditions of Table I are shown in Fig. 6.

Since the non-dimensional interface thickness is seen to be independent of ε, the variation of dimensional interface thickness with terminator voltage gradient (and hence leader voltage gradient because they are proportional) may be determined directly from the definition of x. Thus

$$\Delta = \frac{\delta_1 kT}{qE_A} = \frac{\delta_1 (1-\alpha) kT}{qE_B}$$

Interface thickness is seen to be inversely proportional to the terminator or leader voltage gradients with the temperature of the system as a parameter for a

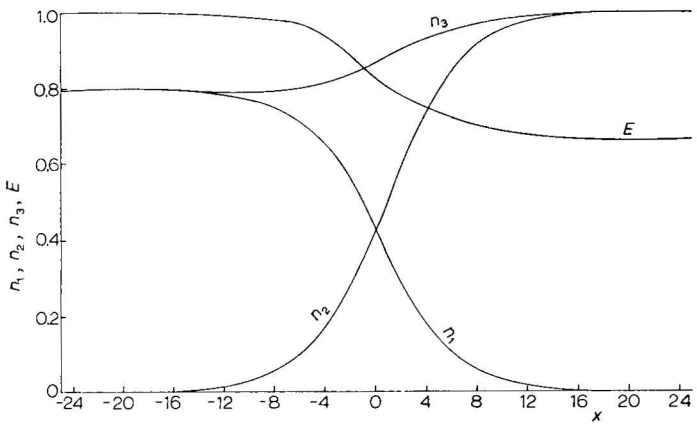

Fig. 6. Concentration and electric field profiles.

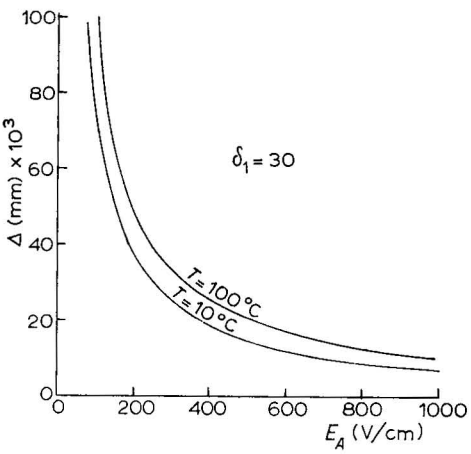

Fig. 7. Variation of interface thickness with terminator voltage gradient.

given δ_1. This variation is depicted in Fig. 7 for a value of δ_1 equal to 30, and is of the form predicted by Hinckley[17] on the grounds previously mentioned.

CONCLUSIONS

The approximate solution given is seen to satisfy the governing equations for the one-dimensional interface in isotachophoresis with a maximum error of 7.5% for values of terminator to leader concentration ratio greater than 0.5. It yields values of interface thickness that are less than those of previous analyses, and this prediction seems to be in line with experimental observations. It is seen that the sum of the concentrations of leader, terminator, and counterion in the region of the interface is very small but not zero, in accordance with Gauss's law. The interface thickness is found to be inversely proportional to the leader voltage gradient as predicted by Hinckley[17].

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Dr. Milan Bier for his interest and comments in discussion. Thanks are also due to J. O. N. Hinckley for suggesting this study and for providing us with previous publications and unpublished material related to the problem.

This study was supported by the National Aeronautics and Space Administration under contract NAS8-29566.

LIST OF SYMBOLS

b_2 = Dimensionless mobility$=\mu_2/\mu_1$
b_3 = Dimensionless mobility$=\mu_3/\mu_1$
C = Integration constant
C_1 = Integration constant (ions$\cdot$cm$^{-2}\cdot$sec^{-1})
C_2 = Integration constant (ions$\cdot$cm$^{-2}\cdot$sec^{-1})
C_3 = Integration constant (ions$\cdot$cm$^{-2}\cdot$sec^{-1})
$\bar{C}_3$ = Integration constant
D_1 = Terminator diffusion coefficient (cm^2/sec)
D_2 = Leader diffusion coefficient (cm^2/sec)
D_3 = Counterion diffusion coefficient (cm^2/sec)
E^* = Electric field (V/cm)
E = Dimensionless electric field$=E^*/E_A$
E_A = Electric field in terminator zone (V/cm)
E_B = Electric field in leader zone (V/cm)
e = Electronic charge (C/electron)
K = Constant
k = Boltzmann's constant (erg/°K)
M = Ionic number density in leader zone (ions/cm^3)
N = Ionic number density in terminator zone (ions/cm^3)
N_1 = Constant, exponent in assumed n_1 solution
n_1^* = Terminator ionic number density (ions/cm^3)
n_2^* = Leader ionic number density (ions/cm^3)
n_3^* = Counterion ionic number density (ions/cm^3)
n_1 = Dimensionless terminator ionic number density
n_2 = Dimensionless leader ionic number density
n_3 = Dimensionless counterion ionic number density
q = Ionic charge (C/ion)
T = Temperature (°K)
t = Time (sec)
U = Speed of migrating ions (cm/sec)
x^* = Distance along axis of tube referred to a fixed coordinate system (cm)
$\tilde{x}$ = Distance along axis of tube referred to a moving coordinate system (cm)
x = Dimensionless distance along axis of tube referred to a moving coordinate system$=\tilde{x}qE_A/kT$
α = Dimensionless parameter$=(b_2-1)/b_2=1-\theta_1$
β = Dimensionless parameter$=(b_3+1)/b_3$

δ_1 = Dimensionless terminator interface thickness
δ_2 = Dimensionless leader interface thickness
δ_3 = Dimensionless counterion interface thickness
δ_E = Dimensionless electric field interface thickness
Δ = Dimensional leader or terminator interface thickness (mm)
ε^* = Dielectric constant of the medium (F/m)
ε = Dimensionless dielectric constant $= E_A^2 \varepsilon^* / kTM$
θ_1 = Dimensionless electric field in leader zone $= E_B/E_A = 1-\alpha$
θ_2 = Dimensionless number density in terminator zone $= N/M = (b_3\theta_1+1)/(b_3+1)$
μ_1 = Mobility of terminator ion ($cm^2 \cdot V^{-1} \cdot sec^{-1}$)
μ_2 = Mobility of leader ion ($cm^2 \cdot V^{-1} \cdot sec^{-1}$)
μ_3 = Mobility of counterion ($cm^2 \cdot V^{-1} \cdot sec^{-1}$)
ϕ = Function of x in assumed n_1 solution
ψ = Function of x in assumed n_1 solution

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Journal of Chromatography, 95 (1974) 147–157

CHROM. 7452

NON-LINEAR FITTING METHOD FOR RECORDED CHROMATOGRAPHIC PEAKS

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(Received January 23rd, 1974)

SUMMARY

A method for fitting mathematical functions to experimental chromatographic peaks is described, with special reference to those gas–solid chromatographic peaks that have particular fitting difficulties owing to their high degree of skewness. The method was used with different functions and with peaks of diverse characteristics. The computer program used for the calculation and the different results obtained are also discussed.

INTRODUCTION

A knowledge of the mathematical functions that describe chromatographic peaks is fundamental towards the optimization of the calculations and for obtaining better results in gas chromatography, such as the calculation of the peak area (when an integrator is not available)[1], the resolution of overlapping peaks[2,3] and physico-chemical studies based on gas chromatography[4,5].

In gas chromatography, as the elution curves give the variation of concentration with time at the exit of the column, they can be represented by curves of density probability following a determined statistical distribution. We have utilized this approach in most of the cases studied.

It is in gas–solid adsorption chromatography in which it is most difficult to know the exact shape of the chromatographic peaks, as the bands are usually very asymmetric, with a vertical front and a very protracted tail. For this reason, the present study of mathematical functions is centred mainly on those that take asymmetry into account as an important factor. This problem has been dealt with by several workers, including Anderson *et al.*[6], Grushka *et al.*[7], Gladney *et al.*[8] and, more recently, Chesler and Gram[9], who employed for the first time a method of adjustment on the basis of a convolution function, the result of which is expressed in analogue form so as to make possible a selective modification of the adjustment parameters; these workers used as many as eight parameters in order to achieve their aim. In the present work, we have obtained an iterative adjustment of peaks on the basis of an automatic non-linear regression, several functions being successively employed. In the method proposed, no special calculation or measurement is required

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TABLE I

SOME FUNCTIONS FOR THE DESCRIPTION OF CHROMATOGRAPHIC PEAKS

Function	*Mathematical expression*	*Function characteristics*	*Applications*
Gauss[10]	$y = h_{max.} \cdot \exp\left[-(t-t_R)^2/2\sigma^2\right]$	Continuous distribution. Peak substantially symmetric.	Linear non-ideal chromatography. Theoretical peak and some hydrocarbons.
Poisson[10]	$y = h_{max.} \cdot \exp(-t_R) \cdot \frac{(t_R)^t}{t!}$	Discontinuous distribution.	Low number of theoretical plates. Liquid–liquid chromatography.
Modified Gauss[11]	$y = h_{max.} \cdot \left(\frac{t_R}{t}\right)^{\frac{1}{2}} \cdot \exp\left[-2t_R(t_R^{\frac{1}{2}} - t^{\frac{1}{2}})^2/2\sigma^2\right]$	Continuous distribution. Slightly skew.	Experimental peaks in GLC.
Convolution Gauss–exponential	(A) Anderson *et al.*[12]: $y = \frac{h_{max.}}{2} \int_0^{\delta} \exp(-t'/\tau) \cdot \exp\left[-\left(\frac{t-t_R-t'}{\sigma}\right)^2\right] \cdot dt'$ (B) Gladney *et al.*[8]: $y = \frac{h_{max.}}{\tau} \cdot \int_{-\infty}^{t} \left[\exp\left(-\frac{(t'-t_R)^2}{2\sigma^2}\right) \cdot \exp\left((-t-t+t_R)/\tau\right)\right] \cdot dt'$	Continuous distributions. A new parameter (asymmetry function) appears. Asymmetry is related to the τ/σ ratio.	Peaks are fitted, from $\tau = 0$ (pure Gauss), to $\sigma = 0$ (pure exponential decay). They can be used for GSC peaks with moderate asymmetry.
Composition Gauss–polynomic function	(A) Grushka *et al.*[7]: $y = \frac{1}{\sigma\sqrt{2\pi}} \cdot \exp\left(\frac{-(t-t_R)^2}{2\sigma^2}\right) \cdot \left(1 + \sum_{i=3} \frac{C_i}{i!} \cdot H_i\left(\frac{t}{\sigma}\right)\right)$ (B) Grubner[13,14]: $y = \frac{1}{\sqrt{2\pi}} \cdot \exp\left(-\frac{(t-t_R)^2}{2\sigma^2}\right) \cdot \left[1 + \frac{S}{6} \cdot \left(\left(\frac{t-t_R}{\sigma}\right)^3 - 3 \cdot \left(\frac{t-t_R}{\sigma}\right)\right) - \frac{E}{24} \cdot \left(\left(\frac{t-t_R}{\sigma}\right)^4 - 6 \cdot \left(\frac{t-t_R}{\sigma}\right)^2 + 3\right)\right]$	There are based statistical moments of the independent variable (t). H_i = Hermite polynomials. $C_3 = S$ = skewness. $C_4 = E$ = excess.	All type of curves are fitted (symmetrical as well as asymmetric).

for the estimation of the initial data of the iteration, nor is any complementary instrument necessary: the recorded graph and the corresponding computer suffice.

The best known functions for the description of a chromatographic peak that we have found in the literature are summarized in Table I.

METHOD OF FITTING

Once the various functions that we have described briefly have been studied, the main problem is to establish which is the most adequate mathematical function for each case (given an actual peak) and which are the values of its parameters. In this section, we describe the mathematical method employed in order to obtain the parameters of each function, in the most exact form possible, thus obtaining a correct mathematical expression for it. With this aim, we considered it convenient to try a fitting method that would provide an equation that adequately describes an actual chromatogram.

The adjustment method selected is that employed by Law and Bailey[15], based on the known method of least squares, which is made applicable to any type of function through a technique of general, non-linear, regression. This method has been employed previously in problems related to gas chromatography by Campreciós and Gassiot[3].

In order to effect the adjustment, a simultaneous variation is made of all the parameters that occur in the function considered, until there are obtained, through successive iterations, some parameter values such that, given the experimental points, the function presents a minimal mean quadratic error.

Consider a function of the type

$$y_i = \varphi(x_{1i}, x_{2i}, x_{3i}, \cdots, x_{mi}, B_1, B_2, B_3, \ldots, B_{np}) \pm e_y \tag{1}$$

where

y_i = dependent variable;
x_{ji} = independent variable;
B_k = function parameters;
i = 1, 2, 3, ..., n=number of points;
j = 1, 2, 3, ..., m=number of independent variables;
k = 1, 2, 3, ..., np=number of parameters;
e_y = experimental error.

If this function is developed into a Taylor series, terminated after the first derivative, and brought in the assimilation $\Delta B_k \simeq \mathrm{d}B_k$, we have:

$$\varphi^{i+1} = \varphi^i + \left(\frac{\partial \varphi}{\partial B_1}\right)^i \cdot \Delta B_1 + \left(\frac{\partial \varphi}{\partial B_2}\right)^i \cdot \Delta B_2 + \ldots + \left(\frac{\partial \varphi}{\partial B_k}\right)^i \Delta B_k + \ldots \tag{2}$$

where i is the iteration order

$$\Delta B_k = B_k{}^{i+1} - B_k{}^i \tag{3}$$

so that, in each iteration there is a linear increment of a non-linear function.

The condition imposed is that the mean quadratic error should be minimal:

$$\frac{\partial \sum_{i=1}^{n} S_i^2}{\partial B_k} = 0 \tag{4}$$

where $S_i = y_i - \varphi_i$ (in each point)

The system of equations to be solved in each iteration is of the type:

$$A\,|b> = |c>\,;\; A^T \cdot A\,|b> = A^T\,|c>\,;\; |b> = (A^T \cdot A)^{-1} \cdot A^T\,|c> \tag{5}$$

where

A = matrix of the elements: $\left(\frac{\partial \varphi}{\partial B_k}\right)_i$;

$|b>$ = vector of the unknown: ΔB_j;

$|c>$ = vector of the errors: S_i.

In order to solve the system, some initial values are given to B_k and, if the scheme converges for values of B_k that minimize ΣS_i^2, the vector $|b>$ represents the ΔB_k values, and therefore eqn. 3 can be solved for $B_k{}^{i+1}$. These new values can then be used in the next iteration, and thus successively, until ΔB_k eventually becomes negligible. The limitation of the method lies in the fact that it may lead to divergent values if the initial parameters calculated are not in the region of the correct values. However, if some "restrictive" factors are introduced, the convergence is always obtained.

APPLICATION OF THE METHOD TO CHROMATOGRAPHIC PEAKS

According to the above discussion, a chromatographic peak is of the type

$$y = \varphi(t, B_1, B_2, \ldots, B_{np})$$

where time (t) is the only independent variable and B are the parameters; in all of the functions presented here, their number varies from 3 to 5, according to whether the shape differs more or less from that of a perfect Gaussian peak.

The nomenclature used is as follows:

B_1 = height of the peak;

B_2 = retention time;

B_3 = standard deviation;

B_4 = skewness;

B_5 = excess.

Hence it is correct to apply to a chromatographic band the method of fit described above.

However, when attempting to work with any of the proposed functions, the problem arises of how to estimate correctly the initial value of the parameters involved in each of them. The answer is simple when there are symmetrical (or virtually symmetrical) peaks, but is more difficult when the asymmetry of the peak is consider-

able, because in such cases even the parameters of direct measurement, such as retention time and peak height, are modified. For this reason, we believe that the best means of calculating the parameters of the different functions is through the statistical moments of the variable, defined as follows.

Statistical moments of the variable with respect to the origin:

$$m_i = \frac{\int_0^\infty c(t) \cdot t^i \mathrm{d}t}{\int_0^\infty c(t) \cdot \mathrm{d}t}$$

Statistical moments of the variable with respect to the mean:

$$\overline{m}_i = \frac{\int_0^\infty c(t) \cdot (t - m_1)^i \mathrm{d}t}{\int_0^\infty c(t) \cdot \mathrm{d}t}$$

where

$c(t)$ = peak profile.

The computer applies the "method of trapezes" for the evaluation of the integrals in the above expression with the same points used in the adjustment.

In a statistical distribution, it is verified that:

$$\bar{x} = m_1$$

$$\sigma^2 = \overline{m}_2$$

$$S = \frac{\overline{m}_3}{\sigma^3}$$

$$E = \frac{\overline{m}_4}{\sigma^4} - 3$$

We use the following nomenclature.

$$B_2 = t_R = m_1$$

$$B_3 = \sigma = \sqrt{\overline{m}_2}$$

$$B_4 = S = \frac{\overline{m}_3}{\sigma^3}$$

$$B_5 = E = \frac{\overline{m}_4}{\sigma^4} - 3$$

We find the height in the middle of the distribution by means of the following equation:

$$y_{\max.} = \varphi(t_R, h_{\max.}, t_R, \sigma, S, E)$$

where $h_{\max.}$ is unknown.

As we have already indicated, the values of both B_2 and B_1 do not correspond exactly to t_R and to the maximum height when the peak is very asymmetric.

PRACTICAL APPLICATION

From the above discussion, one can appreciate the complexity of the calculation involved in this fitting procedure. In order to make it easy to apply, a program has been designed in Fortran IV and operated on an IBM 1130 computer. The program is written in such a way that several alternatives of calculation exist that can be selected by the use of cards or by entering the data by means of a console. The scheme of the program is shown in Fig. 1.

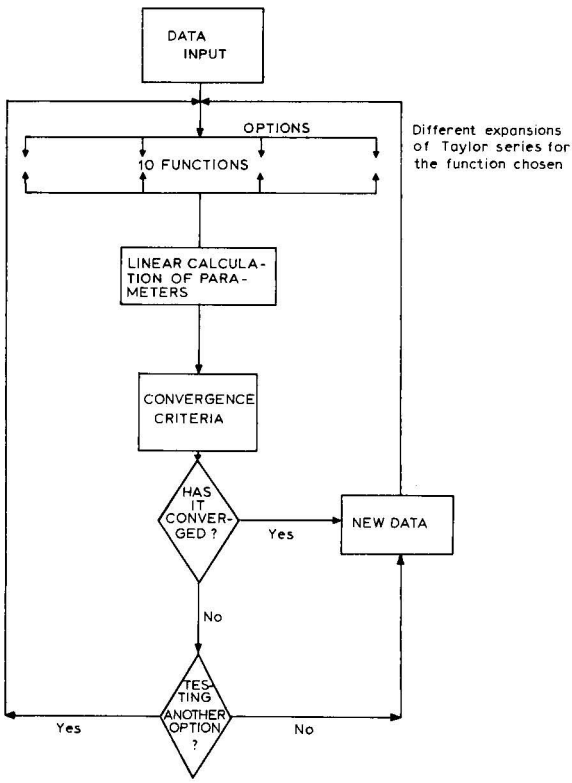

Fig. 1. Scheme of computer program (PAPAT).

The program functions as follows. First, the data are introduced, which consist of peak identification, points on the chromatogram and indicator of the function chosen, followed by the calculation of the statistical moments by means of the above equations. After finding the function which has been indicated to it, the program applies the method of adjustment discussed above, calculates the derivatives, finds the new value of each parameter and compares the result with the maximal error allowed for each function. According to whether the difference between y_i and

φ_i is larger or smaller than this error, it proceeds to work on a new iteration, or else it writes the results and goes on to calculate a new problem.

This program can calculate any type of chromatographic peak, employing as many mathematical functions as may be desired. In fact, it is able to work simultaneously with a maximum of ten different functions. We have worked with only four types of functions, namely the Gauss, Littlewood, Grubner and Gladney types, and, in general, we obtained satisfactory results.

The calculation program is applicable not only to chromatographic peaks, but also to any type of problem in which it may be desired to fit to a set of experimental points a curve following a given statistical distribution.

RESULTS

The results obtained in our work differed according to the type of peak studied. Some individual cases are described briefly below.

For actual peaks obtained in gas–liquid chromatography the result is always coherent, *i.e.*, the curve adjusts itself to the actual points, within a margin of minimal error (Table II). However, with peaks obtained in gas–solid chromatography, the agreement is less satisfactory, as the moments obtained are different from their actual values (Table III).

TABLE II

EXPERIMENTAL PEAK (GAS–LIQUID CHROMATOGRAPHY)

Number of data points: 21. Maximum allowable tolerance of parameters: 0.01.

Parameter	*Initial calculated value*	*Fitted parameters*		
		Gauss: number of iterations = 2	*Littlewood: number of iterations* = 2	*Grubner: number of iterations* = 6
Height	6.030	6.214	6.214	6.139
Retention time	5.648	5.652	5.652	5.666
Standard deviation	0.115	0.106	0.106	0.109
Skewness	0.364	–	–	0.515
Excess	−0.522	–	–	0.051

TABLE III

EXPERIMENTAL PEAK (GAS–SOLID CHROMATOGRAPHY)

Number of data points: 20. Maximum allowable tolerance of parameters: 0.01.

Parameter	*Initial calculated value*	*Fitted parameters*			
		Gauss: number of iterations = 3	*Littlewood: number of iterations* = 3	*Grubner: number of iterations* = 9	*Gladney: number of iterations* = 11
Height	12.327	13.650	13.675	16.632	11.219
Retention time	38.263	37.831	37.849	38.431	35.910
Standard deviation	2.281	2.017	2.015	1.937	0.859
Skewness	1.906	–	–	1.087	1.917
Excess	7.012	–	–	1.325	–

We have also examined theoretical peaks generated by the functions studied, for which fitting by means of other functions presents no problems, so that the correctness of the choice of the functions studied and, at the same time, the acceptability of the method could be confirmed (Tables IV–VI).

TABLE IV

THEORETICAL PEAK (GAUSS)

Number of data points: 15. Maximum allowable tolerance of parameters: 0.01.

Parameter	*Initial calculated value*	*Fitted parameters*			
		Gauss: number of iterations = 1	*Littlewood: number of iterations = 2*	*Grubner: number of iterations = 3*	*Gladney*
Height	20.000	19.998	19.842	19.992	–
Retention time	8.000	7.999	8.064	8.000	–
Standard deviation	1.995	2.000	2.009	1.997	–
Skewness	$0.399 \cdot 10^{-3}$	–	–	$0.683 \cdot 10^{-3}$	–
Excess	−0.035	–	–	$-0.966 \cdot 10^{-3}$	–

TABLE V

THEORETICAL PEAK (GLADNEY)

Number of data points: 28. Maximum allowable tolerance of parameters: 0.05.

Parameter	*Initial calculated value*	*Fitted parameters*			
		Gauss: number of iterations = 2	*Littlewood: number of iterations = 2*	*Grubner: number of iterations = 5*	*Gladney: number of iterations = 5*
Height	22.220	21.750	21.556	21.115	16.342
Retention time	14.719	14.570	14.590	14.902	13.603
Standard deviation	1.398	1.110	1.127	1.219	0.697
Skewness	1.086	–	–	1.054	1.393
Excess	2.029	–	–	0.266	–

TABLE VI

SYMMETRICAL PEAK

Number of data points: 31. Maximum allowable tolerance of parameters: 0.01.

Parameter	*Initial calculated value*	*Fitted parameters*			
		Gauss: number of iterations = 3	*Littlewood: number of iterations = 3*	*Grubner: number of iterations = 4*	*Gladney*
Height	128.297	146.399	145.726	125.973	–
Retention time	30.000	30.000	30.047	30.000	–
Standard deviation	4.190	3.844	3.861	3.309	–
Skewness	$-0.388 \cdot 10^{-3}$	–	–	$-0.105 \cdot 10^{-2}$	–
Excess	−1.482	–	–	−1.521	–

Of the functions studied, that which presents the greatest difficulty of fit is that of Gladney *et al.*[8], even when the peak is almost Gaussian in shape. This is a reasonable result, because the convolution becomes the product of infinite and infinitesimal terms when the skewness is also infinitesimal. From the formal point of view, we can eliminate this indetermination, but this is not possible when purely numerical work is carried out in a computer, where the precision is finite.

Contrary to the function given by Gladney *et al.*[8], the function that shows adjustment, in general, for all peaks with a lower error with regard to the actual values is that of Grubner[13]. This result is logical, as functions of a polynomial type are usually a perfect substitute for any other type of function, if the parameters are adequate and the number of terms in the polynomial is not too small (Table VII and Fig. 2).

For the maximal error allowed, we have established in most cases a value of 0.01 unit. In some cases, however, a convergence for a tolerance of 0.01 unit has not been obtained, while a convergence has been obtained, allowing a larger error of the order of 0.05 unit. Also, in other cases, the contrary occurred, *i.e.*, when the tolerance was reduced to 0.001 unit, the result remained coherent. The discrete nature of the system of adjustment (in the sense of not having a function, but only several points), does not preclude that some local minima with no relation to the chromatographic peaks could be obtained. This effect results in some functional parameters devoid of sense, such as a negative standard deviation.

TABLE VII

COMPARISON OF RESULTS OBTAINED WITH SOME FUNCTIONS

t_R (*experimental*)	*y*				
	Experimental	*Gauss* (*3 iterations*)	*Littlewood* (*3 iterations*)	*Grubner* (*9 iterations*)	*Gladney* (*11 iterations*)
34.00	0.10	2.17	2.05	0.14	0.29
35.00	2.32	4.96	4.91	3.82	3.12
35.50	6.80	6.83	6.86	6.80	6.80
36.00	11.45	8.86	8.95	10.01	10.78
37.05	14.57	12.52	12.65	14.49	15.21
37.50	14.03	13.37	13.46	14.00	14.55
38.00	12.91	13.58	13.59	13.50	12.96
38.50	11.80	12.97	12.92	11.63	11.19
39.00	10.16	11.65	11.57	9.67	9.59
40.00	7.37	7.83	7.79	7.01	6.90
41.00	5.22	4.12	4.18	5.60	5.06
42.00	3.45	1.69	1.87	3.82	3.68
43.00	2.23	0.54	0.63	1.85	2.68
44.00	1.45	0.13	0.18	0.60	1.95
46.00	0.59	0.00	0.00	0.01	1.03
48.00	0.34	0.00	0.00	0.00	0.54
50.00	0.25	0.00	0.00	0.00	0.28
52.00	0.20	0.00	0.00	0.00	0.15
54.00	0.11	0.00	0.00	0.00	0.08
56.00	0.00	0.00	0.00	0.00	0.04

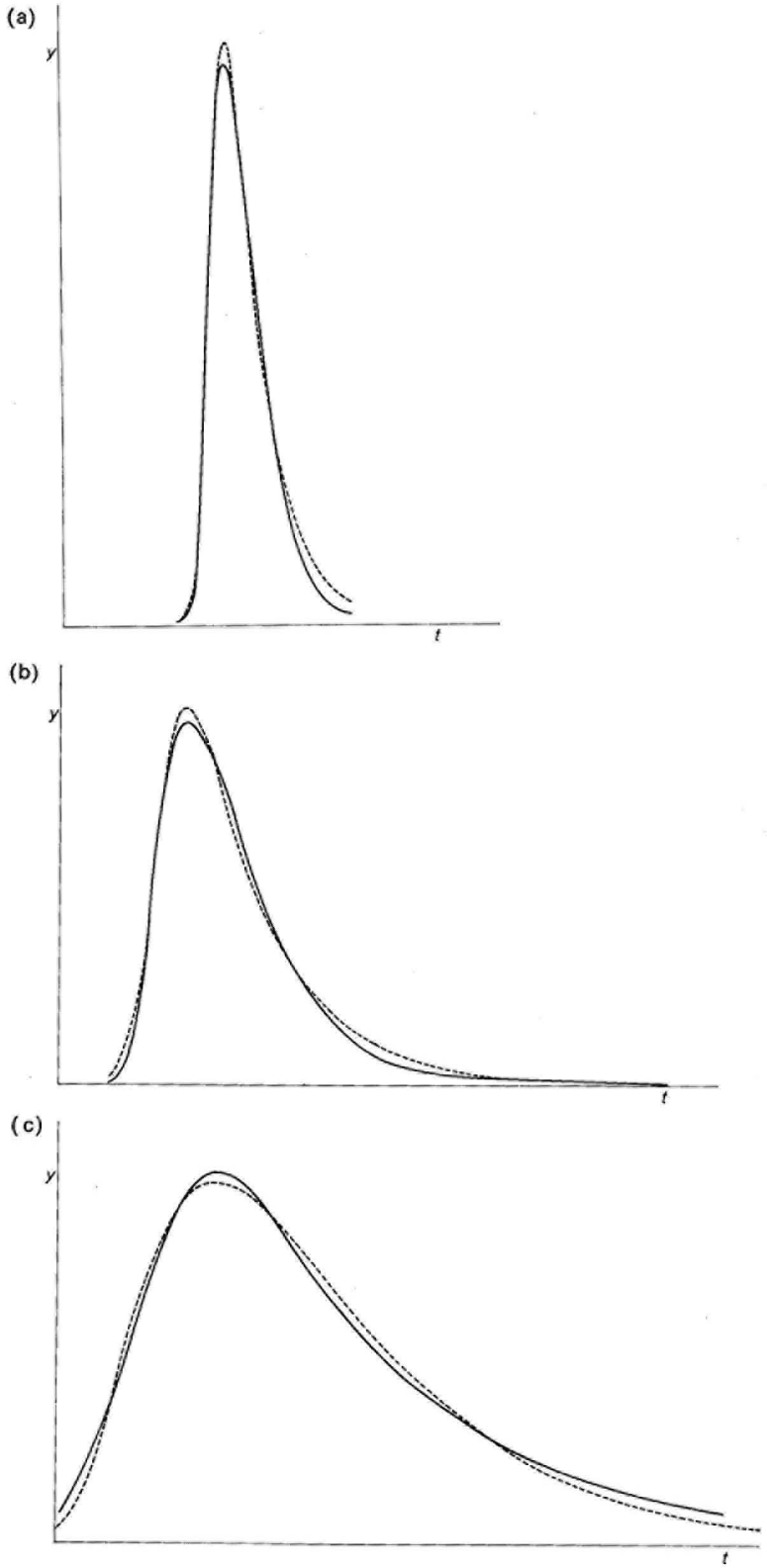

Fig. 2. Shapes of experimental (———) and calculated (– – – –) fitting peaks for acetic acid. Flow-rates of carrier gas: (a), 60 ml/min; (b), 30 ml/min; (c), 15 ml/min.

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Journal of Chromatography, 95 (1974) 159–167

CHROM. 7497

ADSORPTION BEHAVIOUR OF AROMATIC NITRO COMPOUNDS ON SEPHADEX LH-20

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SUMMARY

By classifying the mechanism of adsorption of solutes to gels into two groups, *viz.*, ionic effects (ion exchange, ion exclusion and ion adsorption) and pure adsorption (hydrogen bonding and π-bonding), and by considering the gel structure as hydrochloric acid, potassium hydroxide, or potassium chloride in methanol are adsorbed on to the gels and the surface of the gel becomes positively charged, the adsorption behaviour of dinitrophenyl derivatives on Sephadex LH-20 in methanol, 0.05 *N* hydrochloric acid–methanol, 0.05 *N* potassium hydroxide–methanol and 0.05 *N* potassium chloride–methanol have been explained. The solutes that have no dissociative groups interact with the gel only by pure adsorption. The solutes that have an acidic group interact with the gel by ion adsorption under the dissociation conditions for the solutes, and those that have a basic group by ion exclusion under both the dissociation and non-dissociation conditions. These behaviours are compared with those in methanol.

Benzene or pyridine added to methanol are assumed to block the interaction sites of gels and hence lower K_d values for the solutes are obtained. The presence of two nitro groups and hydroxyl, carboxyl or amino groups on a benzene ring shows the potentiation effect of adsorption to the gel. The dissociative groups favour the ionic effects, which are greater than the sieving effect. A nitro group is assumed to be adsorbed to the gel by hydrogen bonding and this secondary effect is greater than the molecular sieving effect for low-molecular-weight compounds.

INTRODUCTION

The retardation of dinitrophenylated linear monomers and oligomers of polyamides on Sephadex LH-20 was studied under various conditions[1]. The values of K_d for some solutes were greater than unity and the addition of an inorganic electrolyte to the sample solution caused extreme retardation. In general, when a solute departs from the elution volume predicted on the basis of molecular sieving, *viz.*,

by appearing either earlier or later than expected, some other types of interactions between the solute and gel matrix must occur.

Although the molecular sieving properties of gel materials used in gel chromatography usually predominate, other types of interactions between solutes and gel materials have also been observed when Sephadex was used as the bed material. Gelotte[2] described some adsorption properties of Sephadex G-25 towards low-molecular-weight substances such as amino acids and alkaloids, and divided these secondary effects into two groups, namely adsorption to the bed materials and superimposed effects. Adsorption is related to the structure of the solutes and superimposed effects depend upon the conditions of the run, *i.e.*, the ionic strength, composition in and pH of the eluent. Kwon[3] studied the pH-dependent elution of malonaldehyde on Sephadex G-10 and observed adsorption to the gel matrix below pH 4. Janson[4] discussed the adsorption behaviour of some substances on Sephadex in different solvents and found that highly charged substances tend to be excluded at an opposite pH range and that the retardation of aromatic amino acids is markedly decreased on the addition of 1 *M* pyridine. Studies of adsorption phenomena on Sephadex have also been made by other workers[5–8].

Of the adsorption phenomena, the affinity of dextran gel for aromatic and pseudo-aromatic substances is particularly striking in accordance with the affinity characteristics found for adsorption to cellulose. A planar structure and an extended system of conjugated bonds in the solute favour adsorption[4]. The adsorption of aromatic compounds to dextran gel is presumed to result from the π-electron bonding between them. Janson[4] stated that in distilled water, the K_d values for acidic aromatic substances are a compromise between the two opposing effects, aromatic adsorption and ion exclusion, and that adsorption increases with increasing salt concentration, although the ion-exclusion effect, which depends on small amounts of fixed carboxyl groups, is eliminated by the addition of small amounts of an electrolyte to the distilled water. He also considered that the increase in aromatic adsorption is caused either by an increase in the number of adsorption sites available or by an increase in the strength of the interaction due to a decrease in the size of the layer of water of hydration which prevents solute–gel interaction.

The interaction of aromatic molecules with Sephadex LH-20 in organic solvents has also been studied[9]. Streuli[10] found that the adsorption values of planar aromatic hydrocarbons on Sephadex LH-20 were linear functions of the resonance energies of these compounds when methanol was used as the eluent. He concluded that the interaction between these aromatic molecules and the gel involved the π-electron cloud, and that Lewis acid–base complexes were formed. Heteroaromatic and polar substituted compounds, such as phenols and anilines, are more strongly adsorbed than predicted from resonance energies, indicating that hydrogen bonding as well as π-bonding participates in the adsorption.

The role of the solvent employed in the solute–gel interaction has also been discussed[11]. In methanol, π-bonding predominates, but some resonating species are also able to form hydrogen bonds. Dimethylformamide favours sieving, tetrahydrofuran favours hydrogen bonding, and π-bonding is insignificant in both solvents.

The adsorption behaviour of dinitrophenyl derivatives on Sephadex LH-20 in several methanolic eluents has been investigated in this work and the adsorption mechanisms are discussed.

EXPERIMENTAL

The gel used was Sephadex LH-20, which was swollen in the solvent used as the eluent for 24 h prior to being packed in a glass column of 100 cm × 16 mm I.D. The dry gel weight was 45–46 g and the height of gel bed was 91–93 cm. The eluents used were methanol, 0.05 *N* hydrochloric acid–methanol, 0.05 *N* potassium hydroxide–methanol, 0.05 *N* potassium chloride–methanol, 10% benzene–0.05 *N* hydrochloric acid–methanol, 1% benzene–0.05 *N* hydrochloric acid–methanol, 0.5% dinitrobenzene–methanol and 1 *M* pyridine–methanol. Some aromatic nitro compounds were purchased, and others were prepared in this laboratory. All sample solutions were between 0.05 and 0.1% in concentration and 1-ml portions were applied directly to the top of the gel bed.

The effluent was delivered to a flow cell adapted to a Hitachi Model 124 spectrophotometer equipped with an absorbance recorder. The absorbance of the effluent was recorded continuously. The wavelength at which the peak maximum of a solute was 0.5–0.7 in absorbance (D) was selected for each sample. V_0 was determined using Blue Dextran modified as follows: 20 mg of Blue Dextran were dissolved in 1 ml of 10% sodium hydroxide solution and stored overnight, 0.3 ml of dimethyl sulphate was then added, the solution was stirred for 5 h, the sulphuric acid liberated was neutralised with sodium hydroxide solution as soon as possible and the solution was diluted to 10 ml with methanol. V_i was calculated from the values of V_0, the weight of gel and total gel bed volume (V_t). Other experimental conditions and procedures were as described earlier[1].

RESULTS

The partition coefficients, K_d, of several aromatic nitro compounds and other

TABLE I

K_d VALUES FOR VARIOUS SOLUTES ON SEPHADEX LH-20 IN METHANOL AND METHANOLIC SOLUTIONS OF HYDROCHLORIC ACID, POTASSIUM HYDROXIDE AND POTASSIUM CHLORIDE

Solute	K_d			
	Methanol	*0.05 N HCl–methanol*	*0.05 N KOH–methanol*	*0.05 N KCl–methanol*
Benzene	1.09	1.13	1.10	1.14
Nitrobenzene	1.29	1.32	1.26	1.32
m-Dinitrobenzene	1.36	1.37	1.36	1.36
2,4-Dinitrotoluene	1.33	–	–	–
2,4-Dinitrophenetole	1.36	1.35	1.47	1.38
2,4-Dinitrophenol	1.77	1.59	6.17	5.25
2,4-Dinitrobenzoic acid	1.77	1.57	3.22	2.68
2,4-Dinitroaniline	2.05	2.05	2.05	2.01
2,4-Dinitrophenylaminocaproic acid	1.39	1.41	2.54	2.13
Benzoylaminocaproic acid	0.88	0.86	1.63	1.31
ε-Aminocaproic acid	1.11	0.62	1.22	0.65
Benzoic acid	1.25	1.19	2.15	1.84
m-Phenylenediamine	1.60	0.79	1.43	1.46

related aromatic compounds in several eluents are listed in Tables I and II. The K_d values were calculated using equation

$$K_d=(V_e-V_0)/V_i$$

where V_e is the elution volume of the solute, V_0 is the void volume of the gel column, and V_i is the inner volume of the column. In order to discuss the effect of a nitro group and other substituents attached to a benzene ring on the adsorption to the gel some compounds that do not have a nitro substituent were also examined.

TABLE II

K_d VALUES FOR VARIOUS SOLUTES ON SEPHADEX LH-20 IN METHANOLIC SOLUTIONS OF BENZENE, DINITROBENZENE AND PYRIDINE

Solute	K_d			
	0.05 N HCl–1% benzene–methanol	*0.5% Dinitrobenzene–methanol*	*0.05 HCl–10% benzene–methanol*	*1 M pyridine–Methanol*
2,4-Dinitrophenylaminocaproic acid	1.40	1.40	1.16	1.18
2,4-Dinitrophenol	–	1.59	1.48	1.16
2,4-Dinitroaniline	–	1.97	–	1.72
2,4-Dinitrophenetole	–	–	–	1.25
Benzoylaminocaproic acid	0.85	–	–	–

DISCUSSION

In spite of the similar molecular weights of the dinitrophenyl derivatives examined, large differences in K_d values were observed. Besides the substituents on the dinitrophenyl ring, the properties of the eluents also influenced the K_d values.

Elution behaviour of solutes in methanol

The K_d values for all of the solutes examined except benzoylaminocaproic acid are greater than unity, which indicates the participation of adsorptive interactions between the solutes and the gel. The K_d value for benzene is 1.09 and adsorption by π-bonding between the gel and the π-electrons of benzene is considered to occur[12]. Upon substituting a nitro group for a hydrogen atom in benzene (nitrobenzene), the K_d value increases to 1.29, and the introduction of a second nitro group (*m*-dinitrobenzene) increases K_d to 1.36. The high electronegativity of the nitro group favours hydrogen bonding between its oxygen atoms and the hydrogen atoms of the gel, and consequently nitrobenzenes utilize hydrogen bonding as well as π-bonding in the adsorption process.

Depending on the type of substituent with which a hydrogen atom of *m*-dinitrobenzene is replaced, the K_d values for 2,4-dinitrophenyl derivatives increase in the following order:

$$H=-CH_3=-OC_2H_5 \leqslant -NH(CH_2)_5COOH < -OH=-COOH < -NH_2$$

Brook and Munday[13] found that the adsorption by Sephadex dextran gels of *m*- or *p*-monosubstituted phenols, anilines and benzoic acids can be correlated by the Hammett equation. They also suggested that the benzene derivatives might be adsorbed to the hydroxyether cross-linking groups by hydrogen bonding. In the case of the *m*-dinitrobenzene derivatives, the Hammett equation cannot be correlated with the adsorption behaviour, because the *ortho* effect between the substituent and one nitro group at the *ortho* position of the substituent should be considered.

The K_d values for 2,4-dinitrotoluene and 2,4-dinitrophenetole are similar to that of *m*-dinitrobenzene, as the methyl and ethoxy groups have low electronegativities and hydrogen bonding to the gel is negligible. Carboxyl and hydroxyl groups are more electroattractive and have higher electronegativities. The higher K_d values for 2,4-dinitrobenzoic acid and 2,4-dinitrophenol are due to this higher electronegativity and the partial dissociation of these groups in methanol. The influence of these groups on the K_d values of derivatives is shown in Table III. The increases in K_d caused by the substitution of two nitro groups for hydrogen atoms in the benzene rings of benzoic acid and benzoylaminocaproic acid are identical. This increase is larger than that observed on the substitution of two nitro groups into benzene. The increase in K_d values upon the substitution of a carboxylic acid and two nitro groups into benzene is greater than the sum of the increases observed upon separate substitution of a carboxylic group and two nitro groups into benzene. Both a nitro group and a carboxyl group are electroattractive, and when both two nitro groups and a carboxyl group are introcuded the hydrogen bonding is increased due to the potentiation effect.

TABLE III

CHANGES IN K_d VALUES WITH DIFFERENT SUBSTITUENTS USING METHANOL AS ELUENT

Derivative	*Parent compound*	*Substituent*	*Increase in K_d over that of parent compound*
Benzoic acid	Benzene	–COOH	0.16
Nitrobenzene	Benzene	$-NO_2$	0.20
m-Dinitrobenzene	Benzene	$-NO_2$ (×2)	0.27
2,4-Dinitrobenzoic acid	Benzene	–COOH, $-NO_2$ (×2)	0.68
2,4-Dinitrobenzoic acid	*m*-Dinitrobenzene	–COOH	0.41
2,4-Dinitrobenzoic acid	Benzoic acid	$-NO_2$ (×2)	0.52
2,4-Dinitrobenzoylaminocaproic acid	Benzoylaminocaproic acid	$-NO_2$ (×2)	0.51
2,4-Dinitrophenol	*m*-Dinitrobenzene	–OH	0.41

In methanol, the K_d value for benzoylaminocaproic acid is lower than that of ε-aminocaproic acid, suggesting that this compound utilizes the molecular sieving effect more than the adsorption process with π-electrons of the benzene ring and the gel. When two nitro groups are substituted for two hydrogen atoms in the benzene ring of benzoylaminocaproic acid, the K_d value increases considerably owing to the

hydrogen bonding of the nitro groups with the gel. The increase in the K_d value in this instance is identical with that for 2,4-dinitrobenzoic acid.

The influence of amino groups on the adsorption process (*m*-phenylenediamine) is greater than that for nitro groups (*m*-dinitrobenzene).

Elution behaviour of solutes in methanolic solutions of hydrochloric acid, potassium hydroxide or potassium chloride

The changes in K_d values observed upon changing the eluent from methanol alone to 0.05 *N* methanolic solutions of hydrochloric acid, potassium hydroxide and potassium chloride are shown in Table IV. The effects can be classified in six categories:

(a) K_d values for solutes that have no dissociable groups are unchanged (benzene, nitrobenzene and dinitrobenzene).

(b) K_d values for compounds that have an acidic group decrease in the acidic eluent and increase in the alkaline and the neutral eluents (dinitrophenol, dinitrobenzoic acid and benzoic acid).

(c) With dinitrophenylaminocaproic acid and benzoylaminocaproic acid, which have longer chains, the K_d values are unchanged in the acidic eluent although they have an acidic group.

(d) K_d values for ε-aminocaproic acid decrease in hydrochloric acid–methanol

TABLE IV

CHANGE IN K_d VALUES ON CHANGING THE ELUENT FROM METHANOL ALONE TO DIFFERENT METHANOLIC SOLUTIONS

Change in K_d	*Eluent*		
	0.05 N HCl-methanol	*0.05 N KOH-methanol*	*0.05 N KCl-methanol*
Unchanged	Benzene Nitrobenzene *m*-Dinitrobenzene 2,4-Dinitroaniline 2,4-Dinitrophenetole 2,4-Dinitrophenyl-aminocaproic acid Benzoylaminocaproic acid	Benzene Nitrobenzene *m*-Dinitrobenzene 2,4-Dinitroaniline	Benzene Nitrobenzene *m*-Dinitrobenzene 2,4-Dinitroaniline 2,4-Dinitrophenetole
Increased		2,4-Dinitrophenetole 2,4-Dinitrophenyl-aminocaproic acid Benzoylaminocaproic acid 2,4-Dinitrophenol 2,4-Dinitrobenzoic acid ε-Aminocaproic acid Benzoic acid	2,4-Dinitrophenyl-aminocaproic acid Benzoylaminocaproic acid 2,4-Dinitrophenol 2,4-Dinitrobenzoic acid Benzoic acid
Decreased	2,4-Dinitrophenol 2,4-Dinitrobenzoic acid ε-Aminocaproic acid Benzoic acid *m*-Phenylenediamine	*m*-Phenylenediamine	ε-Aminocaproic acid *m*-Phenylenediamine

and potassium chloride–methanol and increase in potassium hydroxide–methanol.

(e) K_d values for dinitroaniline are unchanged in these eluents and those for *m*-phenylenediamine decrease.

(f) K_d values for 2,4-dinitrophenetole increase in the alkaline eluent and are unchanged in other eluents.

The results for the behaviour of acidic aromatic substances in potassium chloride–methanol support Janson's theory[4], but it is impossible to explain other phenomena with his theory. In the studies by Janson[4] and Eaker and Porath[12], the probable role of the solvent, which was water when Sephadex G-10 and other unmodified dextran gels were used, was stressed. The apparent inadequacy of Janson's theory in our study is probably due largely to the fact that the solvent and the gel are not water and Sephadex G-10 but methanol and Sephadex LH-20, respectively. In order to explain the reverse elution behaviour when an electrolyte is included in the eluent, the suggestions of Gelotte[2], Streuli[10,11] and Brook and Munday[13] on adsorption behaviour are insufficient. The organophilic Sephadex LH-20 gel might be more polar than the solvent, methanol, in which case polar solutes might be adsorbed through hydrophilic interactions and, by adding an electrolyte to methanol, the polarity of the eluent might increase with respect to the gel phase. However, this hypothesis is still insufficient in order to explain the behaviour of solutes such as dinitrobenzene which do not have any dissociative group and the K_d values of which were unchanged in the four eluents used.

The elution behaviour of the solutes in group (a) indicates that the strengths of hydrogen bonds and π-bonds between the solutes and the gel do not vary when hydrochloric acid, potassium hydroxide or potassium chloride was added to methanol, implying there is neither an increase in the number of adsorption sites of the gel nor a decrease in the size of the layer of hydration which might prevent solute–gel interactions. The elution behaviour of solutes in group (b) in potassium hydroxide–methanol and potassium chloride–methanol can be explained by the change in the solute–gel interactions due to the dissociation or the suppression of dissociation of the acidic groups, but that in potassium chloride–methanol cannot be explained by the dissociation–non-dissociation theory. The elution behaviour of the solutes in potassium chloride–methanol should be the same as that in methanol alone if the increase in K_d values was caused by the dissociation of the acidic groups.

It might be appropriate to consider that the structure of the gel itself might be altered by the addition of hydrochloric acid, potassium hydroxide or potassium chloride to methanol. It may be postulated that hydrochloric acid, potassium hydroxide or potassium chloride in the eluent interacts with the residual hydroxyl group in the gel through hydrogen bonds of the following types:

$$H^{\delta+}\text{–}Cl^{\delta-} \cdots H^{\delta+}\text{–}O^{\delta-}\text{–gel}$$

$$\begin{array}{l} K^{\delta+}\text{–}O^{\delta-} \cdots H^{\delta+}\text{–}O^{\delta-}\text{–gel} \\ \quad\;\; | \\ \quad\; H^{\delta+} \end{array}$$

$$K^{\delta+}\text{–}Cl^{\delta-} \cdots H^{\delta+}\text{–}O^{\delta-}\text{–gel}$$

As a result of these hydrogen bonds, the outside of the gel becomes positively charged in all three instances.

With regard to mechanism, the adsorptive effects can be divided into two classes: (1) the ionic effects involving electrostatic interactions including ion exchange, ion exclusion and ion adsorption; and (2) the pure adsorptive effects involving hydrogen bonding and π-bonding. The data in Table IV might be explained on the basis of the gel–hydrochloric acid (potassium hydroxide or potassium chloride) interactions suggested above, together with the two mechanisms for the adsorption of solutes on to gels.

The solutes that have no dissociable groups are adsorbed to gels only by pure adsorption in the three eluents. The solutes that have an acidic group are inhibited from dissociation in hydrochloric acid–methanol and the ionic effects due to the dissociation are neglected, so that the elution of the solutes occurs earlier than in methanol. These solutes dissociate in potassium hydroxide–methanol or potassium chloride–methanol and the delay in the elution of the solutes due to the ion adsorption between the ion and the gel increases markedly. The solutes that have a basic group are positively charged in hydrochloric acid–methanol and potassium chloride–methanol and exhibit the ion exclusion effect, which persists even in potassium hydroxide–methanol. Several solutes behave similarly in potassium chloride–methanol, hydrochloric acid–methanol and potassium hydroxide–methanol, and in a different manner to that in methanol alone, indicating the occurrence of ion exclusion or ion adsorption between the solutes and the gel. The observation that the K_d values for dinitroaniline are almost identical in three eluents can be explained on the assumption that the ionic effects are absent owing to the formation of a chelate ring by hydrogen bonding between an amino group and the nitro group in the *ortho* position. We assume from the theory mentioned above and the fact that the K_d values for ε-aminocaproic acid in hydrochloric acid–methanol and potassium chloride–methanol are similar that the carboxyl group in ε-aminocaproic acid does not dissociate in methanol or in potassium chloride–methanol, that the amino group does dissociate, and that the ion exclusion effect occurs in potassium chloride–methanol. The results discussed are summarized in Table V.

Elution behaviour of solutes in methanolic solutions including benzene, dinitrobenzene and pyridine

Hydrogen bonding and bonding by coulombic force are local adsorptions, and the saturation of adsorption by such bonding is determined by the number of interaction sites on the gel surface. The addition of aromatic compounds such as 0.2 *N* sodium salicylate to the eluent in order to saturate the interaction sites in the gel was reported to be rather ineffective[6]. However, dinitrophenyl amino acids are eluted later than the free amino acids in phenol–acetic acid–water (1 : 1 : 1) eluent and earlier in 1 *M* pyridine eluent. Further, the interaction of the gel with aromatic compounds was assumed to be completely eliminated[14].

If specific groups in the gel are assumed to act as the adsorption sites for solutes, an appropriate means of preventing these interactions is to block the sites with other compounds. No differences in K_d values were observed on addition of 1% of benzene or 0.5% of dinitrobenzene to methanol. However, the effects of the addition of 10% of benzene or 1 *M* pyridine to methanol might be ascribed to blockage or "saturation" of the adsorption sites of the gel (Table II). Apparently the sites for π-bonding with solutes are blocked in 10% benzene–methanol and the sites for hy-

drogen bonding in 1 *M* pyridine–methanol. These secondary effects are not completely eliminated by the addition of 10% of benzene or 1 *M* pyridine as the K_d values are still greater than unity.

TABLE V

MAIN SECONDARY EFFECTS CONCERNED IN GEL–SOLUTE INTERACTION FOR ADSORPTION

Abbreviations: P.A. = pure adsorption; I.E. = ionic effects; M.S.E. = molecular sieving effect.

Classification	*Solute*	*Eluant*			
		Methanol	*HCl–methanol*	*KOH–methanol*	*KCl–methanol*
a	Benzene Nitrobenzene *m*-Dinitrobenzene	P.A.	P.A.	P.A.	P.A.
b	2,4-Dinitrophenol 2,4-Dinitrobenzoic acid Benzoic acid	P.A. I.E.	P.A.*	P.A.** I.E.	P.A.** I.E.
c	2,4-Dinitrophenylamino caproic acid Benzoylaminocaproic acid	M.S.E.***	P.A.§	P.A.** I.E.	P.A.** I.E.
d	ε-Aminocaproic acid	I.E.	I.E.§§	I.E.§§§	I.E.
e	*m*-Phenylenediamine	P.A. I.E.	I.E.§§	P.A.§	I.E.§§
f	2,4-Dinitrophenetole	P.A.	P.A.		P.A.

* Inhibited from dissociation.
** Ion adsorption with positive charge of the gel.
*** Mostly M.S.E.
§ Not dissociated.
§§ An $-NH_2$ group dissociates and ion exclusion with positive charge of the gel.
§§§ A –COOH group dissociates and ion adsorption with positive charge of the gel.

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Journal of Chromatography, 95 (1974) 169–174

CHROM. 7445

A SIMPLIFIED DESIGN FOR AN ELECTRON CAPTURE DETECTOR

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(Received January 10th, 1974; revised manuscript received March 3rd, 1974)

SUMMARY

A simple, rugged electron capture detector based on a concentric tube design has been developed. The new detector functions as well as a current detector based on Kovar–glass insulations but has advantages in terms of ruggedness and ease and inexpensive repair.

INTRODUCTION

Electron capture detectors are noted for their extreme sensitivity, partial specificity and ease of contamination. Contamination can be a severe problem when the detector is used under less than optimal conditions[1]. Plating of the electron source by column effluent is a problem particularly when ^{3}H is used as a source due to the rather low operating temperature of the usual titanium tritide foils. Often this factor limits column operating temperature to less than optimum values.

Two means of partially overcoming plating problems have been used. The first is the use of high-temperature radioactive foils, such as ^{63}Ni or most recently $Sc^{3}H$, as the electron source. Detectors using these foils can be operated at much higher temperatures and column effluents are less likely to plate out and more easily revolatilized off of the foils. These detectors still have a problem with material plating out onto the foils which cannot be removed thermally. The second means of overcoming plating problems is through a detector design that enables easy foil maintenance to be carried out. The Varian concentric tube electron capture detector (U.S. patent 3,277,296) is an excellent example and coupled with the cleaning method of Holden and Wheatley[1], a foil may be removed, decontaminated and back in service in a matter of hours. The basic detector design is based on a Kovar–glass–Kovar–glass–Kovar function where the glass serves as an insulator. The relatively thin-walled glass insulator of the Kovar detector is quite fragile and, once broken, is generally beyond repair** and quite costly to replace. We wish to report on a modified concentric tube detector (modular cell) that, while having the advantages mentioned above, is rugged and easy to build or repair.

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** Two professional glass blowers, when requested to repair a broken Kovar detector, were not successful.

CONSTRUCTION OF THE DETECTOR CELL AND METHODOLOGY

The basic design features of the modular cell are shown in Fig. 1. The stainless-steel vent cap (I) the stainless-steel cathode (II) and the stainless-steel anode wafer (V) are easily machined out of stainless-steel stock. The anode tubing in this instance is made out of 17-gauge, stainless-steel syringe needle tubing (obtained from a 17-gauge hypodermic needle) and is friction-fitted into the hole in the stainless-steel anode wafer. The actual gauge size is relatively unimportant but must snugly fit the glass capillary tube section (IV and VI). With the large number of sizes of hypodermic needles available, the selection of glass capillary stock to give a snug fit is readily accomplished. The cathode–anode Pyrex insulator (IV) is made of stock Pyrex capillary tubing. A glass saw is used to cut the ends of the Pyrex tubing off square and the small depression in the insulator is made by grinding with a

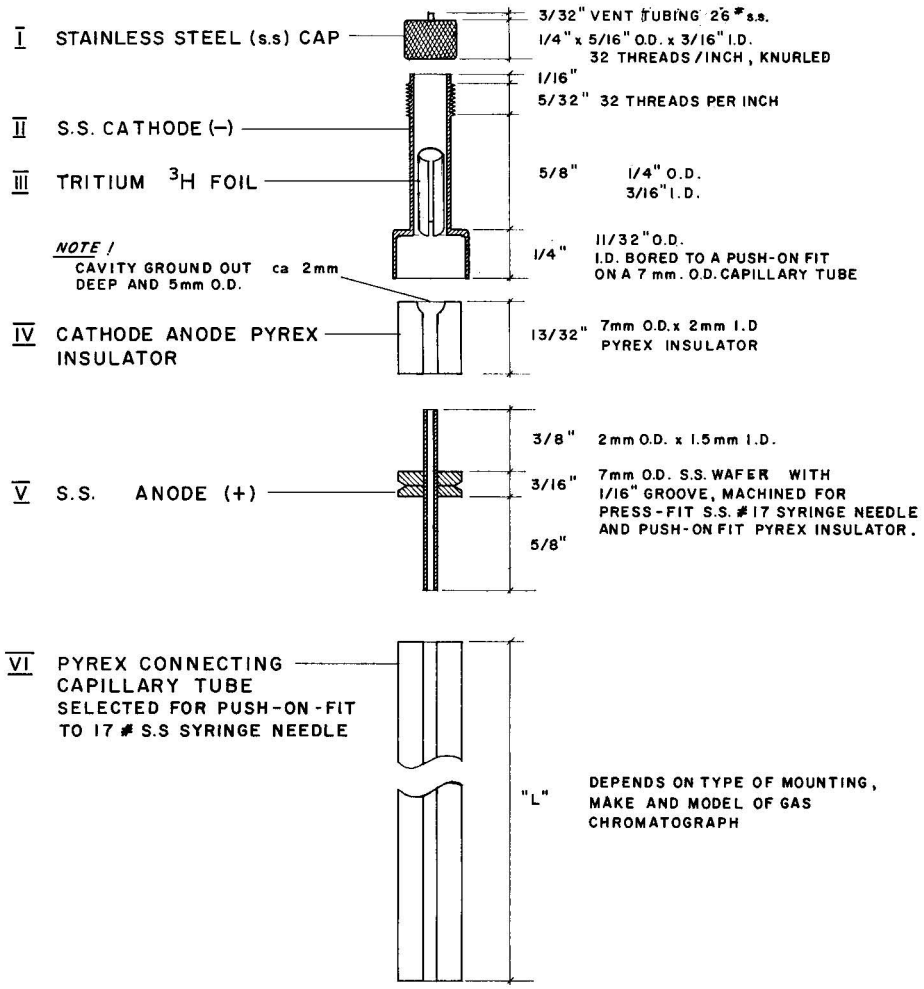

Fig. 1. Exploded view showing design features of individual components of the modular cell electron capture detector.

rounded Pyrex rod and carborundum powder. The detector uses a Varian 250-mCi Ti^3H_2 foil and, when assembled, is stacked onto a vertical glass capillary column effluent tube analogous to that described by Uthe *et al.*[2]. In this study the detector was mounted on a Microtek MT 220 Gas Chromatograph fitted with a 6 ft. $\times$ 0.25 in. I.D. glass column packed with 3% OV-1 on Chromosorb W (80–100 mesh) using nitrogen as a carrier gas at a flow-rate of 90 ml/min. The operating temperatures were 230°, 180° and 245° for the injection ports, column and detector block, respectively. All tests of detector operation were carried out using hexane solutions of lindane. In all cases comparison between the Kovar cell and the modular cell were carried out using the same column and same radioactive foil. Following the completion of a test, the foil was transferred back to the original detector and its response again determined to ensure stability of all operating parameters especially with regard to foil contamination.

The detector castle was a standard Varian design and Varian clip-on signal cables were used. The castle was mounted on a heating block turned out of aluminum stock. The heater block and the castle were insulated by asbestos paper overlaid with aluminum foil. The thermocouple and the heater cartridge were mounted on opposite sides of the heater block. The detector foil temperature was calibrated against the heater block temperature by removing the ^3H foil and placing a thermocouple inside the cell. For this particular design a heater block temperature of 245° gave a foil operating temperature of 214°.

RESULTS AND DISCUSSION

The linearity of response and the sensitivity of both the modular cell and the Kovar cell are shown in Fig. 2. The modular cell was slightly more sensitive than the Kovar cell, but both responses were within the same order of magnitude. No significant differences in the linearity of response were noticed between the two cells. There was no noticeable difference in noise when the detectors were operated at maximum sensitivity (Fig. 3). No evidence for oxygen contamination of the detector used in this study was found because the standing current equalled that of the sealed Kovar cell at operating voltages. This was rather surprising for it had been assumed that small leaks would be present in a friction-fit arrangement as used in the modular cell and would lead to increased noise over that present in a Swaged system such as the Kovar cell. The small upward baseline drift in the modular cell tracing is due to operation of the detector only a short time after placement onto the column.

The effect of varying the collection (d.c.) voltage on detector response is shown in Fig. 4. Again little difference was found between the two cells, both of them showing maximal response around 5 V and very similar response profiles. Standing current *vs.* voltage profiles are shown in Fig. 5. The Kovar cell is appreciably better at lower voltages, but at maximum current no great difference exists between the cells and at operational voltages (90 V) the standing currents of both cells are essentially the same. The small increase in current in the modular cell at 90 V d.c. as compared to the Kovar cell is probably due to the closer promixity of the anode to the cathode in the modular cell. This also indicates that no problem of current suppression due to oxygen level diffusion[3] through the friction-fit fittings occurred.

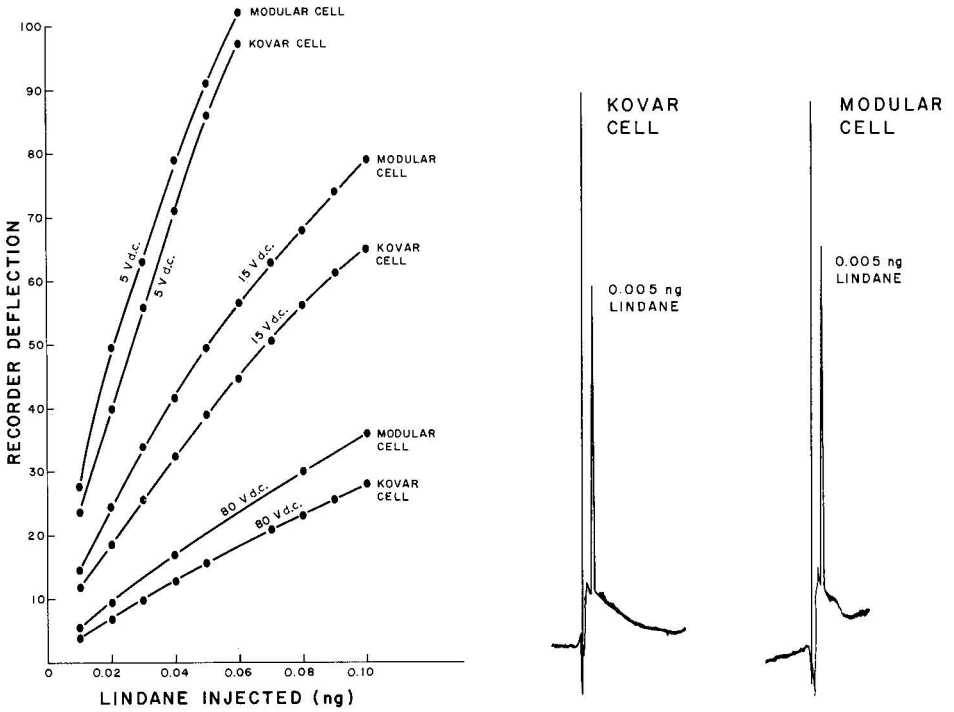

Fig. 2. Comparison of linearity of responses and sensitivity of response of the Kovar cell *vs.* modular cell electron capture detector.

Fig. 3. Comparison of recorder noise levels observed with Kovar cell and modular cell electron capture detectors operated in the maximum sensitivity ranges.

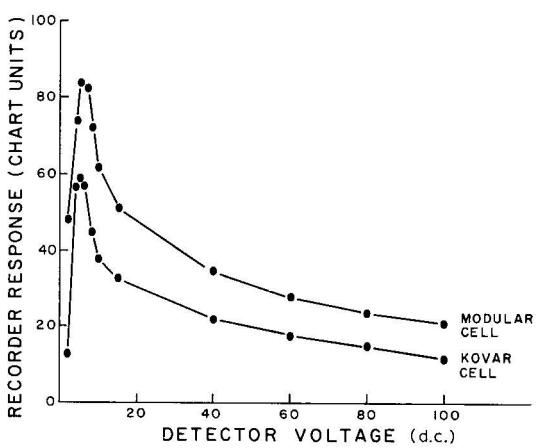

Fig. 4. Effects of varying collection voltage on Kovar and modular cell electron capture detectors.

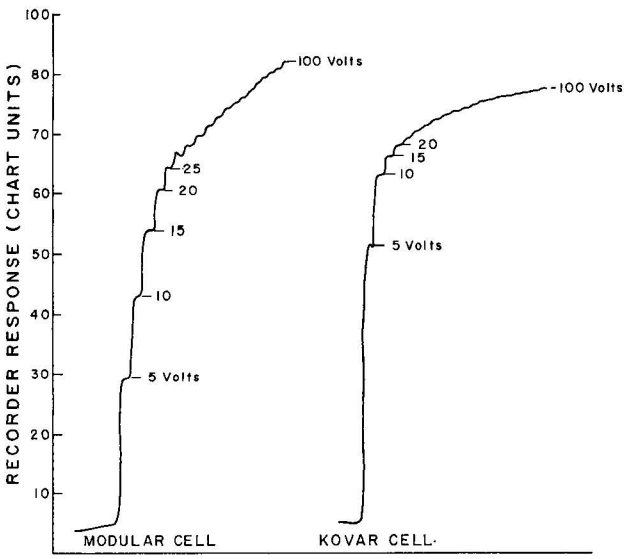

Fig. 5. Standing current (d.c. voltage) profiles of Kovar and modular cell electron capture detectors.

Use of both detectors in routine analysis of organochlorine residues has shown the modular cell to function as well as the Kovar cell. The frequency at which foils had to be removed and cleaned to restore detector performance was about the same. The ease with which the foil could be removed from the cathode (it is open at both ends) made maintenance relatively simple.

In summary, the modular cell functions as well as the Kovar cell. It has, however, certain advantages in terms of manufacture, ruggedness and serviceability. No evidence was found to suggest that joints tighter than friction-fit were necessary for good performance. The modular cell with a friction fit between the inner thin-walled stainless-steel tube and the outer thick Pyrex insulation tubes, when heated, would tend to form a tighter and tighter seal because the thermal expansion of stainless steel is greater than that of Pyrex. If a leak were a problem, a seal made with an electron-capture grade grease would probably suffice since pressures within the detector are only slightly above atmospheric pressure. The modular cell was not tested with any of the high-temperature foils, but there does not appear to be any major obstacle to their use, as is the case with the Kovar cell, at temperatures up to 300° (ref. 4) and use of a silica (quartz) cathode–anode insulator would extend the temperature range further[5]. No attempt was made to operate the modular cell in a pulsed mode as there seems to be little to be gained in operating concentric tube electron capture detectors in this mode.

Although no specific fragility tests were carried out, the use of a much thicker walled glass insulator in the modular cell as compared to that of the Kovar cell would indicate decreased fragility of the modular cell. The friction-fit functions of the modular cell would also not transmit imposed stress to the glass insulator from the non-glass portions as readily as would be the case with the Kovar cell.

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Journal of Chromatography, 95 (1974) 175–180

CHROM. 7500

THIN-LAYER CHROMATOGRAPHIC DETERMINATION OF THE STABILITY OF COMPLEXES OF SUBSTITUTED STYRENES ON SILVER NITRATE-IMPREGNATED SILICA

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(First received December 27th, 1973; revised manuscript received April 9th, 1974)

SUMMARY

A quantitative approach is presented for the determination of complexing strengths by means of thin-layer chromatography. This approach has been applied to the silver (I) complexes of a series of 3- and 4-substituted styrenes. The results are considered in terms of a Hammett–Yukawa relationship with a ρ value of -0.8, which is close to the results obtained from solubility and distribution measurements.

INTRODUCTION

Of the various methods for measuring the strenght of interaction between a metal and a ligand, recently reviewed by Hartley[1] for complexes between unsaturated hydrocarbons and transition metals, the use of thin-layer[2] and column liquid chromatography[3] has received little attention. In particular, these chromatographic methods may offer a convenient and rapid means for the study of the stability of complexes between a metal ion or atom and a ligand in solution. Furthermore, liquid chromatography may be complementary to the well developed gas chromatographic method in the case of non-volatile ligands.

In this paper, we present a quantitative approach to the study of metal–ligand interactions by means of thin-layer chromatography (TLC). In order to test the method, we studied the silver(I) complexes of a series of 3- and 4-substituted styrenes on silver nitrate-impregnated silica. Comparison is made with literature results obtained from direct solubility and distribution measurements.

EXPERIMENTAL

The styrenes were commercial samples or preparations made by standard procedures[4,5]. The pre-coated silica gel plates (20 × 20 cm) were purchased from Merck, Darmstadt, G.F.R., (Silicagel 60, layer thickness 0.25 mm). The silica area was 260 m^2/g (nitrogen adsorption), pore volume 1.6 ml/g, mean pore radius 140 Å (mercury penetration), and water content 8% after heating for 1 h at 120° (loss of weight after subsequent heating of the silica for 65 h at 400°).

The silver nitrate-impregnated silica gel plates were prepared by immersing the pre-coated plates in a solution of silver nitrate in acetonitrile for 30 min, followed by

drying for 60 min at 120°. The use of solutions containing 0, 1, 3, 6, 10 and 20% (w/v) of silver nitrate yielded plates with a loading of 0.00, 0.025, 0.07, 0.15, 0.23 and 0.47 g of silver nitrate per gram of silica.

Samples (6 μl) of a 1% solution of the styrenes in *n*-heptane were spotted on the plates, developed with chloroform at room temperature and rendered visible with phosphomolybdic acid[6] (20% solution in ethanol).

THEORETICAL

Consider the complexation of a ligand L in solution with metal ions M impregnated on a support S under TLC conditions. The stationary phase consists of both M and S and the interactions of the ligand with the stationary phase have to be described in terms of both a complex constant K_M and an adsorption equilibrium constant K_S.

For the transfer of 1 mole of L from the stationary phase to the mobile phase, the reversible work due to complexation of L with M (denoted by $W_{rev,M}$) is given by[7]

$$W_{rev,M} = RT\left(\ln \frac{C_m}{C_M} - \ln K_M\right) \tag{1}$$

where C_m and C_M are the amounts of the mobile phase and M, respectively*. When n molecules of L are transferred from the stationary to the mobile phase, or when one molecule of L is transferred n times from the stationary to the mobile phase, the reversible work delivered is $n/N \cdot W_{rev,M}$ (N = Avogadro constant). Comparison of a ligand L_0 with some other ligand L_1 under the same conditions leads to

$$\frac{n}{N}(W_{rev,M})_{L_1} - \frac{n}{N}(W_{rev,M})_{L_0} = -\frac{n}{N} RT \ln \frac{(K_M)_{L_1}}{(K_M)_{L_0}} = \frac{n}{N}[(\Delta G_M)_{L_1} - (\Delta G_M)_{L_0}] \tag{2}$$

where ΔG_M is the free energy of complex formation between the ligand and M. The K_M values are given by[7]

$$K_M = \frac{C_m}{C_M}(1/R_F^M - 1) \tag{3}$$

where R_F^M denotes the R_F value of the ligand developed on an imaginary TLC plate with just M as the stationary phase and $n=N$. Combining eqns. 1 and 3 gives

$$W_{rev,M} = -RT \ln (1/R_F^M - 1) \tag{4}$$

However, the experimental R_F values are determined by both M and S complex formation. Furthermore, these R_F values count for n instead of N interactions between the ligand and M, *i.e.*, they refer to $n/N \cdot W_{rev,M}$ rather than $W_{rev,M}$. The following expression can be written:

$$[(W_{rev,MS})_{L_1} - (W_{rev,MS})_{L_0}] - \theta[(W_{rev,S})_{L_1} - (W_{rev,S})_{L_0}] = \frac{n}{N}[(W_{rev,M})_{L_1} - (W_{rev,M})_{L_0}] \tag{5}$$

where $W_{rev,MS}$ and $W_{rev,S}$ are the reversible work pertaining to the M-impregnated

* The ratio C_m/C_M may vary along the length of the chromatogram[8]. However, this phenomenon is eliminated later by the use of relative K values (*cf.*, eqns. 8, 10 and 12).

S and the S stationary phase, respectively, wheras $1-\theta$ $(0 \leqslant \theta \leqslant 1)$ is the fraction of the surface area of S covered by M. By analogy with eqn. 4, we can write

$$(W_{\text{rev,MS}}) = -RT \ln (1/R_F^{\text{MS}} - 1) \tag{6}$$

and

$$(W_{\text{rev,S}}) = -RT \ln (1/R_F^{\text{S}} - 1) \tag{7}$$

where R_F^{MS} and R_F^{S} are the respective R_F values of the ligand on the plates. Substitution of eqns. 6 and 7 for L_0 and L_1 in eqns. 2 and 5 gives

$$\frac{n}{N}[(W_{\text{rev,M}})_{L_1} - (W_{\text{rev,M}})_{L_0}] = RT \ln \left[\frac{(1/R_F^{\text{MS}} - 1)_{L_1}}{(1/R_F^{\text{MS}} - 1)_{L_0}}\right] - \theta RT \ln \left[\frac{(1/R_F^{\text{S}} - 1)_{L_1}}{(1/R_F^{\text{S}} - 1)_{L_0}}\right]$$

$$= -\frac{n}{N} RT \ln \frac{(K_{\text{M}})_{L_1}}{(K_{\text{M}})_{L_0}} = \frac{n}{N}[(\Delta G_{\text{M}})_{L_1} - (\Delta G_{\text{M}})_{L_0}] \tag{8}$$

In the case of structurally related ligands L_1 and L_0, *e.g.*, 3- and 4-substituted styrenes, for which the Hammett relationship[9] is applicable, we can write, assuming that complexation is caused almost solely by the interaction of the olefinic bond with the silver ions as found in homogeneous silver(I) complexation[10,11],

$$(\Delta G_{\text{M}})_{L_1} - (\Delta G_{\text{M}})_{L_0} = -2.3 RT \rho\sigma \tag{9}$$

where L_0 refers to the unsubstituted ligand. Combination of eqns. 8 and 9 results in

$$\log \left[\frac{(1/R_F^{\text{MS}} - 1)_{L_1}}{(1/R_F^{\text{S}} - 1)_{L_1}^{\theta}}\right] = \frac{n}{N}\rho\sigma + \log \left[\frac{(1/R_F^{\text{MS}} - 1)_{L_0}}{(1/R_F^{\text{S}} - 1)_{L_0}^{\theta}}\right] \tag{10}$$

However, the plot of the logarithmic term *versus* σ gives a slope that is dependent on n/N. In order to determine the true ρ value, we must eliminate this factor. To a first approximation, we assume that R_F^{M} is correlated with R_F^{MS} and R_F^{S} according to the relationship

$$(1/R_F^{\text{M}} - 1) \approx \frac{N}{n}[(1/R_F^{\text{MS}} - 1) - \theta(1/R_F^{\text{S}} - 1)] \tag{11}$$

Substitution of eqn. 11 into eqn. 10 results in the following final expression:

$$\log [(1/R_F^{\text{MS}} - 1)_{L_1} - \theta(1/R_F^{\text{S}} - 1)_{L_1}] \approx \rho\sigma + \log \left[\frac{n}{N}(1/R_F^{\text{M}} - 1)_{L_0}\right] \tag{12}$$

Eqn. 10 can be best used to test the linear free energy relationship, whereas eqn. 12 has to be applied to obtain the correct slope of the relationship found. Finally, $\theta = 1$ can be used in most instances, because most often $(1/R_F^{\text{MS}} - 1)_{\text{L}} \gg (1/R_F^{\text{S}} - 1)$ and/or loading by M is small.

RESULTS AND DISCUSSION

In the preparation of the silver nitrate-impregnated silica gel plates by immersing pre-coated silica gel plates in a solution of silver nitrate in acetonitrile, complete removal of the acetonitrile, as shown by infrared spectroscopy, was effected by drying the plates at 120° for 1 h. The blank silica gel plates were obtained in the same manner

using pure acetonitrile. Table I shows some data for the various silver nitrate-impregnated silica gel plates used.

TABLE I

DATA FOR SILVER NITRATE-IMPREGNATED SILICA GEL PLATES

Loading (*g $AgNO_3$/g silica*)	*Solid $AgNO_3$** (*relative figures*)	*Surface area*** (m^2/*g silica*)
0.00		260
0.025		250
0.07	1	240
0.15	1	250
0.23	2.5	220
0.47	10	220

* From X-ray diffraction measurements.
** Nitrogen adsorption (± 10%).

The small decrease in the surface area of the stationary phase at the higher loadings of silver nitrate is the result of blocking of the micro-pores (<30 Å) of the silica by silver nitrate crystallites, as shown by the mercury penetration measurements and the fact that the surface area returns to its original value of 260 m^2/g after extraction of the silver nitrate by acetonitrile.

X-ray diffraction measurements indicate the presence of silver nitrate crystallites larger than 10^3 Å. The relative intensities of the diffraction lines are not in agreement with those expected from the loading precentages and indicate the occurrence of an aqueous silver nitrate solution on the silica gel. This is well understood because the silica gel dried at 120° still contains 8% of water, which can dissolve 0.18 g of silver nitrate per gram of silica at room temperature. This result explains the large increase in the amount of solid silver nitrate at higher loadings (Table I). It is clear that the interaction of the ligand with the solid silver nitrate present is of minor importance because of its much smaller surface area (estimated silver nitrate surface area < 6 m^2 per gram of silica for the highest loading). Consequently, the complexation phenomena to be studied must be ascribed almost solely to the interaction of the ligand with the aqueous silver nitrate phase on the silica gel.

A series of 3- and 4-substituted styrenes was developed on the various silver nitrate-impregnated silica gel plates at 25° with chloroform as the eluent. The results are summarized in Table II.

Table III shows the reaction parameters $n/N \cdot \rho$ and ρ, which were obtained from the data in Table II using eqns. 10 and 12 with $\theta=1$. The σ values used were derived from the Yukawa expression $\sigma=\sigma^n+r\Delta\sigma_R^+$ (ref. 12) with $r=0.44$, using σ^n and $\Delta\sigma_R^+$ values of Wepster and co-workers[13,14]. The choice of a resonance parameter r of 0.44 is somewhat arbitrary, *viz.*, the mean value of r obtained from eqn. 10. The use of eqn. 12 in order to obtain the true ρ values gives a lower correlation coefficient than the use of eqn. 10, owing to both the approximation made in eqn. 11 to eliminate the n/N factor and the greater relative error in the term $1/R_F^{MS}-1/R_F^S$ with respect to $(1/R_F^{MS}-1)/(1/R_F^S-1)$. This latter cause is well demonstrated by the correlation co-

TABLE II

TLC DATA FOR SUBSTITUTED STYRENES ON SILVER NITRATE-IMPREGNATED SILICA GEL (R_F^{MS}) AND SILICA GEL (R_F^S) WITH CHLOROFORM AS THE ELUENT

	Loading (g $AgNO_3$/g silica)							
	0.025		*0.07*		*0.23*		*0.47*	
Substituent	R_F^{MS}	R_F^S	R_F^{MS}	R_F^S	R_F^{MS}	R_F^S	R_F^{MS}	R_F^S
4-OMe	0.57	0.75	0.34	0.75	0.34	0.71	0.24	0.71
4-Me	0.66	0.81	0.47	0.79	0.44	0.78	0.35	0.76
H	0.68	0.79	0.51	0.77	0.48	0.77	0.39	0.74
3-OMe	0.61	0.74	0.48	0.75	0.45	0.71	0.37	0.71
3-Cl	0.73	0.80	0.65	0.79	0.65	0.78	0.58	0.77
3-NO_2	0.67	0.73	0.61	0.73	0.62	0.70	0.55	0.69

TABLE III

$n/N\cdot\rho$ AND ρ VALUES FOR THE SILVER(I) COMPLEXATION OF 3- AND 4-SUBSTITUTED STYRENES ON SILVER NITRATE-IMPREGNATED SILICA

Correlation coefficients are given in parentheses.

		Loading (g $AgNO_3$/g silica)			
Equation	*Parameter*	*0.025*	*0.07*	*0.23*	*0.47*
10	$n/N\cdot\rho$	−0.22 (0.98)	−0.49 (0.99)	−0.52 (0.99)	−0.59 (0.99)
12	ρ	−0.5 (0.89)	−0.74 (0.96)	−0.88 (0.97)	−0.82 (0.98)

efficients of the relationships from the silica impregnated with 0.025 g/g of silver nitrate, in which the R_F^{MS} and R_F^S values differ only slightly. The "true" ρ value from these data is therefore unreliable.

In conclusion, the results indicate a ρ value of -0.8 ± 0.1 for the complexation equilibria of substituted styrenes with silver(I). This value is very close to the ρ value of -0.77 obtained by Fuens *et al.*[10] for the silver(I) complexation of substituted styrenes from direct solubility and distribution measurements. The results presented indicate that TLC is a useful additional technique for the quantitative determination of the strenghts of complexes[1,8].

ACKNOWLEDGEMENTS

Nitrogen adsorption measurements were carried out by Mr. A. M. van Wijk of this Laboratory. Mercury penetration measurements were carried out by Mrs. L. A. de Wit and Mr. N. van Westen of the Laboratory of Chemical Technology. X-ray diffraction measurements were carried out by Mr. N. M. van der Pers of the Laboratory of Metallurgy.

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Journal of Chromatography, 95 (1974) 181–188

CHROM. 7504

THIN-LAYER CHROMATOGRAPHY IN THE DETECTION OF POISONING BY PESTICIDES

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(First received January 2nd, 1973; revised manuscript received April 16th, 1974)

SUMMARY

The relative efficiencies of various chromogenic reagents used in thin-layer chromatography (TLC) for the detection of commonly used pesticides in India have been evaluated. Combined diagnostic techniques using two sets of chromogenic reagents on a single TLC plate are suggested for the detection of certain organochlorine and organophosphorus pesticides.

INTRODUCTION

About 50–80% of the poisons detected in toxicology cases in India (homicides, suicides and accidents as referred to by the police) are organophosphorus and organochlorine pesticides. The main chemicals involved are parathion, malathion, endrin, diazinon, etc[1]. The detection of common pesticides, therefore, is a problem frequently faced by forensic toxicologists in India. There is an extensive literature on both the qualitative and quantitative analysis of pesticides and their metabolites[2–8]. Of the methods described a variety of thin-layer chromatographic (TLC) techniques have been found to be both sensitive and economical for the detection of individual members of different groups of pesticides[3–6,9–20]. Attempts have also been made with some success to achieve the multiple detection of pesticides[3,7,9,19,21,22]. The TLC procedures available for organophosphorus and organochlorine pesticides have been re-evaluated critically for the detection of any of the common pesticides on a single plate using one or more developing reagents. The resultant method could then be routinely adopted in toxicological analysis.

EXPERIMENTAL

Chromatographic procedure

Glass plates (20 × 20 cm) were spread with a 0.25-mm layer of a slurry of silica gel in water (type I) or of silica gel–alumina (7:3) in water (type II). The plates were activated at 120° and spotted with about 2 μl of a 2% solution of aldrin, dieldrin, DDT, chlordane, endrin, lindane, malathion, parathion, diazinon, dimethoate and phosphamidon, as well as a control extract of viscera in diethyl ether. The plates were then developed to a height of 11 cm with various solvent mixtures (Tables I, II and III) and dried, and the following chromogenic reagents were used as sprays.

A (i) 0.5% silver nitrate in ethanol[23].
(ii) Expose to ultraviolet (UV) light for 1 h.

B (i) 0.2% brilliant green in acetone[24].
(ii) Expose to bromine vapour.

C (i) 0.5% rhodamine B solution followed by 10% sodium carbonate solution[3].
(ii) Expose to UV light for 15 min.

D (i) Malachite green–1% benzoperpurine–isopropanol–1 *N* sodium hydroxide (4:1:7:5)[25].
(ii) Expose to UV light for 1 h (or to sunlight until the plate becomes green).

E (i) To 0.1 g of silver nitrate dissolved in 1 ml of water, add 20 ml of 2-phenoxyethanol, dilute to 200 ml with acetone and add a drop of 30% hydrogen peroxide[13].
(ii) Expose to UV light for 1 h after heating the plate for 20 min at 110°.

F Freshly prepared 2:1 mixture of 10% zinc chloride solution in acetone and 20% diphenylamine solution in acetone[11,26].

G (i) 0.625% *o*-dianisidine in ethanol[12].
(ii) Heat the plate at 90° for 10 min.
(iii) Expose to UV light for 30 min.

H (i) 0.5% chloramine T followed by 0.5% congo red solution in 1% ethanol[9].
(ii) Heat to 110° for 10 min.

J 0.5% palladium chloride solution in 18% hydrochloric acid[27].

K 0.1% mercuric nitrate followed by 0.5% diphenylcarbazone solution in ethanol[9].

L 0.05% g of bromophenol blue in 10 ml of acetone diluted to 100 ml with 1% silver nitrate solution in 3:1 water–acetone[14].

M (i) 0.5% congo red in 1% ethanol[28].
(ii) Expose plate to bromine vapour.

N (i) 20% dimethylformamide in diethyl ether.
(ii) Heat to 100° for 5 min.
(iii) 1% tetrabromophenolphthalein ethyl ester in acetone[10].
(iv) 0.5% silver nitrate in ethanol followed by 5% citric acid solution.

O (i) 0.5% 2,6-dichloroquinone-4-chlorimide in cyclohexane[15,16].
(ii) Heat to 100° for 10 min.

P (i) Mixture of 1 ml of 0.25% fluorescein solution in dimethylformamide and 49 ml of ethanol[13].
(ii) Expose to UV light for 7 min.

Q (i) 2% *p*-nitrobenzylpyridine in acetone[17,18].
(ii) Heat at 110° for 10 min.
(iii) Spray with 10% tetraethylenepentamine in acetone.

Extraction procedure

The extraction procedure for all of the pesticides involved direct extraction with diethyl ether of a neutral homogenate of the biological material. The residue obtained on evaporation was usually suitable for direct spotting on TLC plates and did not require an intermediate clean-up procedure. Alternatively, steam distillation of the acidified viscera sample gave volatile pesticides in the aqueous distillate, from

which they were extracted with *n*-hexane. The residue after distillation was filtered and extracted with acetone to give non-volatile organochlorine pesticides.

RESULTS AND DISCUSSION

Typical results obtained with the different chromogenic reagents used for the detection of six common organochlorine pesticides (Table I) indicated that the best separation was achieved on silica gel plates with *n*-hexane–acetone (4:1) as the mobile phase. However, when silver nitrate is used as chromogenic reagent[13], a silica gel–alumina adsorbent mixture (II) gave better spots for the chlorinated pesticides. The chromogenic reagents A, B, C and D gave false positive results with the control viscera extract, in addition to their failure to detect some of the pesticides in this group. With several of the chromogenic reagents studied, UV irradiation was necessary. Although silver nitrate[13] in some form was the common chromogenic reagent with a reported sensitivity of 0.05 μg, *o*-dianisidine[12] also served to identify all members of this class. With zinc chloride–diphenylamine reagent[26], the spots were rendered visible more easily by the characteristic colour difference for each member of this group. Aldrin and lindane, however, did not respond to this reagent, but they are only infrequently encountered in toxicological cases.

The results presented in Table II show the response of five commonly used organophosphorus pesticides to twelve chromogenic reagents. Not all the reagents gave similar responses to all members of this group. Chromogenic reagent N did not respond to phosphamidon although the other members could be detected. The dark background with reagents B, L, O and P–A made it difficult to identify the spots with certainty, although all five pesticides could be detected with these reagents. *p*-Nitrobenzylpyridine (reagent Q) and *o*-dianisidine (reagent G) were, however, found to be the most suitable for diagnostic work on organophosphorus pesticides. The spots obtained were distinct and persistent on a light background and the test was claimed to be sensitive to about 0.5 μg amounts of organophosphorus pesticides[17,18]. Silica gel plates were found to be preferable in this case, while *n*-hexane–acetone (4:1) was generally suitable as the mobile solvent phase.

Several procedures and chromogenic reagents listed in Tables I and II were combined for rapid screening and multiple detection of common organochlorine and phosphorus pesticides. It was observed that organochlorine compounds were more difficult to detect; several combinations of chromogenic reagents listed in Tables I and II were tried and the significant results obtained are given in Table III. In other instances, satisfactory results could not be obtained. Silver nitrate–2-phenoxyethanol reagent (E) was sensitive for all of the pesticides, except for aldrin, on a silica gel–alumina plate (type II). The identification was difficult, however, as the background turned black when an adequate exposure to UV light was made. This drawback made this reagent less suitable for general screening work. With reagents G and G–Q, although both classes of pesticides responded fairly well, the organophosphorus spots were not distinct with G and the dark background precluded the use of G–Q. By use of zinc chloride–diphenylamine reagent followed by *p*-nitrobenzylpyridine reagent (F–Q in Table III), organochlorine pesticides appeared first with the ircharacteristic colours while the organophosphorus compounds became visible later with a purple-blue colour. A lack of response to aldrin and lindane with this reagent combination.

TABLE I

DETECTION OF ORGANOCHLORINE PESTICIDES BY TLC

R_F values are given in parentheses where relevant.

No.	*Chromogenic reagent*		*Adsorbent type* *	*Solvent system*	*Aldrin*	*Dieldrin*	*DDT*	*Chlordane*	*Endrin*	*Lindane*	*Control (viscera extract)*	*Colour of spots observed*
	Code	*Components*										
1	A	Silver nitrate in ethanol	I	*n*-Hexane–liquid paraffin–dioxane (7:2:1)	–	–	–	+	–	–	+	Black spots on grey background
2	B	Brilliant green	I	*n*-Hexane–acetone (4:1)	–	–	+	+	+	–	+	Light yellow spots on green background
3	C	Rhodamine B	I	*n*-Hexane–acetone (4:1)	+	–	+	+	+	+	+	Violet spots on pink background
4	D	Malachite green	I	*n*-Hexane	+	–	+	+	+	+	+	Dark green on green background
5	E	Silver nitrate–2-phenoxy ethanol	II	Acetone–*n*-heptane (2:98)	–	+ (0.76)	+ (0.75)	+ (0.75)	+ (0.38)	+ (0.38)	–	Black spots on dark brown background
6	F	Zinc chloride–diphenylamine	II	*n*-Hexane–acetone (4:1)	–	+ (0.64)	+ (0.69; 0.36)	+ (0.72)	+ (0.6)	–	–	Different colours (purple, orange, green, etc.) on light blue background
7	G	*o*-Dianisidine	I	*n*-Hexane	+ (0.81)	+ (0.81)	+ (0.89; 0.77)	+ (0.86)	+ (0.16)	+ (0.29)	–	Brown spots on dirty white background (dark spots in UV light)

* I = Silica gel; II = silica gel–alumina (7:3).

TABLE II

DETECTION OF ORGANOPHOSPHORUS PESTICIDES BY TLC

R_F values are given in parentheses where relevant.

No.	Chromogenic reagent		Adsorbent type*	Solvent system	Malathion	Parathion	Diazinon	Dimethoate	Phosphamidon	Control (viscera extract)	Colour of spots observed
	Code	Components									
1	H	Chloramine T	I	*n*-Hexane–acetone (4:1)	–	–	–	–	–	–	–
2	C	Rhodamine B	I	*n*-Hexane acetone (4:1)	–	+	–	–	–	–	Violet spots on pink background in UV light
3	J	Palladium (II) chloride	I	*n*-Hexane–acetone (4:1)	+	–	+	–	–	–	Yellow spots on dirty white background
4	K	Mercury(II) nitrate–diphenylcarbazone	I	*n*-Hexane–acetone (4:1)	+	+	+	–	–	–	White spots on violet background
5	M	Congo red	I	*n*-Hexane–acetone (4:1)	+	+	+	–	–	–	Brown spots on light brown background
6	B	Brilliant green	I	*n*-Hexane–acetone (4:1)	+	+	+	+	+	–	Yellow spots on grey background
7	N	Tetrabromophenol-phthalein ethyl ester–silver nitrate	II	Methylcyclohexane	+	+	+	+	–	–	Blue spots on yellow background
8	L	Bromophenol blue	I	*n*-Hexane–acetone (4:1)	+	+	+	+	+	–	Yellow spots that turn violet with time on dark background
9	O	2,6-Dichloroquinone-4-chloroimide	I	*n*-Hexane–acetone (4:1)	+	+	+	+	+	–	Purple on dark background
10	P–A	Fluorescein and silver nitrate	II	*n*-Hexane–acetone (4:1)	+	+	+	+	+	–	Pinkish spots in UV light White spots on brown background
11	Q	*p*-Nitrobenzyl-pyridine–tetraethylenepentamine	I	*n*-Hexane–acetone (4:1)	+ (0.43)	+ (0.41)	+ (0.65)	+ (0.0)	+ (0.08)	–	Purple-blue spots on light brown background
12	G	*o*-Dianisidine	I	*n*-Hexane–acetone (4:1)	+	+	+	+	+	–	White spots on light yellow background

* I = Silica gel; II = silica gel–alumina (7:3).

TABLE III

SIMULTANEOUS DETECTION OF ORGANOCHLORINE AND ORGANOPHOSPHORUS PESTICIDES BY TLC

R_F values are given in parentheses where relevant.

No.	*Chromogenic reagents*		*Adsorbent type**	*Solvent system*	*Aldrin*	*Dieldrin*	*DDT*	*Chlordane*
	Code	*Components*						
1	E	Silver nitrate–2-phenoxyethanol	I	*n*-Hexane–acetone (4:1)	–	–	–	+
2	E–P	Silver nitrate–2-phenoxyethanol and fluorescein	II	Acetone–*n*-heptane (2:98)	–	+ (0.84)	+ (0.82)	+ (0.84)
3	F–L	Zinc chloride–diphenylamine and bromophenol blue	I	*n*-Hexane–acetone (4:1)	–	+	+	+
4	F–Q	Zinc chloride–diphenylamine and *p*-nitrobenzylpyridine	II	*n*-Hexane–acetone (4:1)	–	+ (0.68)	+ (0.73)	+ (0.76)
5	G	*o*-Dianisidine	I	*n*-Hexane–acetone (4:1)	+	+	+	+
6	G–O	*o*-Dianisidine and 2,6-dichloroquinone-4-chloroimide	I or II	*n*-Hexane–acetone (4:1)	+	+	+	+
7	G–Q	*o*-Dianisidine and *p*-nitrobenzylpyridine–tetraethylenepentamine	I	*n*-Hexane–acetone (4:1)	+ (0.96; 0.32)	+ (0.96; 0.76)	+ (0.85)	+ (0.88)
8	G–Q	As for 7	II	*n*-Hexane–acetone (4:1)	+ (0.92)	+ (0.78)	+ (0.82)	+ (0.90)

* I=silica gel; II=silica gel–alumina (7:3).

however, limited its general applicability, although the technique was otherwise acceptable.

o-Dianisidine followed by *p*-nitrobenzylpyridine (G–Q in Table III) as a double spray technique on silica gel plates using *n*-hexane–acetone (4:1) as the mobile phase was found to be more useful. It revealed all of the organochlorine pesticides as brown-coloured spots on exposure to UV light and a purple-blue colour was finally obtained with organophosphorus compounds. This double spray procedure could be used as the basis for general screening work. A silica gel–alumina plate (type II), however, was not found to be satisfactory with this technique as the organochlorine pesticides sometimes tended to move with the solvent front. The thin-layer chromatogram obtained by method No. 7 (reagent G–Q in Table III) is shown in Fig. 1.

The results obtained for a given pesticide should be further confirmed by comparison with known standards using one or more of the specific chromogenic reagents already discussed.

n	*Lindane*	*Malathion*	*Parathion*	*Diazinon*	*Dimethoate*	*Phosphamidon*	*Colour of spots observed*
	−	+	+	+	+	+	Dark spots on UV exposure
	+	+	+	+	+	+	E: black spots on UV exposure
	(0.62)	(0.2)	(0.41)	(0.49)	(0.0)	(0.1)	P: white spots on brown background (violet in UV light)
	+	+	+	+	+	+	F: different colours L: yellow spots that turn purple on dark grey background
	−	+	+	+	+	+	F: different colours
		(0.51)	(0.66)	(0.74)	(0.09)	(0.15)	Q: purple-blue on light brown background
	+	+	+	+	+	+	G: light brown (violet in UV light); white on light brown background for phosphorus compounds
	+	+	+	+	+	+	G: brown spots on dark background O: purple spots on dark background
	+	+	+	+	+	+	G: brown spots on light brown background
	(0.62)	(0.3)	(0.32)	(0.56)	(0.0)	(0.0)	Q: purple-blue spots on light brown background
	+	+	+	+	+	+	As for 7
	(0.67)	(0.37)	(0.58)	(0.65)	(0.0)	(0.0)	

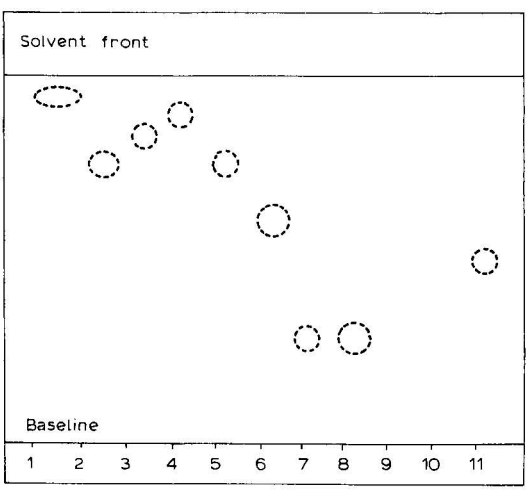

Fig. 1. Thin-layer chromatogram of six organochlorine and five organophosphorus pesticides using the double reagent G–Q (see No. 7 in Table III). 1 = Aldrin; 2 = dieldrin; 3 = DDT; 4 = chlordane; 5 = endrin; 6 = lindane; 7 = malathion; 8 = parathion; 9 = phosphamidon; 10 = dimethoate; 11 = diazinon.

CONCLUSION

Analytical techniques based on advanced gas–liquid chromatographic (GLC) equipment are increasingly becoming the methods of choice for the quantitative determination of pesticides at even nanogram levels[4,8]. The application of infrared spectrophotometry[4] as a specific characterisation technique requires a larger and purer sample, even though this can be coupled with GLC. TLC is an alternative inexpensive but sensitive technique for the rapid screening and multiple detection of pesticide residues at even 0.5 μg levels[6,29]. An appraisal of the existing techniques and a combination of the spray reagents has resulted in a rapid diagnostic survey of pesticides commonly involved in human poisoning in India. Considering that a large volume of work has to be carried out by the toxicologist in this field, a simpler and more economical technique is of great use for preliminary screening, which can be further supported by other qualitative and quantitative techniques.

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Journal of Chromatography, 95 (1974) 189–212

CHROM. 7482

DETERMINATION OF AMINO ACID PROFILES IN BIOLOGICAL SAMPLES BY GAS CHROMATOGRAPHY

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(Received March 11th, 1974)

SUMMARY

Based on solvent-free gas chromatography of the *n*-propyl, N-acetyl derivatives, procedures are described for the routine determination of amino acid profiles in biological samples including protein hydrolysates, plant tissue extracts, urines and sera. Derivatization is carried out in a 25-min two-step procedure. Use of a basic acylation environment allows derivatization of arginine and histidine. Chromatography using capsule injection on to a polar column with temperature programming gives resolution of 21 common amino acids within 15 min. Norleucine used as the internal standard provides quantitative capability. Sensitivity of the method permits quantitation at the nanomole level. Recoveries of amino acids added to sera ranged over 90–99%; an exception was arginine which gave 78%. Typical reproducibility data indicate that a coefficient of variation of 2–5% is attainable.

INTRODUCTION

The use of gas chromatography (GC) for the determination of amino acids is attractive because it provides quantitative results at high sensitivity, at a speed unequalled by other procedures. Indeed several GC procedures are detailed in the literature (*e.g.*, refs. 1–6). Common to these is the requirement to derivatize the amino acids in order to make them volatile, but stable enough for the GC analysis. The relative ease with which amino acids may be chemically modified to give many different derivatives[7,8] often leads to difficulty in choosing a derivative for a particular purpose. Where a particular amino acid is to be analyzed it is possible to select a derivative which will provide remarkable simplicity and speed in the analysis, as, for example, the procedure of Halpern *et al.*[9] for serum phenylalanine. Other possible derivatives, for example the N-dimethylaminomethylene alkyl esters[10], have great potential for amino acid analysis but insufficient evaluation has been published on their utility for the analysis of biological samples.

For routine determination of amino acid profiles, especially where accuracy, rapidity, economy, and simplicity of manipulation are prime requirements, the *n*-propyl, N-acetyl derivatives[4,5,8,11] warrant serious consideration. The reagents required are readily obtainable at high purity, are inexpensive and are not unduly sensitive to moisture. The derivatives are stable and are prepared in a two-step pro-

cedure that may be carried out in a screw-capped test tube. The use of a capped tube simplifies the manipulation. A single addition is made of the propylation reagent. In conjunction with a standard heating block there will be a temperature gradient along the tube which induces a refluxing action in the reagent. Furthermore, the pressure developed in the tube assists the reaction. Problems associated with moisture originating in the sample or from the reaction are obviated by use of a small amount of 2,2-dimethoxypropane as a water scavenger. An additional advantage is the commercial availability of pure derivatives allowing ready monitoring of recoveries and optimization of GC conditions.

The propylation is effected using propanolic HCl. The acylation reagent used is a more basic reagent than previously used for this purpose[5], consisting of acetic anhydride, triethylamine and acetone. The reagent effects acylation rapidly (within 2 min) and completely for most amino acids. Arginine and histidine, which have strongly bound HCl on the molecule following the propylation step, are also acylated with the mixed reagent used in the procedures that follow, thereby obviating the need for alternate procedures to determine these amino acids[5]. During the esterification, asparagine and glutamine are converted to the corresponding acids. Cystine is analyzed as cysteine, a reducing agent being used to effect preliminary reduction to cysteine in the sample. Organic reducing agents such as thioglycol are suitable but for this report stannous chloride was used.

Although dried protein hydrolysates may be derivatized and analyzed directly, most other biological samples require pretreatment to separate the amino acids from the matrix which may include protein, carbohydrates, salts, urea, lipids and peptides. Of several methods[9,11–14] most emphasize deproteinization. For this report a method is used based on that by Harris *et al.*[15] utilizing a cation-exchange resin for removal of cations present in the sample followed by preferential elution of the amino acids using an aqueous solution of ammonium hydroxide. The cation-exchange resin which was selected has high cross-linkage characteristics enabling deproteinization of the sample to be achieved by molecular exclusion chromatography.

GC of amino acid samples on capillary columns will be undoubtedly of increasing importance because of the speed and resolution of these columns. An indication of the potential inherent in the use of this type of column is given in the study by Jönsson *et al.*[16]. However, packed columns were used for this report.

GC on a single packed column of the *n*-propyl, N-acetyl amino acids has been considerably improved by the availability of a stable, polar, silicone liquid phase. Previously, complete resolution of the *n*-propyl, N-acetyl derivatives of all the protein amino acids was difficult to achieve on a routine basis, because it was necessary to use liquid phase mixtures[4,5,11] which were unsatisfactory for long-term routine use because of temperature limitations. The column used for this report was 3 ft.$\times$1/8 in. O.D. stainless steel. The packing was 0.31% Carbowax 20M, 0.28% Silar 5CP and 0.06% Lexan on Chromosorb W AW, 120–140 mesh. The temperature limit of this combination is better than 250° and allows repeated use of the column to this temperature without visible loss of resolution.

Injection of samples using solvent-free capsule injection (Model MS-41 Capsule Sampler)[17] was employed because of its advantages over syringe injection. Amongst others, these advantages include elimination of the septum and solvent, and greatly improved quantitative reproducibility for the compounds analyzed.

Additionally, it is possible to transfer, by successive washings, the total sample to the capsule for injection, thereby providing improved sensitivity for small samples.

The procedures were studied to determine derivatization reproducibility of pure amino acids and recoveries of amino acids from biological materials. Results obtained by the procedures were applied to a wide range of biological materials including protein hydrolysates, plant extracts and physiological samples. Emphasis has been given to analysis of sera and urine for amino acid metabolic disorders. GC analyses were computerized, which provided considerable flexibility and precision in handling the data. The final reports were expressed automatically in micromoles or milligrams for amino acid concentrations, as referenced against a common standard.

MATERIAL AND APPARATUS

Reagents

The following reagents were used: Dowex 50-X8 (H^+) 100–120 mesh (Sigma, St. Louis, Mo., U.S.A.); stannous chloride, analyzed reagent grade (Matheson, Coleman & Bell (East Rutherford, N.J., U.S.A.); ammonium hydroxide, analyzed reagent grade (Matheson, Coleman & Bell); dry air or nitrogen; ethyl acetate, spectroquality (Matheson, Coleman & Bell); acetic acid, glacial, analyzed reagent grade (J.T. Baker, Phillipsburgh, N.J., U.S.A.); acetic anhydride, 99+% (Matheson, Coleman & Bell); 2,2-dimethoxypropane, 99+% (Aldrich, Milwaukee, Wisc., U.S.A.); acetone, spectroquality (Matheson, Coleman & Bell); triethylamine (Matheson, Coleman & Bell); *n*-propyl alcohol* (Matheson, Coleman & Bell); anhydrous HCl*, technical grade (Scientific Gas Products, Edison, N.J., U.S.A.); amino acids, A grade (Calbiochem, La Jolla, Calif., U.S.A.); norleucine, A grade (Calbiochem) (for use as an internal standard); *n*-propyl, N-acetyl amino acids (Graff Ass., Santa Clara, Calif., U.S.A.); α-chymotrypsin protein (bovine pancreas, cryst.), A grade (Calbiochem.); ovalbumin, cryst., grade V (Sigma); histone (calf thymus), Type II (Sigma); lysozyme, 3×cryst. (egg white), A grade (Calbiochem); ribonuclease, 5×cryst. (bovine pancreas), A grade (Calbiochem.); collagen (calf skin, acid soluble) (Sigma).

pH adjusting solution. A 50% solution of acetic acid in deionized water and containing 5.0 mg $SnCl_2$/100 ml was used for adjustment of sample pH. (The stannous chloride is introduced with this solution to reduce cystine present in the sample to cysteine.)

Propylation reagent. The propylation reagent, propanolic HCl, was made by passing anhydrous HCl into 200 g cooled *n*-propyl alcohol until the reagent was 8 *M* in HCl on a w/w basis. Stored at 4° in a polypropylene bottle the reagent lasts several months.

Acylation reagent. A mixture of acetone, triethylamine and acetic anhydride (5:2:1) was prepared daily. This reagent may discolor on standing but this will not have any effect on the acylation.

Preparation of resin column

The column was made by pushing a small quantity of glass wool well down

* See *Propylation reagent*, below.

into the stem of a pasteur pipet. The glass wool was not too firmly compacted to avoid reducing the flow-rate. 50 mg of Dowex 50-X8 (H^+) 100–120 mesh resin was washed (using deionized water) on to the glass wool. It is important that high-quality moist resin be used for the column, and that it should be in the H^+ form. Commercially available resin proved adequate without special regeneration being required but the stock container was kept tightly sealed to prevent loss of exchange capacity due to adsorption of ammonia from the laboratory air. Expended resin was regenerated by passing 2 ml 1 *N* HCl through the resin column at about three drops per second then washing the resin with deionized water to pH 6–7. A schematic of the column showing approximate positioning of the resin is given in Fig. 1. 50 mg of resin will provide approximately 150 μequiv. exchange capacity. This is adequate for treatment of most types of samples. For example, 100 μl serum contains approximately 1 μmole amino acids and 15 μequiv. of inorganic cations[15]. 50 mg of resin with 150 μequiv. capacity represent a tenfold excess over requirements.

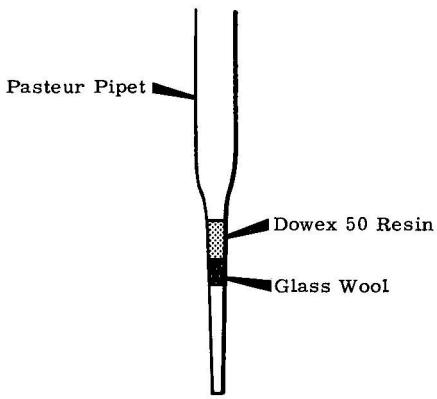

Fig. 1. Schematic of resin column illustrating positioning of the resin.

Standards. A stock solution of each amino acid including norleucine was made up in aqueous 0.1 *N* HCl at a level of 0.1 m*M*/ml. A working solution containing all the amino acids except norleucine was made up by combining equal aliquots of the individual amino acids, evaporating down the mixture to a small volume then diluting this with 0.01 *N* HCl to provide a level of 10 μM/ml for each amino acid.

Internal standard. Norleucine was used as the most convenient pure internal standard available. Working standards in 0.01 *N* HCl were prepared at a level of 10 μM norleucine/ml and at a level of 2 mg/ml. For results to be expressed in μmoles, the former is used, for results to be expressed in mg, the latter is used.

Standards were stored in capped polypropylene bottles and maintained at 4°.

Glassware

Glassware included 1-ml graduated pipets, 10-μl disposable micropipets, 15×85 mm disposable glass culture tubes, 16×75 mm glass culture tubes with screw-cap (PTFE-lined) closures (Kimax No. 45066A) and 145-mm disposable pasteur pipets. For 10-μl serum analyses, it was found useful to employ special reaction tubes, Reactivials, 3 ml, supplied by Pierce.

Equipment

The following equipment was used: heating block, Temp-blok (Scientific Products); manifold (for evaporation of derivatized samples); rotary evaporator, Buchi Rotavapor-R (Brinkman).

Gas chromatography

A Perkin-Elmer Model 3920 gas chromatograph with dual flame ionization detectors and linear temperature programming was used together with a Model 56 1-mV recorder. Injection was carried out with the Model MS-41 solvent-free capsule injection system. The PEP-2 Data Processor was used for automatic data reduction.

Column

The column used was 3 ft. × 1/8 in. O.D. stainless steel. This was packed with a mixed polar packing consisting of 0.31% Carbowax 20M (Perkin-Elmer), 0.28% Silar 5CP (Applied Science Labs., Rochester, N.Y., U.S.A.) and 0.06% Lexan (Perkin-Elmer) on Chromosorb W AW, 120–140 mesh. Prior to coating, the Chromosorb was heated to remove moisture at 400° for 1 h, then cooled. The packing was prepared by combining the required quantity of liquid phases in 140 ml of a chloroform–methanol mixture (85:15) and pouring the mixture on to 50 g of Chromosorb W AW contained in a 500-ml round-bottom flask. Then the solvent was cautiously evaporated off using a rotary evaporator adjusted to the lowest speed and a water-bath set at 90°. After filling the column it was conditioned with a helium flow of 12 ml/min at 250° for 2 h, then 16 h at 220°, or until the baseline was stable.

PROCEDURES

Sample requirements

Protein hydrolyzates. Acid hydrolyzates of pure protein were evaporated down with added internal standard (norleucine) to a dry residue and the derivatization procedure given below carried out. With a suitable quantity of protein (about 1–10 mg), it was possible to carry out the hydrolysis in a screw-cap tube used for all subsequent chemical manipulation, thereby obviating transfers.

Serum and urine samples. As with all complex samples, the amino acids require separation from some of the biological matrix. The method employed depends on adsorption of cations from a solution at pH 2–2.5 on to a Dowex 50 cation-exchange resin column. Non-ionic material and anions are washed through the column. Amino acids are preferentially eluted from the resin using 2 *N* NH_4OH. The procedure used was as follows.

Sample pretreatment

Serum or urine. (1) Transfer 0.1 ml of serum or 0.5 ml urine, 10 μl (=20 μg) of 2 mg/ml norleucine internal standard (see *Internal standard*) and 0.1 ml pH adjusting solution to a 16 × 75 mm tube. Mix gently. The pH should be 2–2.5 and is checked by spotting a minute amount of the mixture on to universal indicator paper. (2) Transfer mixture to resin column using a pasteur pipet for the transfer. Allow to pass through column at about one drop per 5 sec using very little air pres-

sure as, for example, from a rubber bulb. *Caution.* Do not allow column to run dry! (3) Wash sample tube with 0.5 ml deionized water and transfer the washing to the column. Pass through the resin and immediately follow this with 0.5 ml of deionized water. The flow-rate may be increased to about one drop per second. (4) The resin is eluted using 2 ml 2 *N* NH_4OH passed through the column at about one drop per second. The eluate is collected in a 16×75 mm screw-cap culture tube and evaporated to dryness using a rotary evaporator and a water-bath set at 90°. (A 4-cm length of 1-cm Tygon tubing is suitable for connecting the tube to the rotary evaporator.)

Plant samples. Leaf, root tissues and pulses were first extracted and cellulose and other fibrous or particulate material was removed. The sample was extracted with 70% ethanol. In this case, the plant samples were extracted by pulverizing a weighed quantity (1–10 g) of tissue with several volumes of aqueous 70% ethanol. The combined filtered extracts were flash-evaporated to a small volume and the volume made up with 70% ethanol so that a 1-ml extract was equal to 1 g of the original tissue. 100 µl of the extract were taken to dryness and 10 µl (20 µg) of internal standard were added. The residue was taken up in 0.5 ml deionized water and 0.5 ml pH adjusting solution. The pH was checked (pH 2.0–2.5) and processed as for serum.

Where a buffer (*e.g.*, phosphate buffer) containing cations is employed for the extraction, due consideration must be given to the exchange capacity of the resin. Generally, the resin quantity will be sufficient to take up cations in the sample. It is advisable to increase the resin quantity where other cations (*e.g.*, in a buffer) are added. The appropriate milliequivalent exchange capacity of the batch of resin used will provide the information for calculating this quantity.

Plant juices. Following centrifugation or filtration to remove debris, plant juices may be processed through the column directly after addition of internal standard and adjustment to pH 2–2.5. Because of species and varietal variation, citrus juices should be adjusted to the correct pH using 10% acetic acid which is added dropwise until the correct pH range is attained. A suitable volume of juice to use is 0.1 ml. The sample is processed through the resin and the eluate taken to dryness.

Derivatization

(1) To the dry eluate residue contained in a screw-cap 16×75 mm culture tube, add 50 µl (one drop) dimethoxypropane followed by 1 ml of propylating reagent. The dimethoxypropane scavenges moisture that may be present in the residue and removes water formed during esterification.

(2) Cap the tube firmly and heat it in a heating block at 110° for 20 min. Although several hundred reactions of this type have been processed without tube breakage, it is advisable to use a safety screen at this stage.

(3) Cool the tube briefly and open. Evaporate off excess reagent at 110° using a current of dry air or nitrogen. It is useful to hold moistened universal indicator paper just above the mouth of the tube to determine that all acid vapors have been evaporated off. Residual acid can react with the acylating reagent to produce an insoluble salt. This interferes mechanically with subsequent transfers and reduces the amount of reagent available for the reaction.

(4) Cool the tube briefly. Add 1 ml of acylating reagent. Cap the tube firmly.

Warm the tube for 30 sec at 60°. Cool the tube briefly. Evaporate the excess reagents at 60° using a current of dry air or nitrogen. (*Caution.* Derivatized material at this point is very volatile. Do not exceed the recommended temperature and use a gas flow not greater than 50 ml/min! Higher temperatures and gas flows will cause losses of several amino acids, particularly alanine and valine.)

(5) On completion of evaporation, no strong odor of acetic anhydride should be apparent. Cool the tube, take up the residue in 0.1 ml of anhydrous ethyl acetate. Cap the tube. The derivatized material is stable for several days at room temperature and several months when stored at 4°. 2- to 10-μl aliquots of the derivatized material are taken for the GC analysis.

GC analysis

The following instrument settings were used. The helium carrier gas flow-rate was 12 ml/min at an inlet pressure of 70 p.s.i. The injector temperature was 250° and the detector temperature was 300°. The oven was temperature programmed from 125° to 180° at 8°/min and 180° to 250° at 32°/min. Attenuation was set at 64×10. Injections were made by Perkin-Elmer MS-41 capsule system[17], evaporating off the solvent from the capsule before sealing and injecting. The capsule system was chosen because of its several advantages, including improved resolution, improved reproducibility and avoidance of solubility problems associated with the use of a solvent.

The GC performance was tested for resolution and sensitivity by injecting 1 μl of a mixture of pure derivatized amino acids containing 4 nmoles/μl of each derivative. A typical chromatogram is given in Fig. 2. Derivatized tryptophan was chromatographed isothermally at 250° on columns made from three different batches of packing in order to provide HETP data. Plate numbers for the 3-ft. columns ranged from 2,250 to 2,400 plates for the tryptophan derivative. The resolution of the amino acid standard was visually identical on the columns except that retention times on one column were somewhat greater for the later peaks.

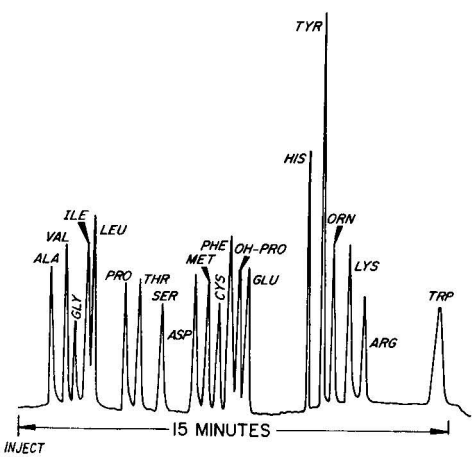

Fig. 2. Chromatogram obtained by injecting a mixture containing 4 nmoles of each derivatized amino acid.

The GC analyses were carried out using the GC parameters described above. Three columns were used for the analyses, it being convenient to use one for sera and urine analyses on a continuous basis, the others for plant extracts and protein hydrolysates. There was minimal variation in performance among columns. Throughout all the analyses reported here norleucine was used as an internal standard to provide quantitation capability and identification of other amino acids using retention times relative to the norleucine derivative. The analytical method for the computer used for the data reduction was obtained using standard amino acid mixtures taken through the complete procedures and gas chromatographed. The computer calculated the relative retention times (RRT) and response factors for each amino acid and applied this information for data reduction of all subsequent analyses.

It is of paramount importance to ensure that reagents used do not contribute unwanted volatiles to the GC analysis. Hence reagent blank runs were made to determine the suitability of the reagents used for this type of analysis. The purest obtainable amino acids were used for standards. It was found that several have other amino acids as impurities. Typical of this situation is the Grade A arginine used for the work reported here. When derivatized and chromatographed, it gave a small secondary peak corresponding in retention time to derivatized ornithine, as shown in Fig. 3. To determine whether some decomposition of arginine had occurred during derivatization, the free arginine was chromatographed by two-dimensional thin-layer chromatography (TLC) on silica. The chromatograms showed a small ninhydrin-positive, Sakaguchi-negative spot with R_F characteristics similar to ornithine. It was

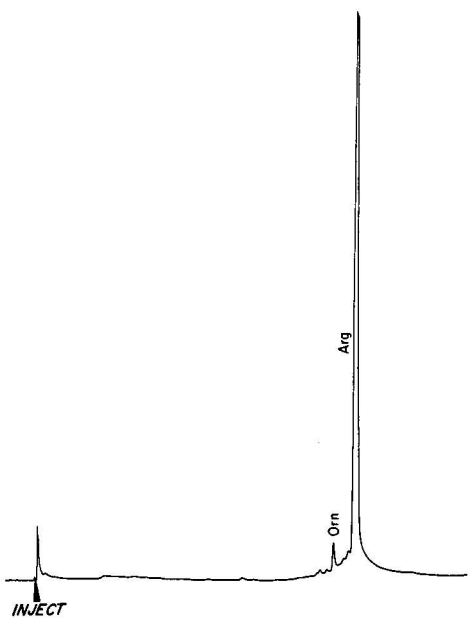

Fig. 3. Chromatogram of derivatized arginine. Injected sample size was 2μg. Ornithine was present as a contaminant in the arginine. The remainder of the background indicates the low level of interference contributed by the reagents.

concluded that the secondary peak on the chromatogram was ornithine originating as an arginine impurity. The remainder of the chromatogram illustrates the type of background obtained with the reagents and procedure used for this report. Although several small peaks are discernible, they provide no serious interference problems.

RESULTS

Derivatization

To ascertain reproducibility of derivatization, fifteen aliquots of a mixed amino acid standard solution were transferred to the reaction tubes and the solution evaporated to dryness using a heating block at 100° and a gentle current of dry air. The residues were carried through the complete derivatization procedure and 2-μl aliquots of the final ethyl acetate solution were gas chromatographed. Peak areas against an internal standard (*n*-propyl, N-acetyl norleucine) added just prior to injection were calculated automatically by the PEP-2 data processor and the results analyzed statistically. Results for some amino acids are given in Table I.

TABLE I

REPRODUCIBILITY OF DERIVATIZATION

Fifteen aliquots of aqueous amino acids, each at a level of 8 μM, were derivatized without pretreatment. Levels were calculated against those from an additional analysis used as an 8-μM standard.

	Amino acid							
	Val	*Ile*	*Thr*	*Asp*	*Phe*	*Tyr*	*Trp*	*His*
Mean ($\bar{X}$)	8.06	7.99	8.12	8.00	8.05	8.01	7.98	7.85
Standard deviation (SD)	0.17	0.18	0.20	0.12	0.16	0.40	0.19	0.40
Coefficient of variation (CV), %	2.1	2.2	2.5	1.5	2.0	5.0	2.4	5.1

Derivatization efficiency was studied by derivatizing different levels of pure amino acid solutions adding an injection standard immediately prior to analysis and comparing peak areas with corresponding areas of pure distilled derivatives. Results obtained for eight replicate analyses are given in Table II. Most amino acids derivatize over a 0.1-μg to 10-μg range with at least 96% efficiency. An anomaly is arginine, which derivatizes at about 78% efficiency. This is due to the incomplete removal of HCl from the guanido group. Acylation of the *n*-propyl arginine may be improved by repeating the acylation procedure. However, the standard procedure is highly reproducible (the coefficient of variation is about 2.5%) and, provided this small loss of sensitivity for arginine is acceptable, little purpose is served by increasing the total time of analysis.

Pretreatment recoveries

Recoveries were determined using the resin pretreatment procedure. Eight separate 0.1-ml aliquots of a standard amino acid mixture, containing 1 μg of each

TABLE II

DERIVATIZATION DATA FOR AMINO ACIDS

Aqueous amino acid solutions were evaporated to dryness and derivatized. Peak areas are expressed as % of peak areas obtained with pure distilled derivatives. N = Number of samples.

	Amino acid						
	Val	*Ile*	*Thr*	*Phe*	*Lys*	*Arg*	*Trp*
Level 0.1 μg, $N=6$							
$\bar{X}$	98.7	99.3	98.7	98.8	98.3	79.0	99.8
SD	1.6	2.3	1.4	1.9	2.1	1.8	2.4
CV, %	1.7	2.3	1.5	1.9	2.1	2.3	2.4
Level 1μg, $N=6$							
$\bar{X}$	98.0	97.7	98.1	98.7	99.9	77.9	99.1
SD	0.8	1.1	1.7	2.2	1.6	1.8	2.5
CV, %	0.8	1.1	1.7	2.2	1.6	2.3	2.5
Level 10 μg, $N=6$							
$\bar{X}$	97.8	96.8	97.5	97.6	97.5	77.8	97.6
SD	0.9	0.9	1.0	0.8	1.7	1.8	1.3
CV, %	0.9	1.0	1.1	0.8	1.8	2.3	2.4

amino acid, were adjusted to pH 2.2, processed through the resin, subsequently derivatized and gas chromatographed. Immediately prior to the injection a known amount of pure norleucine derivative was added as injection standard. Peak areas were computed and the peak area of each amino acid expressed as a percentage of the corresponding chromatographic area of the amino acid derivatized but not subjected to resin pretreatment. Results are given in Table III.

TABLE III

PRETREATMENT RECOVERIES

Aqueous solutions of amino acid subjected to the complete procedure. Pure norleucine derivative was added to processed sample as an injection standard and peak areas of each amino acid were compared with amino acids derivatized but not subjected to pretreatment. $N=8$.

	Amino acid						
	Val	*Ile*	*Ser*	*Thr*	*Asp*	*Met*	*Phe*
$\bar{X}$	0.98	0.98	0.97	0.96	0.98	0.92	0.97
SD	0.02	0.02	0.03	0.03	0.03	0.04	0.02
CV, %	2.0	2.0	3.1	2.9	2.9	4.4	2.1
	Glu	*His*	*Tyr*	*Orn*	*Lys*	*Arg*	*Trp*
$\bar{X}$	0.97	0.91	0.94	0.96	0.97	0.79	0.97
SD	0.02	0.05	0.03	0.03	0.03	0.02	0.02
CV, %	2.1	5.5	3.2	3.1	3.1	2.5	2.1

Serum recoveries

To determine recoveries from serum, where it could be expected that some absorption of amino acids by protein might occur, dialyzed serum with added amino acids was subjected to the complete procedure. Eight 0.1-ml aliquots of dialyzed serum were taken and the amino acids were added at the 1 mg/ml level. Allowance for residual amino acids was made by taking six aliquots of the serum through the procedure and computing peak area means, corresponding to residual amino acids, to provide a background correction for the recovery experiments. Results obtained from the recovery experiments were expressed as percentages of the corresponding areas of individual amino acids in runs made with amino acid solutions processed without serum. The results are given in Table IV. Recoveries are close to 100% with an excellent coefficient of variation.

TABLE IV

RECOVERIES OF AMINO ACIDS ADDED TO DIALYZED SERUM

100-μl aliquots of serum with amino acids were added at the 1-mg/dl level and subjected to the procedure. Peak areas of amino acids were compared with those of amino acids processed without serum. Values were corrected for residual amino acids in serum before additions. $N=8$.

	Amino acid						
	Val	*Ile*	*Ser*	*Thr*	*Asp*	*Met*	*Phe*
$\bar{X}$	0.96	0.99	0.97	0.97	0.99	0.93	0.96
SD	0.02	0.03	0.03	0.03	0.03	0.04	0.02
CV, %	2.1	3.0	3.1	3.1	3.0	4.3	2.1
	Glu	*His*	*Tyr*	*Orn*	*Lys*	*Arg*	*Trp*
$\bar{X}$	0.96	0.90	0.97	0.96	0.96	0.78	0.95
SD	0.03	0.05	0.04	0.03	0.05	0.02	0.02
CV, %	3.1	5.6	4.1	3.1	5.2	2.6	2.0

Physiological samples

A typical chromatogram obtained from a normal urine is illustrated in Fig. 4. The chromatogram shows the presence of most amino acids and has very little background interference indicating that the pretreatment procedure provides adequate separation of amino acids from this type of sample matrix.

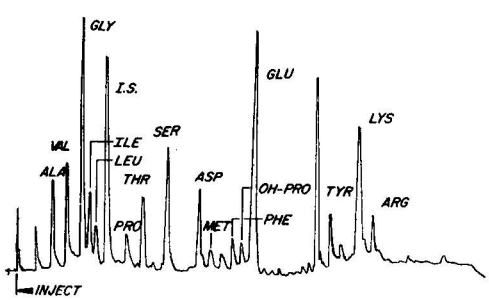

Fig. 4. Chromatogram of a normal adult urine sample subjected to the complete procedure.

The application of the procedure to a number of normal serum and urine samples was investigated. For 32 normal sera and 15 normal urines, a range of values for each amino acid was obtained. These values are given in Table V. Asparagine and glutamine are converted to their respective acids during the propylation step and these contribute to the aspartic and glutamic acid values given. The range of values corresponds reasonably well with results reported for the procedures of Stein and Moore[12] and Stein[18].

TABLE V

PHYSIOLOGICAL LEVELS OF URINARY AND SERUM AMINO ACIDS

Normal adult subjects were used for the complete procedure, $N=15$ for urines and $N=32$ for sera. Values for aspartic and glutamic acids include asparagine and glutamine.

Amino acid	*GC procedure*		*Moore and Stein procedure**		
	Urine (mg/24h) range	*Serum (mg/dl) range*	*Urine (mg/24h)*		*Serum (mg/dl) Av.*
			Range	*Av.*	
Alanine	14–65	2.9–4.3	20–70	46	3.41
Valine	2–8	2.1–4.1	–	< 10	2.88
Glycine	54–160	0.9–2.8	70–200	132	1.69
Isoleucine	12–35	0.3–1.7	10–30	18	1.54
Leucine	20–72	0.7–2.1	10–25	14	1.69
Proline	1–7	1.9–3.0	–	< 10	2.36
Threonine	18–55	1.0–1.7	15–50	28	1.39
Serine	30–70	0.7–1.9	25–75	43	1.12
Aspartic acid	14–52	0.2–1.5	–	< 10	0.03
Methionine	2–6	0.3–0.8	–	< 10	0.38
Cysteine and cystine	6–15	0.6–1.9	10–20	10	1.18
Phenylalanine	5–34	0.3–2.2	10–30	18	0.84
Hydroxyproline	2–12	1.0–0.8	–	–	–
Glutamic acid	20–80	4.8–12.9	–	< 10	0.70
Histidine	80–225	0.6–1.8	110–320	216	1.15
Tyrosine	10–42	0.6–1.5	15–50	35	1.03
Ornithine	1–4	0.1–1.2	–	< 10	0.72
Lysine	8–36	1.9–4.1	10–50	19	2.72
Arginine	1–6	0.9–2.7	–	< 10	1.51
Tryptophan	0–4	0.5–2.4	–	–	1.11

* Values reprinted from R. H. S. Thompson and I. D. P. Wootton (Editors), *Biochemical Disorders in Human Disease*, Academic Press, New York, 3rd ed., 1970.

Urine specimens from cystinuric subjects were obtained and analyzed. A chromatogram, including the relevant portion of the data processor report, of one of these is shown in Fig. 5. Compared with the normal, there is a dramatic increase in excretion of lysine, ornithine and arginine. It should be noted that automatic data reduction greatly simplifies screening procedures. The computer in this example has been instructed to suppress normal levels of amino acids from the report, printing out those that are abnormal. Cystine, analyzed here as cysteine, is increased above normal. The values are given in mg/24 h.

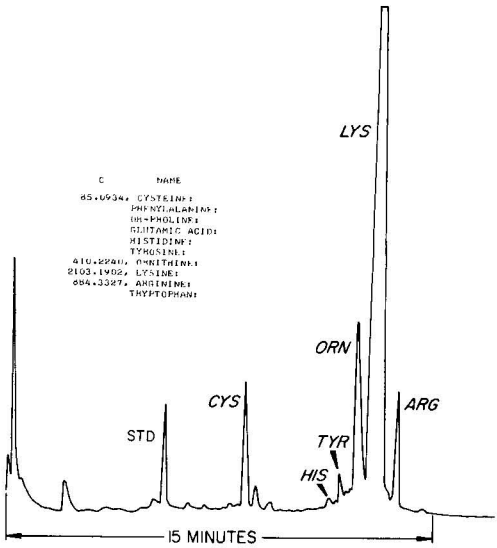

Fig. 5. Chromatogram of a cystinuric urine together with a portion of the computer report.

Several maple syrup urine samples were processed. A chromatogram obtained from a maple syrup urine is given in Fig. 6 compared with a normal urine processed identically. In the case of the maple syrup urine chromatogram, the attenuation of the GC instrument was increased to emphasize abnormal amino acid levels. In this case valine, isoleucine and leucine are clearly abnormal. The computer report for this analysis is given in Table VI compared with the report for a normal urine. The report for the abnormal urine gives only abnormalities, normal values being suppressed. In the report for the normal urine, the computer has been instructed to report all values.

Several PKU sera from both treated and untreated subjects were processed. Fig. 7 compares a chromatogram obtained from a PKU serum with that obtained from a normal serum. The level of abnormal phenylalanine was found to be 28.5

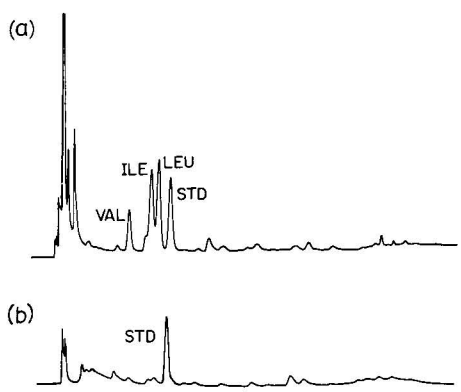

Fig. 6. Chromatogram of a maple syrup urine (a) compared with that of a normal urine (b).

TABLE VI

COMPUTER REPORTS FOR MAPLE SYRUP URINE AND NORMAL URINE SAMPLES

The normal report gives all amino acid levels. The MSU report suppresses all but abnormal values. 100 mg/dl internal standard was used for this analysis.

Run 7: Normal urine		*Run 5: MSU sample*	
C	*Name*	*C*	*Name*
40.4653,	Alanine:	.0000,	!
2.9314,	Valine:	.0000,	!
86.0321,	Glycine:	.0000,	!
16.0516,	Isoleucine:	.0000,	!
14.6568,	Leucine:	.0000,	!
100.0000,	Std:	.0000,	!
2.9931,	Proline:		Alanine:
29.9315,	Threonine:	121.3916,	Valine:
.0000,	!		Glycine:
48.0404,	Serine:	243.7520,	Isoleucine:
.0000,	!	146.9675,	Leucine:
.0000,	!	100.0000,	Std:
45.9353,	Aspartic:		Proline:
7.1788,	Methionine:		Threonine:
6.7231,	Cysteine:	.0000,	!
8.3375,	Phenylalanine:		Serine:
4.5112,	OH-Proline:		Aspartic:
34.3415,	Glutamic acid:		Methionine:
.0000,	!		Cysteine:
.0000,	!		Phenylalanine:
.0000,	!		OH-Proline:
.0000,	!		Glutamic acid:
91.0959,	Histidine:	.0000,	!
19.0444,	Tyrosine:	.0000,	!
8.1906,	Ornithine:	.0000,	!
12.2176,	Lysine:		Histidine:
5.4813,	Arginine:		Tyrosine:
	Tryptophan:		Ornithine:
			Lysine:
			Arginine:
			Tryptophan:

mg/dl. The procedure gives results that may be correlated closely with other phenylalanine procedures. It was possible to compare phenylalanine levels by the described procedures and a standard fluorimetric procedure[19] for nine identical sera. The results are compared in Table VII. The results are in excellent agreement, although in the case of the two lowest phenylalanine levels, the GC procedure gave somewhat higher values.

It is possible to use 10-μl samples of serum provided the sample is diluted to 100-μl volume with deionized water before adding the acetic acid solution. To maintain good recoveries at this level it is advisable to use micro-reaction vials such as the Reacti-vial (Pierce) in the 3-ml size for the chemical procedures and to reduce reagent volumes by half. A typical chromatogram obtained from a 10-μl PKU serum

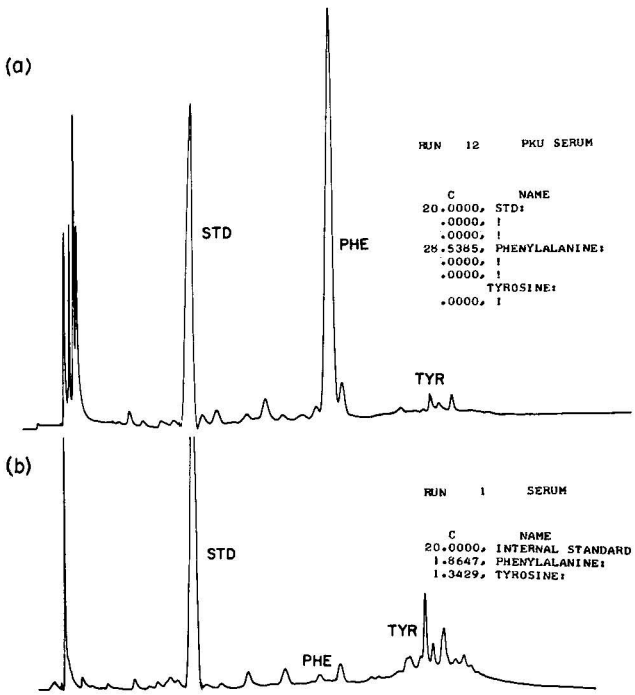

Fig. 7. Chromatogram obtained from a serum from a PKU subject (a) compared with a normal serum (b). Computer reports for each sample are included.

TABLE VII

COMPARISON OF FLUORIMETRIC AND GC METHODS

All results are the average of duplicate analyses.

Serum	*Phenylalanine (mg/dl)*	
	Fluorimetric	*GC*
1	39.5	38.7
2	25.0	25.4
3	23.0	22.8
4	17.5	16.6
5	22.5	22.7
6	13.9	13.6
7	2.0	2.4
8	2.0	2.5
9	27.6	27.2

is illustrated in Fig. 8. To maintain adequate sensitivity, the total derivatized sample was transferred to an MS-41 injection capsule, the solvent evaporated off and the GC analysis carried out. The level of phenylalanine reported was 38.7 mg/dl, a figure in close agreement with the value of 39.5 mg/dl found by the fluorimetric procedure (sample No. 1 in Table VII).

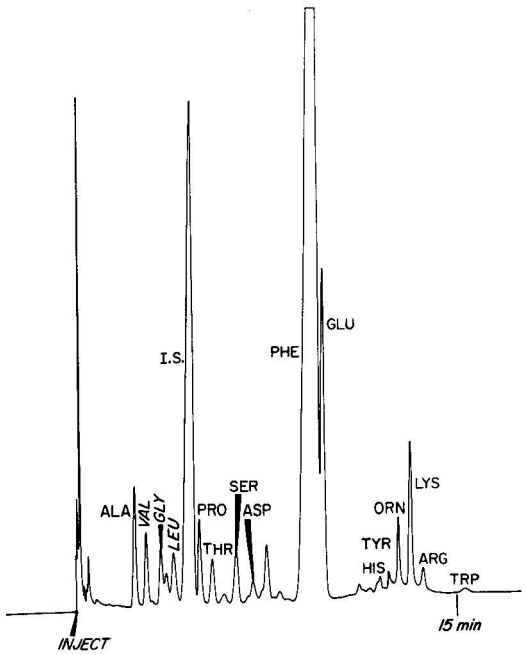

Fig. 8. Chromatogram obtained following processing of a 10-μl serum sample. The serum was obtained from a PKU subject.

Plant tissues. Several plant samples were processed in order to determine the suitability of the procedure for amino acid profiling of soluble amino acids in this type of sample. Throughout these studies, 70% ethanol extracts of the tissues were prepared following the procedure given above. A chromatogram is given in Fig. 9 from an analysis carried out on spinach leaves as representative of leaf tissue. Fig. 10 gives the chromatogram obtained from grape juice, Fig. 11 that from apple juice and Fig. 12 that from orange juice. The juices were processed without any prior extraction, the juices being centrifuged only to remove cellular debris before subjecting them to the resin column. Pigments present in the spinach and grape samples did not interfere with the analysis. Some pigment adsorbed on to the resin but did not alter its capacity to any significant degree.

Protein hydrolyzates. Protein aliquots of 1 to 10 mg were weighed into a reaction tube. 2 ml of 6 *N* HCl were added together with the 20 μg of norleucine internal standard. After flushing with nitrogen, the tube was capped and heated for 22 h at $110° \pm 1°$. After rotary evaporation to dryness, the hydrolyzates were derivatized and gas chromatographed. A chromatogram for ribonuclease hydrolyzate prepared as above is illustrated in Fig. 13.

Six individual aliquots of ribonuclease were hydrolyzed, simultaneously processed and analyzed for amino acid content. The chromatographic data for the six runs are summarized in Table VIII and indicate the reproducibility attainable for this type of sample. No correction has been made for low recoveries of some amino acids due to the hydrolysis conditions used, the data being presented as typical of reproducibility data for the procedures.

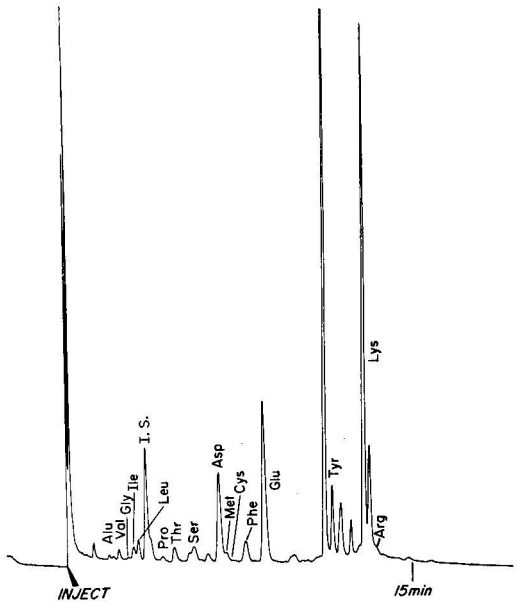

Fig. 9. Chromatogram of soluble amino acids obtained following processing of an ethanolic extract of spinach leaves. Extract equivalent to 100 mg leaf tissue was processed and 1/50 of the derivatized material was chromatographed.

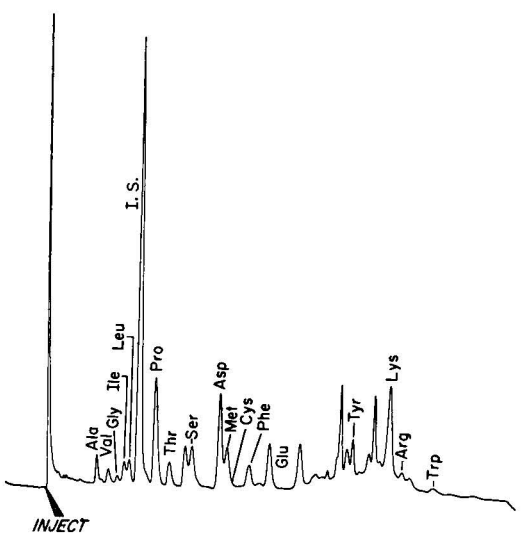

Fig. 10. Chromatogram obtained from grape juice. Injection aliquot equivalent to 20 μl of the original sample.

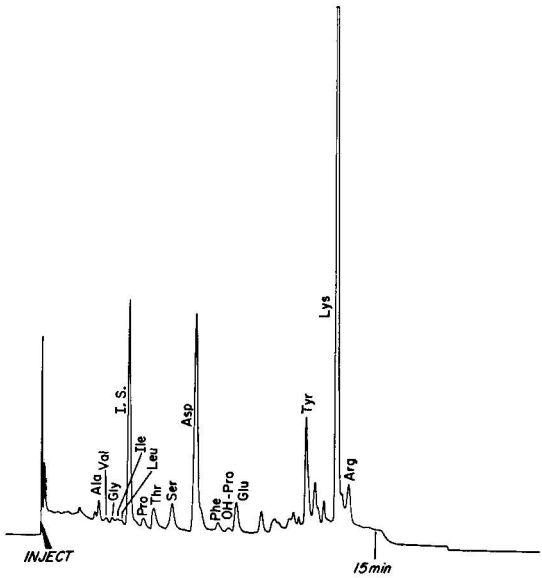

Fig. 11. Chromatogram of apple juice. Injection aliquot equivalent to 20 μl of the original sample of apple juice.

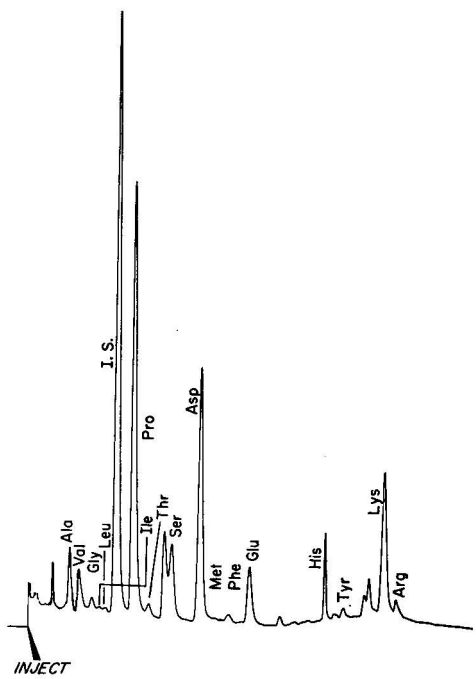

Fig. 12. Chromatogram of orange juice. Injection aliquot equivalent to 20 μl of the original juice.

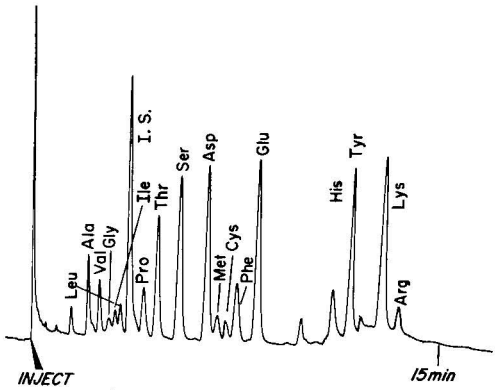

Fig. 13. Chromatogram of hydrolyzate of ribonuclease. 2 mg protein hydrolyzed, derivatized and taken up in 100 μl of ethyl acetate. 1 μl (equivalent to 20 μg protein) injected.

TABLE VIII

AMINO ACID ANALYSIS OF RIBONUCLEASE HYDROLYZATES

Reproducibility data are provided for different samples. The number of hydrolyzate samples was 6.

	$\bar{X}$	*SD*	*CV* (%)
Alanine	7.40	0.14	1.9
Valine	7.18	0.18	2.3
Glycine	1.71	0.04	2.3
Leucine	1.93	0.04	2.1
Isoleucine	1.76	0.04	2.3
Proline	3.50	0.07	2.0
Threonine	7.44	0.20	2.7
Serine	9.76	0.21	2.2
Aspartic acid	13.7	0.39	2.9
Methionine	3.53	0.09	2.6
Cysteine	4.97	0.19	3.8
Phenylalanine	3.19	0.09	2.8
Glutamic acid	12.37	0.29	2.3
Histidine	3.60	0.15	4.2
Tyrosine	6.75	0.14	2.1
Lysine	10.06	0.44	4.2
Arginine	4.16	0.14	3.4

Chromatograms for hydrolyzates of histone, ovalbumin and collagen are given in Figs. 14, 15 and 16, respectively. The amino acid content for these analyses is given in Table IX.

Stability of derivatized samples

The *n*-propyl, N-acetyl derivatives in solution are very stable under normal laboratory conditions. Stored at 4° in capped reaction tubes, the derivatized samples are stable for several weeks. Stability studies were undertaken by taking 0.1-ml

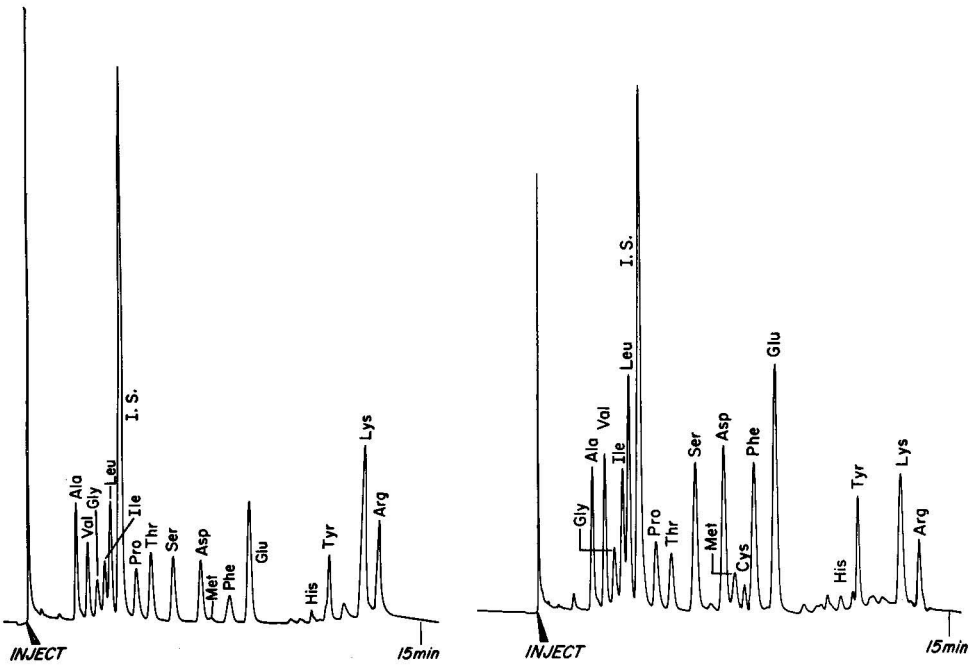

Fig. 14. Chromatogram of histone hydrolyzate. Injection aliquot equivalent to 20 μg protein.

Fig. 15. Chromatogram of ovalbumin hydrolyzate. Injection aliquot equal to 20 μg protein.

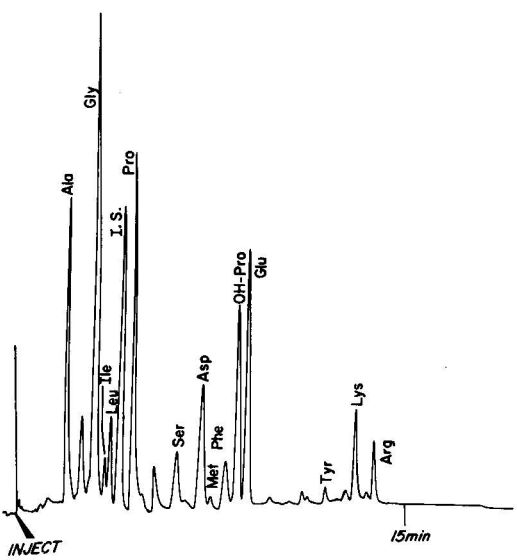

Fig. 16. Chromatogram of 20 μg derivatized collagen hydrolyzate. The hydroxyproline derivative resolves adequately from the phenylalanine and glutamic acid peaks.

TABLE IX

DATA OBTAINED FROM THE ANALYSES OF PROTEIN HYDROLYZATES

No correction has been made for losses due to the hydrolysis conditions used. The results are the average of duplicate analyses.

Amino acid	*g amino acid/100 g protein*		
	Collagen	*Histone*	*Ovalbumin*
Alanine	9.3	8.6	2.2
Valine	2.1	7.4	3.2
Glycine	25.6	5.6	1.2
Isoleucine	1.0	3.4	3.3
Leucine	2.8	6.5	0.4
Proline	11.9	4.2	1.5
Threonine	1.8	6.0	1.2
Serine	3.8	4.3	3.1
Aspartic acid	5.4	4.0	4.0
Methionine	0.6	0.3	2.2
Phenylalanine	1.9	3.0	3.4
Hydroxyproline	13.0	0	0
Glutamic acid	11.5	10.3	7.4
Histidine	0.2	3.2	18.9
Tyrosine	0.4	1.7	15.3
Lysine	3.4	15.2	24.2
Arginine	7.9	9.1	22.1
Cysteine	0	0	0.7

aliquots of dialyzed serum. Levels of phenylalanine equal to 5, 10, 20 and 50 mg/dl were added to duplicate samples and the serum samples carried through the complete procedure. The samples were gas chromatographed immediately after processing. The samples were resealed and stored at 4° for 14 days, then gas chromatographed a second time. Results on duplicates for each time of analysis were averaged. The levels that were found are given in Table X. The results are in excellent agreement.

The results obtained for a serum analysis immediately after processing were compared with an analysis carried out on the same derivatized sample but after sample storage for 4 weeks and 8 weeks at 4°. This comparison is given in Table XI

TABLE X

STABILITY OF DERIVATIZED SERUM SAMPLES CONTAINING ADDED PHENYLALANINE

Samples were analyzed immediately after processing and after 14 days' storage at 4°.

	Added phenylalanine level (mg/dl)			
	5	*10*	*20*	*50*
Analyzed immediately after processing	4.94	10.02	19.86	48.95
Analyzed after 14 days' storage	4.87	9.97	19.94	48.20

TABLE XI

STABILITY OF DERIVATIZED SERUM SAMPLES

The values in mg/dl were obtained by chromatographing immediately on processing and after 4 and 8 weeks' storage at 4°

Amino acid	*Storage time (weeks)*		
	0	*4*	*8*
Alanine	3.7	3.5	3.6
Glycine	1.1	1.2	1.2
Leucine	1.8	1.7	1.8
Proline	2.2	2.4	2.2
Threonine	1.3	1.2	1.2
Cysteine	0.9	1.0	1.0
Phenylalanine	0.8	0.8	0.8
Hydroxyproline	0.4	0.4	0.5
Histidine	1.2	0.9	0.8
Lysine	2.3	2.4	2.4
Arginine	1.9	1.8	1.8
Tryptophan	1.8	1.9	1.8

for several of the amino acids. The histidine level is considerably lower for the stored sample. The chromatograms showed an extra peak between ornithine and lysine. By adding small amounts of water to derivatized histidine, it was subsequently found that *n*-propyl diacetyl histidine is partially deacylated to the monoacetyl ester in the presence of moisture. The monoacetyl ester chromatographs between the ornithine and lysine derivatives. The deacylation is largely eliminated by use of anhydrous ethyl acetate in taking up the final residue. Also the histidine derivative is most stable in solution, the dry residue following derivatization apparently being more sensitive to traces of moisture. Immediate solubilization of the residue in anhydrous ethyl acetate is therefore essential if stability of the histidine derivative is a prime consideration.

DISCUSSION AND CONCLUSION

The described procedures offer a convenient addition to other methods for routine amino acid analysis. Without imposing too great a demand on operator skill or demanding sophisticated equipment, the procedures are capable of producing excellent reproducible results on a wide range of samples. The derivatives have the advantage of being highly stable under average laboratory conditions. Overall analysis time for a single sample using the complete procedure is about 1 h. Considerable savings in time may be made by processing several samples simultaneously. GC does not, of course, provide positive identification for any particular peak. It does, however, in combination with a suitable sample pretreatment procedure, provide for considerable reliability in identification by using retention time characteristics. The pretreatment step using cation-exchange resin with a high cross-linkage is effective in removing anions, large molecules and non-ionic material such as sugars, lipids, polypeptides and protein from the sample. However, it would

be expected that amines and some small peptides would be present in the final material. It is clear from the results that these, for the samples investigated, do not pose a serious problem. It is to be anticipated that some peptide material would derivatize and have retention characteristics similar to those of some of the common amino acids and could therefore contribute to the level reported for an amino acid. In the case of sera and urine analyses, it would be expected that the wide difference between normal and abnormal values would lead to no confusion of interpretation. Indeed, this has been the experience of this worker.

A possible source of interference resides in the less common amino acids. To determine the retention characteristics of several of these, samples of the pure amino acids were processed and chromatographed. The relative retention time of each compared with neighboring amino acids is given in Table XII. Several of these are of interest as they do occur and may be identified in biological samples. Asparagine and glutamine will, when these procedures are used, be analyzed as aspartic and glutamic acids. Citrulline is not analyzed by these procedures. The resin pretreatment step loses taurine and cysteic acid in the effluent.

TABLE XII

RRT CHARACTERISTICS OF SOME COMPOUNDS THAT MAY BE PROCESSED WITH THE COMMON AMINO ACIDS

The RRT is measured relative to norleucine = 1.000.

Compound	*RRT*	
α-Aminoisobutyric acid	0.524	
	0.547	Alanine
β-Amino-*n*-butyric acid	0.827	
	0.837	Isoleucine
β-Aminoisobutyric acid	0.887	
γ-Amino-*n*-butyric acid	1.494	
	1.510	Serine
Asparagine	1.823	Aspartic acid
ε-Amino-*n*-caproic acid	2.106	
	2.154	Phenylalanine
Glutamine	2.290	Glutamic acid
Aminoadipic acid	2.383	
α-Aminopimelic acid	2.773	
	3.034	Histidine

The sensitivity of flame ionization detectors used in GC analysis provides the capability of quantitating levels of a pure compound down to 10^{-12} mole. In practice, the sensitivity attainable is dependent on factors including column performance, background of the sample, and the molar response of the particular amino acid to be quantitated. The results obtained for the sera samples using 100-μl sample size and taking 10-μl aliquots for the GC analyses indicate that, for those amino acids with excellent detector response such as hydroxyproline, it is readily possible to quantitate the equivalent of 50 ng of free amino acid.

ACKNOWLEDGEMENTS

My thanks are due to Ms. Phyllis Taylor (State Department of Health, Hartford, Conn.), Dr. John Meola (Albany Medical Center, Albany, N.Y.), and Graff Associates (Santa Clara, Calif.) for generous provision of abnormal clinical samples. John Meola carried out the fluorimetric procedures for many of the serum phenylalanine levels used in this report. Mr. Gary Schmidt provided able technical assistance. I thank W. Slavin for constructive advice with this manuscript.

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Journal of Chromatography, 95 (1974) 213–221

CHROM. 7508

SEPARATION OF PHENOLIC O-GLUCURONIDES AND PHENOLIC SULPHATE ESTERS BY MULTIPLE LIQUID–LIQUID PARTITION

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(Received February 26th, 1974)

SUMMARY

A liquid–liquid partition method is described for the separation of phenolic O-glucuronides from the corresponding phenolic sulphate esters and of the different compounds within the two classes of conjugates. The method, which involves a countercurrent technique with continuous flow of the solvents, is suitable for the isolation of these metabolic conjugates from biological fluids (bile and urine).

INTRODUCTION

One of the steps involved in the metabolic transformation of many foreign compounds and endogenous substances in mammals is their conjugation with glucuronic acid (UDPGA) and/or sulphuric acid (PAPS). Interest in these conjugates because of their metabolic and physiological significance, together with the difficulties encountered in their chemical synthesis, has stimulated attempts to set up preparative methods suitable for their isolation from biological fluids[1–5]. For this purpose, we have studied the application of the liquid–liquid partition (countercurrent distribution) to the separation of phenolic O-glucuronides and phenolic sulphate esters.

The advantages of this method compared with other available methods are its selectivity, the chemical "inertia" and its effectiveness for preparative purposes.

EXPERIMENTAL

Materials

6-Bromo-2-naphthyl-D-glucuronide (acid form) was obtained from Sigma (St. Louis, Mo., U.S.A.), and *p*-nitrophenyl-D-glucuronide (acid form), *p*-nitrophenyl sulphate (potassium salt) and 6-bromo-2-naphthyl sulphate (sodium salt) were purchased from Serva (Heidelberg, G.F.R.).

The solvents used were analytical-grade reagents and all were distilled before use. In particular, *n*-butanol was pre-treated with neutral alumina so as to eliminate UV-absorbing impurities.

Apparatus

Instead of the conventional Craig machine, the countercurrent apparatus developed by Hietala[6] was used. The apparatus including the glassware was manufac-

tured by Karpinnen Oy (Helsinki, Finland) and the mechanical parts by the Laboratory of Technology, Lepetit (Milan, Italy). The apparatus is a continuous flow countercurrent system with one stationary and one moving phase (upper or lower). The phase ratio $\alpha = v_m/v_s$ (v_m and v_s are the volumes of the moving and stationary phases, respectively) is chosen before each fractionation and can be decreased to 0.2–0.3, depending on the solvent system employed.

The apparatus consists of 150 glass units (unit volume=13.5 ml) connected in series of ten with PTFE tubing. Shaking is accomplished by the rotation of the distribution train axle by $\pm 45°$. A DLC peristaltic pump for organic solvents (E.A. Hughes, Epsom, Great Britain) and an LKB (Stockholm, Sweden) Model 7000 Ultrorac fraction collector are used as ancillary equipment.

Selection of the operating conditions

Because the separation is at a maximum in the Martin–Synge distribution[7] when $v_m/v_s=0$, the lowest phase ratio ought to be selected in order to achieve a high resolution. In our partition studies, the phase ratios were chosen between 0.42 and 0.28. A shaking frequency of 30 cycles per minute, an amplitude of $\pm 45°$ and a flow-rate of 1–2 ml/min were used in these experiments.

Fractionation

At the beginning of a fractionation, the conjugate mixture was dissolved in the stationary phase and loaded into the first tubes (0, 1, 2 and 3) of the apparatus, keeping the phase ratio the same as that to be used in the distribution train. Two different methods were used for developing and analysing the concentration profiles: in the first, the absorbance of the upper or lower phase in the distribution units was measured, while in the second, the effluent collected from the apparatus was analysed spectrophotometrically.

The calculations of the theoretical distribution curves and of the partition coefficients were made as reported by Ellfolk and Hynninen[8], according to the theory of Martin and Synge[7].

A Uvichem Model H-1620 spectrophotometer was used to measure single absorbances. Before and after each fractionation experiment, the identities and the purities of the separated compounds were controlled by measuring their UV spectra with a Beckman Model DB-GT instrument and their IR spectra with a Perkin-Elmer 157 instrument, and evaluating their thin-layer chromatographic R_F values on Merck silica gel F_{254}.

Choice of the solvent systems

Phenolic O-glucuronides and phenolic sulphate esters are generally polar compounds, particularly the former because of the high hydrophilicity of the glucuronic acid molecule; moreover, the phenolic O-glucuronides are weak acids (pK_a of glucuronic acid=5.8), while the corresponding sulphates are strongly acidic. As a result of these properties, the salts of phenolic O-glucuronides and phenolic sulphate esters show, at neutral pH, low partition coefficients between organic and aqueous phases even with good solvents such as *n*-butanol and isoamyl alcohol. A better partition of the phenolic O-glucuronides is possible under moderately acidic conditions (pH 5.0–4.0), whereas lower pH values (3.0–2.0) are required in order to

extract the phenolic sulphates from the aqueous phase. As both phenolic O-glucuronides and phenolic sulphate esters show a higher partition coefficient as free acids, the separation of these conjugates is more easily performed after further transformation from the salt from into the acidic form. In the case of the sulphate esters, the salt–acid conversion is easily accomplished using a strongly cationic exchanger such as Amberlite IR-120 (H^+). For the glucuronides, which are often labile, a rapid acid extraction is preferable.

These considerations, and the physico-chemical properties of the phenolic O-glucuronides and phenolic sulphate esters in the single solvent systems adopted (solubility, reactivity, surface phenomena, etc.), were taken into account when choosing partition systems suitable for their fractionation.

In addition to the required partition properties, the solvent systems selected satisfy other requirements: they are intentionally neutral and virtually inert so as to avoid any possible degradation of the compounds to be fractionated, have a high dissolving capacity as the semi-preparative purposes require, permit UV analysis at wavelengths above 260 nm and, finally, they can be easily modified as necessary for a particular problem.

RESULTS

Taking into account the special difficulties that arise when working with biological material, and in order to evaluate all possible cases, the following types of separations were studied.

(1) Separation of phenolic O-glucuronides (salts) from the corresponding phenolic sulphate esters (salts).

(2) Separation of phenolic O-glucuronides (acids) from the corresponding phenolic sulphate esters (acids).

(3) Fractionation of a mixture of phenolic sulphate esters (salts).

(4) Fractionation of a mixture of phenolic sulphate esters (acids).

(5) Fractionation of a mixture of O-glucuronides (acids).

As reference compounds, the phenolic O-glucuronides and the phenolic sulphates of *p*-nitrophenol and 6-bromo-2-naphthol were used.

Separation of phenolic O-glucuronides (salts) from the corresponding phenolic sulphate esters (salts)

Phenolic O-glucuronides and phenolic sulphate esters are excreted in the urine as salts. Therefore, it is often useful to separate the two classes of conjugates already in this form. For this separation, of the solvent systems studied, one of the most satisfactory was the following:

Sodium phosphate buffer, 0.01 *M*, pH 6.8	4.0 (v/v)	System I
n-Butanol	1.0 (v/v)	
n-Propanol	1.0 (v/v)	
Ethyl acetate	3.0 (v/v)	

The use of a buffered system is necessary in order to avoid dissociation of the phenolic O-glucuronide salts, while *n*-propanol improves the separation between the two phases.

The result of the fractionation, utilising a 70-tube distribution train, is shown in Fig. 1. It can be seen that phenolic sulphate esters are easily eluted from the apparatus with experimental partition coefficients $K_1=4.25$ ($V_m=0.38$ l) and $K_2=1.16$ ($V_m=0.83$ l), V_m= elution volume of the moving phase; on the other hand the corresponding phenolic O-glucuronides show partition coefficients less than 1 ($K_3=0.527$ and $K_4=0.081$), so that their elution from the apparatus will require calculated volumes of moving phase of 1.569 and 8.995 l, respectively.

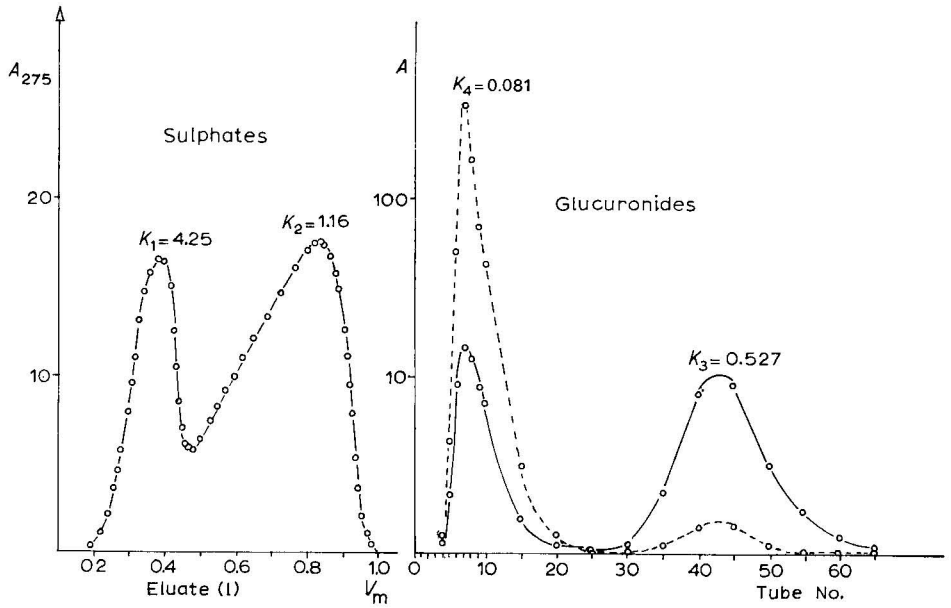


Fig. 1. Separation of a mixture of phenolic O-glucuronides and phenolic sulphate esters in the salt form using solvent system I. K_1=6-bromo-2-naphthyl sulphate; K_2=*p*-nitrophenyl sulphate; K_3=6-bromo-2-naphthyl-β-D-glucuronide; K_4=*p*-nitrophenyl-β-D-glucuronide. A 25-mg amount of each compound was dissolved in 20 ml of the stationary phase and the mixture was loaded into the first four tubes of the distribution apparatus. The number of tubes used in the process was 70. $v_m=3.2$ ml; $v_s=10.3$ ml; $\alpha=v_m/v_s=0.31$; $V_m=1$ l; flow-rate=1 ml/min. Experimental curves obtained by measuring the absorbance, *A*, of the moving phase in the eluate at 275 nm, and of the stationary phase in the distribution train at 275 (———) and 300 (– – – –) nm.

The partition profile of the phenolic sulphate esters deviates considerably from the theoretical profile. This can probably be attributed to the surfactant properties of these compounds, which result in emulsions that affect the phase ratio of the solvent system.

Separation of phenolic O-glucuronides (acids) from the corresponding phenolic sulphate esters (acids)

The phenolic sulphate esters in their acid form can be easily separated from the corresponding phenolic O-glucuronides by using the following partition system:

Water	2.5 (v/v)	System II
Formamide	2.5 (v/v)	
Diisopropyl ether	3.7 (v/v)	
Benzene	1.0 (v/v)	

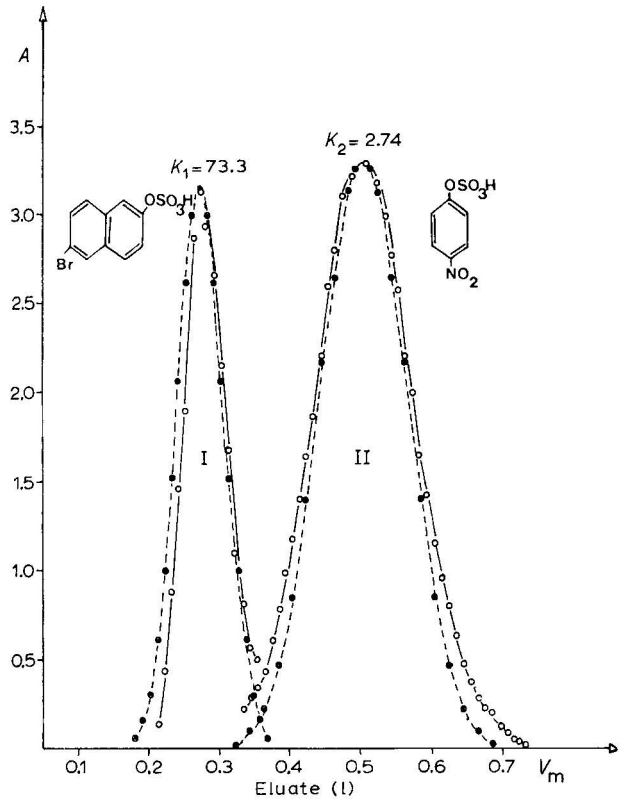


Fig. 2. Separation of phenolic sulphate esters from the corresponding phenolic O-glucuronides in the acid form using solvent system II. K_1 = 6-bromo-2-naphthyl sulphate; K_2 = *p*-nitrophenyl sulphate. A 25-mg amount of each compound was dissolved in 10 ml of the stationary phase and the mixture was loaded into the first four tubes of the distribution apparatus. $v_m = 4.0$ ml; $v_s = 9.5$ ml; $\alpha = v_m/v_s = 0.42$; $V_m = 0.75$ l. The number of tubes used in the process was 50. ○, Experimental curve obtained by measuring the absorbance, *A*, of the moving phase at 288 nm (I) and 308 nm (II); ●, theoretical curve.

In this way, the phenolic sulphate esters are rapidly eluted (Fig. 2), while the phenolic O-glucuronides remain in the first tubes of the apparatus. In this case, the deviation from theory of the distribution curve is negligible because in their acid form the phenolic sulphate esters lose their surfactant properties.

Fractionation of a mixture of phenolic sulphate esters (salts)

The surfactant properties of the phenolic sulphate salts complicate their fractionation by liquid–liquid partition. As an example, the separation of the 6-bromo-2-naphthyl sulphate from the *p*-nitrophenyl sulphate is shown in Fig. 3; the solvent system used was as follows:

Sodium phosphate buffer, 0.01*M*, pH 7.0	4.0 (v/v)	System III
n-Butanol	1.0 (v/v)	
Ethyl acetate	3.0 (v/v)	

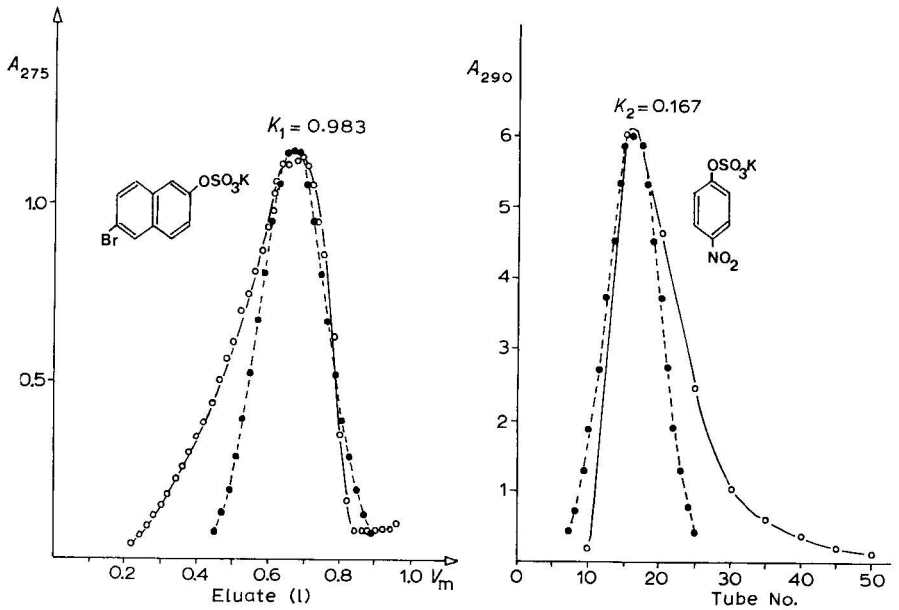


Fig. 3. Separation of phenolic sulphate esters salt mixture using solvent system III. K_1 = 6-bromo-2-naphthyl sulphate; K_2 = *p*-nitrophenyl sulphate. A 25-mg amount of each compound was dissolved in 10 ml of the lower phase and the mixture was loaded into the first two tubes of the distribution apparatus. The number of tubes used in the process was 70. $v_m = 3.2$ ml; $v_s = 10.3$ ml; $\alpha = v_m/v_s = 0.36$; $V_m = 1$ l; flow-rate = 1.5 ml/min. ○, Experimental curve obtained by measuring the absorbance, A, of the moving phase at 275 nm and of the stationary phase at 290 nm; ●, theoretical curve.

The partition coefficients of the two sulphates are different ($K_1 = 0.983$; $K_2 = 0.167$), resulting in a good resolution, and consequently the fractionation can even be accelerated by adding, for example, small amounts of *n*-propanol.

The expected deviation of the experimental curve results in large tails in front of the peaks, indicating the appearance of the lower phase emulsified with the upper phase.

Fractionation of a mixture of phenolic sulphate esters (acids)

In their acid form, the phenolic sulphate esters are easily extracted from the aqueous phase, and a moving phase with low polarity can be used for the partition. For example, solvent system IV gave a good fractionation of the sulphates studied, in agreement with the theoretical result (Fig. 4).

Water	3.0 (v/v)	System IV
Formamide	2.0 (v/v)	
Diisopropyl ether	3.0 (v/v)	
Benzene	1.4 (v/v)	

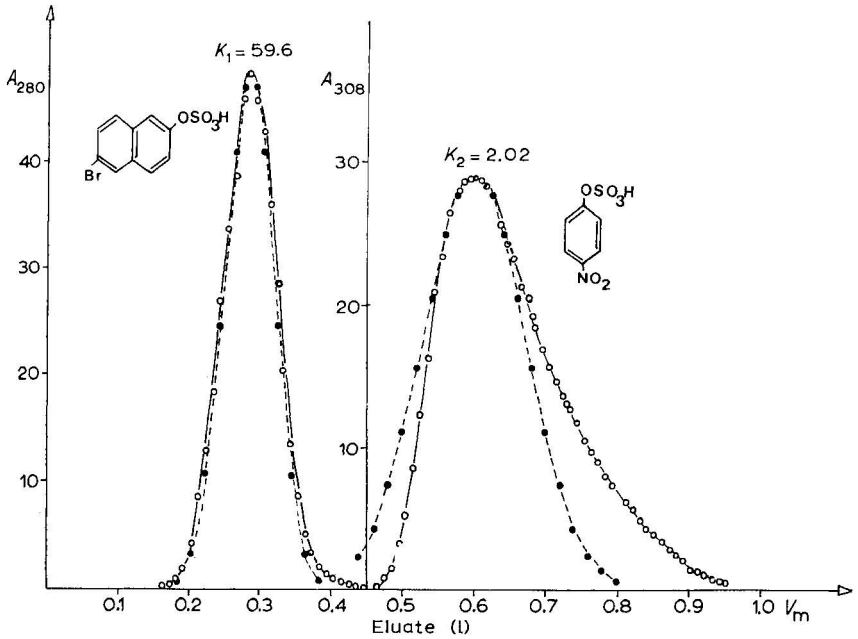


Fig. 4. Separation of phenolic sulphate ester mixture (acid form) using solvent system IV K_1=6-bromo-2-naphthyl sulphate; K_2=*p*-nitrophenyl sulphate. A 25-mg amount of each com pound was dissolved in 10 ml of the stationary phase and the mixture was loaded into the firs two tubes of the distribution apparatus. The number of tubes used in the process was 70. v_m=4.0 ml; v_s=9.5 ml; $\alpha = v_m/v_s = 0.42$; V_m=1 l; flow-rate=1.5 ml/min. ○, Experimental curve obtained by measuring the absorbance, *A*, of the moving phase at 280 and 308 nm; ●, theoretical curve.

This solvent system can be further improved by lowering the partition coefficients of the components being fractionated by decreasing the water:formamide ratio or the diisopropyl ether:benzene ratio.

Fractionation of a mixture of phenolic O-glucuronides (acids)

Of the solvent systems considered, the following was the most satisfactory:

Water	3.7 (v/v)	System V
N,N-dimethylformamide	1.2 (v/v)	
n-Butanol	0.7 (v/v)	
Diisopropyl ether	1.0 (v/v)	
Ethyl acetate	2.3 (v/v)	

This system shows a high resolution capacity, which can be increased by decreasing the water:N,N-dimethylformamide ratio or the ethyl acetate:diisopropyl ether ratio. The deviation of the experimental curve from the theoretical curve (Fig. 5) is attributed only to the low value of the phase ratio, which changes little during the partition.

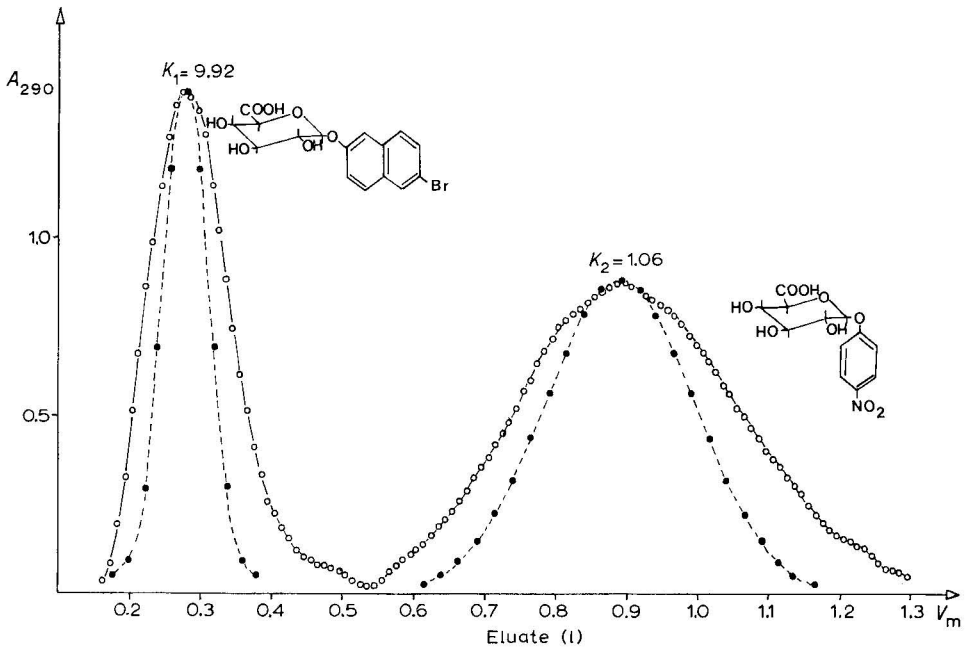


Fig. 5. Separation of a phenolic O-glucuronide mixture (acid forms) using solvent system V. K_1 = 6-bromo-2-naphthyl-β-D-glucuronide; K_2 = *p*-nitrophenyl-β-D-glucuronide. A 25-mg amount of each compound was dissolved in 10 ml of the stationary phase and the mixture was loaded into the first two tubes of the apparatus. The number of tubes used in the process was 70. v_m = 3.0 ml; v_s = 10.5 ml; $\alpha = v_m/v_s = 0.286$; V_m = 1.3 l; flow-rate = 1 ml/min. ○, Experimental curve obtained by measuring the absorbance, *A*, of the moving phase at 290 nm; ●, theoretical curve.

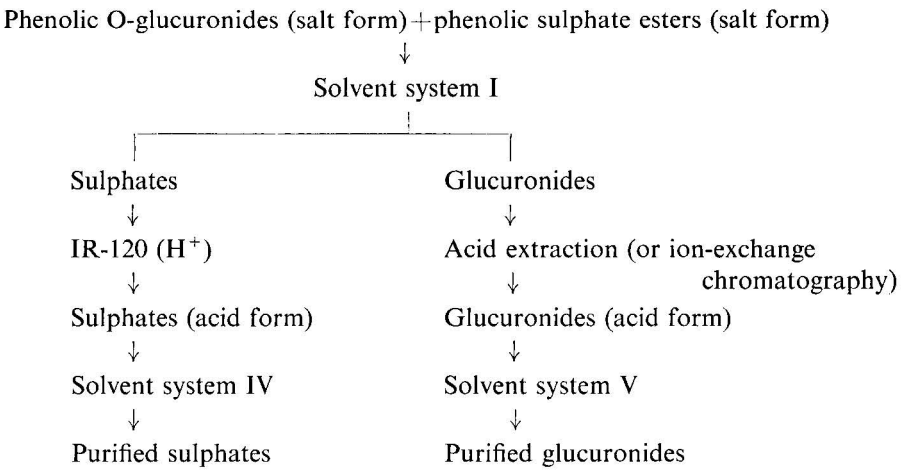
DISCUSSION

The results obtained demonstrated the possibility of separating by multiple liquid–liquid partition phenolic O-glucuronides from the corresponding phenolic sulphate esters and the different compounds within the two classes of conjugates.

Obviously, the isolation of these compounds from biological fluids (urine and bile) requires pre-purification of the crude material[3] before the countercurrent fractionation; in fact, of the various impurities present in urine or bile, salts interfere with the partition systems, affecting the ratio between the two phases, and the other contaminants may affect the analytical measurements. The choice of the analytical method to be used to follow the partition profiles is particularly difficult when the compounds to be isolated have low molar extinction coefficients at wavelengths above 280 nm, because in the far UV region interferences by impurities and by the solvent systems used are very high.

These difficulties can be prevented by the use of labelled compounds. Both from our earlier experience and the results of the present work, we suggest the following scheme for isolating phenolic O-glucuronides and phenolic sulphate esters from biological fluids.

Pre-purified material containing:



ACKNOWLEDGEMENTS

The authors are indebted to Prof. G. Semenza and Prof. T. Tenconi for helpful suggestions and discussions concerning the work presented in this manuscript.

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Journal of Chromatography, 95 (1974) 223–228

CHROM. 7481

THIN-LAYER CHROMATOGRAPHIC SEPARATION OF THE Z AND E ROTATIONAL ISOMERS OF α-N-NITROSO-N-ALKYLAMINO ACIDS

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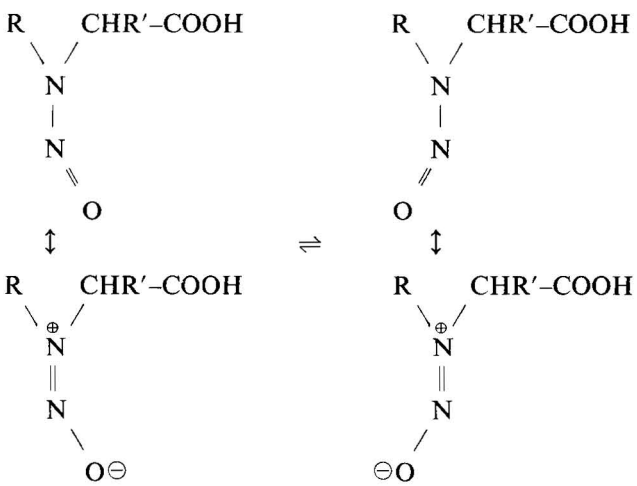
SUMMARY

Thin-layer chromatography at 0–2 °C affords a simple and rapid method for the detection and separation of the Z and E isomers of N-nitroso-N-alkylamino acids. The procedure enables conformational purity of the crystals to be determined and conformational Z⇌E interconversions to be studied.

INTRODUCTION

The partial double bond character of the N–N linkage in N-nitroso-N-alkylamino acids leads to the formation of the Z and E isomers*, which are in dynamic equilibrium[1–4]:



Interconverting rotational isomers can be separated if their mean lifetime is of the order of at least several hours. This means that the free enthalpy of activation ΔG^*, which must be supplied in order to convert the Z isomer into the E isomer or *vice-versa*, is greater than 20–23 kcal/mole (refs. 5 and 6). Several instances of success-

* The Z and E system for specifying double bond isomers unambiguously is employed[6].

ful separations of the Z and E isomers of amides, thioamides and N-nitrosoamines have appeared in the literature[2,4,7–16].

Chromatographic separations of the Z and E isomers of N-nitroso-N-alkylamino acids have been studied on thin layers[4]. It was found that thin-layer chromatography (TLC) offers not only a rapid and simple method for the detection and separation of the isomers, but also a method for the determination of their conformational purity and for the study of conformational interconversions.

EXPERIMENTAL

Thin-layer chromatographic plates

The TLC plates consisted of a 0.25-mm layer of silica gel G (Merck, Darmstadt, G.F.R.) coated on glass supports (20 × 10 cm).

Developing solvents

The following solvent systems were used: S1, *n*-butanol–25% ammonia–water (75:6:75, upper layer); S2, *n*-butanol–25% ammonia (6:1); S3, light petroleum (b.p. 45–58°)–ethyl acetate (5:2); S4, light petroleum (b.p. 45–58°)–ethyl acetate (3:2); S5, light petroleum (b.p. 45–58°)–ethyl acetate (1:1). Solvent systems S3–S5 were used for the separation of isomers of esters of N-nitroso-N-alkylamino acids.

Detection reagents

A 0.1% solution of bromocresol green in methanol was used for spraying chromatograms of N-nitroso-N-alkylamino acids. An iodine tank was used for N-nitrosoamino acids and their esters.

Temperature

In order to obtain compact and well defined spots, it is advisable to run chromatograms at 0–2 °C by placing the tank in a refrigerator. Variations in temperature during the run cause deterioration of the chromatogram. The higher the development, the more diffuse are the spots and the more tailing occurs.

Procedures

Solid recrystallized N-nitroso-N-alkylamino acids were dissolved in cold (0 °C) water, chloroform or acetone and applied with a micropipette 3 cm from the lower edge of the TLC plate. For checking the conformational purity of the crystals, the materials were applied to TLC plates immediately after dissolution. Each solution of a sample was then stored at room temperature for equilibration of the isomers and aliquots, withdrawn at appropriate intervals, and applied to the TLC plate for further development.

Solid recrystallized *p*-nitrobenzyl esters of N-nitrosoamino acids were dissolved in cold (0 °C) ethyl acetate, and immediately after dissolution were applied to the TLC plate. Each solution was then stored at room temperature for equilibration of the isomers and aliquots were withdrawn at appropriate intervals for further development.

RESULTS AND DISCUSSION

The results of the chromatographic separation of Z and E isomers are presented in Tables I and II.

R_F values for the Z and E isomers of eleven N-nitroso-N-alkylamino acids are shown in Table I and for four *p*-nitrobenzyl esters in Table II. All separation experiments were performed with solid starting materials, which were recrystallized before development and which had previously been studied by us at least by nuclear magnetic resonance (NMR) spectroscopy[2–4]. Hence the results presented for the separation of Z and E isomers by TLC can be discussed jointly with the NMR spectroscopic evidence.

Based on the NMR spectroscopic evidence, the TLC results for the separation of the isomers are grouped into five categories (A, B, C, D and F) for discussion.

TABLE I

R_F VALUES OF Z AND E ISOMERS OF N-NITROSO-N-ALKYLAMINO ACIDS ON SILICA GEL G PLATES

Solvent systems: S1 = *n*-butanol–25% ammonia–water (75:6:75); S2 = *n*-butanol–25% ammonia (6:1). Temperature, 0–2 °C.

Compound	*NMR category*	*S1*		*S2*	
		Z	*E*	*Z*	*E*
N-nitrososarcosine	A	–	–	0.19	0.24
N-nitroso-N-ethylglycine	A	–	–	0.19	0.24
N-nitroso-N-propylglycine	A	0.21	0.31	–	–
N-nitroso-N-isopropylglycine	C	0.20	–	–	–
N-nitroso-N-methyl-DL-alanine	B	0.20	0.25	–	–
N-nitroso-N-ethyl-DL-alanine	A	0.22	0.32	–	–
N-nitroso-N-propyl-DL-alanine	A	0.24	0.32	–	–
N-nitroso-N-isopropyl-DL-alanine	C	0.21	–	–	–
N-nitroso-N-methyl-DL-phenylalanine	D	–	–	0.29	0.35
N-nitroso-N-methyl-L-valine	D	–	–	0.25	0.31
N-nitroso-L-proline	F	–	–	0.16	0.20

TABLE II

R_F VALUES OF Z AND E ISOMERS OF *p*-NITROBENZYL ESTERS OF N-NITROSO-N-ALKYLAMINO ACIDS ON SILICA GEL G PLATES

Solvent systems: S3 = light petroleum–ethyl acetate (5:2); S4 = light petroleum–ethyl acetate (3:2); S5 = light petroleum–ethyl acetate (1:1). Temperature, 0–2 °C.

p-Nitrobenzyl ester	*NMR category*	*S3*		*S4*		*S5*	
		Z	*E*	*Z*	*E*	*Z*	*E*
N-nitrososarcosine	B	0.11	0.16	0.24	0.35	–	–
N-nitroso-N-ethylglycine	F	0.21	0.29	–	–	–	–
N-nitroso-N-methyl-DL-alanine	F	0.18	0.25	–	–	–	–
N-nitroso-L-proline	F	–	–	–	–	0.36	0.42

Some of the N-nitroso-N-alkylamino acids studied show a tendency to assume the Z or E conformation in the crystalline form. On dissolution of the original isomer present in the crystal, partial conversion into the other isomer gradually takes place until equilibrium is established[1–4].

According to the NMR spectroscopic evidence, compounds in category A in Tables I and II crystallized in the Z conformation. Chromatograms run on a freshly prepared solution of a sample revealed only a single spot with a lower R_F value. On standing at room temperature, all of these solutions showed the gradual appearance of an upper spot of the E isomer produced during the approach to equilibrium. The ratio of the areas and of the intensities of the colours of the upper and lower spots gradually increased until equilibrium was established.

Compounds in category B crystallized in the E conformation. On dissolution, the Z isomer was gradually produced from the original E isomer until equilibrium was established. Chromatograms run on freshly prepared solutions of these B compounds revealed the presence of only the upper spot. On standing at room temperature, these solutions showed the gradual appearance of a second spot with a lower R_F value. The ratio of the areas and of the intensities of the colours of the lower and upper spots gradually increased until equilibrium was established.

Comparative studies revealed that TLC at 0–2 °C offers a more sensitive method of checking the conformational purity of isomers than does NMR spectroscopy; both for compounds that crystallize in the Z conformation (category A) and those that crystallize in the E conformation (category B), a small extent of contamination by the other isomer could be detected more easily by TLC than by NMR spectroscopy.

N-Nitroso-N-isopropylglycine and N-nitroso-N-isopropyl-DL-alanine (category C) are conformationally pure in the crystal. NMR spectroscopic studies revealed that after dissolution of the original isomer present in the crystal, this isomer was not converted into another isomer. Chemical shifts in the resonances indicated that the Z conformation was present. Both category C compounds gave only one spot on thin-layer chromatograms, and no traces of a second spot were visible even after leaving an aqueous solution of the sample at room temperature for 7 days or heating this solution for 2 h at 90 °C. R_F values for the "lower" spots are in agreement with the NMR evidence that these compounds exist only as the Z isomers.

The first NMR spectrum after dissolution of a sample of N-nitroso-N-methyl-DL-phenylalanine in perdeuterated acetone revealed the presence of a major proportion of the Z isomer and a minor proportion of the E isomer[3]. The proportions of the isomers in solution did not change even after prolonged (7 days) storage at room temperature. The same results were obtained for N-nitroso-N-methyl-L-valine; the proportions of the isomers did not change on storing the solution but according to the NMR spectroscopic evidence a major proportion exists in the form of E isomer and a minor proportion as the Z isomer[3]. Both of these compounds, which are in category D in Table I, gave two spots in each chromatogram and the ratio of their areas and of the intensities of the colours did not change even on prolonged storage of the solution at room temperature. In agreement with the NMR spectroscopic evidence, the main spot for N-nitroso-N-methyl-DL-phenylalanine was that with the lower R_F value, while for N-nitroso-N-methyl-L-valine it was that with the higher R_F value.

The compounds in category F showed a preference for crystallization in the Z conformation. Contrary to the category A compounds these compounds were not usually conformationally pure in the crystal, and immediately after dissolution of a sample of a solid material only a solution with the equilibrium displaced towards the Z isomer resulted. The approach to equilibrium, involving gradual interconversion of the Z into the E isomer, could be studied by repetitive scanning of the NMR spectra[2–4]. Thin-layer chromatograms run for freshly prepared solutions of these category F compounds revealed two spots. These solutions, when stored at room temperature, showed a gradual increase in the ratio of the areas and of the intensities of the colours of the upper and lower spots until equilibrium was established.

It is evident that the Z (*syn*) isomers always gave spots with lower R_F values, and this applied both to N-nitroso-N-alkylamino acids and their *p*-nitrobenzyl esters.

Some oily N-nitroso-N-alkylamino acids and some oily alkyl esters of N-nitrosoamino acids were studied by TLC, but the results of their separation into two spots are not presented here as such oily compounds were usually mixtures of both Z and E isomers and their isomerism has not been studied in detail by other techniques.

The most suitable solvents for the separation of the Z and E isomers of N-nitroso-N-alkylamino acids contained ammonia. The presence of ammonia in the separating solvent systems is of the utmost importance as it was found that interconversions of the Z and E isomers are substantially slower for salts[4]. Thus, in the solvent systems S1 and S2, which gave the best separation results, the Z and E isomers of N-nitroso-N-alkylamino acids are separated mainly in the form of carboxylate anions, and the mean lifetimes of the Z and E anions are substantially longer than those of the corresponding unionized species.

In conclusion, it can be stated that TLC at low temperatures is capable not only of separating the Z and E isomers of N-nitroso-N-alkylamino acids and their esters, but also, because of the high separation efficiency, it enables the conformational purity to be checked, very small amounts of "conformational" contamination of the Z isomer by the E isomer or *vice-versa* to be detected, and the dynamic aspect of the conformational isomerism of this class of compounds to be studied.

Cautionary note

While N-nitroso-N-alkylamino acids are easy to prepare and to handle, it should be remembered that many N-nitrosamines are potent carcinogens. Proper precautions against contact with the skin should therefore always be taken.

ACKNOWLEDGEMENT

We thank the Polish Academy of Science for financial assistance (Grant No. 119/III/74).

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Journal of Chromatography, 95 (1974) 229–231

CHROM. 7496

Note

An ultrasonic method for producing graphite-coated glass capillary columns

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(Received April 26th, 1974)

A simple procedure for coating the inner wall of glass capillary columns with a graphite layer is reported. The procedure consists in coating columns essentially by the dynamic method[1] using a colloidal graphite solution. This solution, which is stable for several days, is obtained by submitting a suspension of graphite (Sterling MT) in dichloromethane to ultrasonic waves, produced from a magnetostrictor as suggested by Crawford[2]. After the removal of the solvent, the graphite layer remaining on the wall becomes visible.

The graphite-coated capillary columns readily permit the use of a wide range of stationary phases. These columns therefore do not suffer the main disadvantage observed when the carbon layer is obtained by the method according to Grob[3], such columns being suitable only for a restricted range of moderately polar phases[4]. Apart from this aspect, Grob's columns require some critical steps in their preparation (speed of pyrolysis, uniform heat transfer, etc.) and are deactivated by moisture[5]. More sophisticated methods for the treatment of inner glass walls have been described (for a review see ref. 5), but according to Novotný[6] a universal method has not yet been found.

Although research is yet at an early stage, our graphite-coated capillary columns have the following properties.

(1) The layer is not subject to mechanical or chemical disturbances. We did not observe any blockage when the columns were subjected to repeated washing with polar and non-polar solvents. Hence a single column can be used for different stationary phases.

(2) The columns were coated with phases of different polarity (SE-30, SE-52, Dexsil, Carbowax 20M, PMPE and Apiezon L) and, although no special care was taken with the coating procedure, very high efficiencies were observed. Typical results are given in Table I.

TABLE I

CHARACTERISTICS OF SOME GRAPHITE COATED GLASS CAPILLARY COLUMNS

I.D. of columns = 0.5 mm.

Column number	1	2	3	4
Liquid phase	Apiezon L	SE-52	SE-30	Carbowax 20M
Column length (m)	14	4	16	13
Number of effective theoretical plates	20,000*	4000**	16,500**	13,800*
HETP (mm)	0.70	1.00	0.97	0.94
$\bar{u}$ (cm/sec)	6.8	10.0	9.0	7.5

* For methyl elaidinate (oven temperature 150°).

** For *n*-hexadecane (oven temperature 120°).

It should be added that coating can be carried out using any solid material that is capable of giving a colloidal solution by ultrasonics; in the first instance graphite was chosen because, according to Brodasky[7], it seems to be an excellent support material for gas–liquid chromatography. The method, in general, can be used to obtain coated open tubular capillary columns suitable for both gas–liquid and gas–solid chromatography. Capillary columns coated with the required material by ultrasonics could find applications in liquid chromatography also, as observed with alkali-treated glass capillary columns[8].

EXPERIMENTAL

The capillary columns were drawn out by the method of Desty *et al.*[9]. The colloidal graphite solution was obtained by irradiating a 1% solution of Sterling MT carbon black (Cabot Corp., Boston, Mass., U.S.A.) in methylene chloride for 15 min in the probe (Model L667) of an ultrasonic generator (Model MSE-386, Measuring & Scientific Equipment Ltd., London, Great Britain), using waves of 24 kHz frequency and 2 μ amplitude. The colloidal graphite was introduced into the capillary by applying a reduced pressure to one end by a water pump. When the column had been filled, the reservoir was removed and suction was continued so as to empty completely the capillary. The solvent was finally removed by a stream of nitrogen. The appropriate liquid film was then deposited by the dynamic procedure[1]. Experiments were carried out using a Carlo Erba (Milan, Italy) Model 2400 gas chromatograph equipped with a flame ionization detector.

ACKNOWLEDGEMENT

We thank the C.N.R. for financial support.

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Journal of Chromatography, 95 (1974) 232–234

CHROM. 7486

Note

Gruppentrennung von Steroidhormonen und Stilbenen aus biologischem Material

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(Eingegangen am 18. März 1974)

Bei der Bestimmung von freien Steroiden und Stilbenen in biologischem Gewebe treten —je nach Herkunft des Materials— erhebliche Schwierigkeiten durch störende Begleitkomponenten auf. So werden z.B. aus Lebergewebe auch grosse Mengen an lipidhaltigen Substanzen (Phospholipide), Gallensäuren und Gallenfarbstoffen mitextrahiert, die sowohl die späteren Trennungen als auch Farbreaktionen stören können.

In der Lebensmittelanalytik werden zudem Methoden benötigt, mit denen im Fleisch von Schlachttieren Androgene und Östrogene nachgewiesen und bestimmt werden können, die auf eine Behandlung der Tiere mit Steroidhormonen hinweisen. Da häuftig an Stelle von Östrogenen Anabolika verfüttert werden, wobei der Uterus-Wachstumstest versagt, müssen die komplexen Systeme erst vorgetrennt werden, wenn sie gaschromatographisch bzw. dünnschichtchromatographisch–densitometrisch analysiert werden sollen.

Bei männlichen Tieren müssen neben den natürlicherweise vorhandenen Androgenen eventuell applizierte Östrogene und bei weiblichen entsprechend neben Östrogenen die applizierten Androgene erfasst werden. Für die Beurteilung sind neben den Hormonen jedoch auch ihre Metaboliten wichtig, bei Testosteron also z.B. Androsteron und Ätiocholanon. Die flüssigchromatographische Auftrennung bis zu den einzelnen Steroiden, wie sie mit Hilfe von Sephadex LH-20 (Pharmacia, Uppsala, Schweden) im Nanogrammbereich mittels radioaktiv markierten Derivaten realisiert wurde[1–3], ist für die Praxis viel zu aufwendig. Es genügt ein Trennverfahren, das zwischen den Androgenen und Östrogenen differenziert, damit anschliessend innerhalb einer Gruppe gaschromatographisch analysiert werden kann. Das hier beschriebene einfache Trennverfahren eliminiert die im biologischen Material in grossem Überschuss vorhandenen Störkomponenten (Triglyceride, Phospholipide, Gallensäuren, Gallenfarbstoffe usw.) und liefert eine Gruppentrennung zwischen Androgenen und Östrogenen.

EXPERIMENTELLES

Vortrennung zur Entfernung der Phospholipide

Da Phospholipide und Triglyceride die Gruppentrennung stören, müssen sie entfernt werden. Bei Triglyceriden geschieht dies durch günstige Wahl des Ex-

traktionsmediums Äthanol. In Extrakten aus Muskelfleisch ist dann der restliche Anteil an störenden Lipiden so gering, dass sie direkt zur Gruppentrennung eingesetzt werden können. Leberextrakte werden dagegen erst in Tetrahydrofuran (THF) gelöst auf eine mit THF äquilibrierte Polyvinylacetatsäule (Merckogel 6000, Merck, Darmstadt, B.R.D.) aufgebracht und mit dem gleichen Lösungsmittel eluiert. Steroide und Stilbene laufen gemeinsam. Publikation in Vorbereitung.

Gruppentrennung mit Sephadex LH-20

7 g Sephadex LH-20 werden in das Elutionsmedium 1 (EM 1) (Heptan–Chloroform–Methanol–Wasser, 100:100:1:1; modifiziert nach Murphy[1]) eingerührt und mindestens 2.5 h gequollen, bevor sie in ein Trennrohr von etwa 20 mm Durchmesser eingefüllt werden. Der Rohrdurchmesser sollte 15 mm nicht unterschreiten, da sonst beim Übergang auf das 2. Elutionsmedium (Dichlormethan–Methanol, 95:5) Änderungen im Quellgrad zu Rissen im Gel führen. Von dem Extrakt bzw. dem eingeengten Eluat der Vortrennung werden 2 ml auf die Füllung aufgebracht und mit 50 ml EM 1 die Androgene gemeinsam mit den Gestagenen und anschliessend mit 60 ml EM 2 die Östrogene aus der Säule eluiert.

ERGEBNISSE

Wie die dünnschichtchromatographische Analyse der in einzelnen Portionen aufgefangenen beiden Elutionsfraktionen zeigt (Fig. 1), sind die Östrogene vollständig von den beiden anderen Hormongruppen getrennt worden. Für Routineuntersuchungen werden jedoch nur die beiden Gesamtfraktionen gesondert aufgefan-

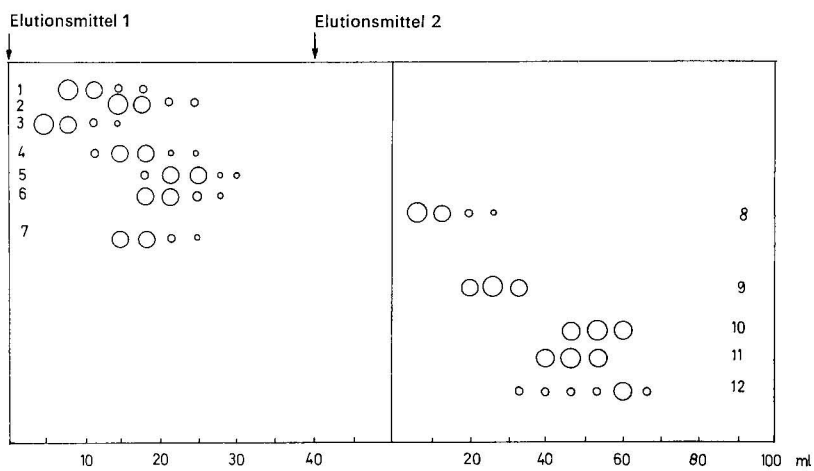

Fig. 1. Dünnschichtchromatographische Kontrolle der Gruppentrennung Androgene/Östrogene nach Chromatographie mit Sephadex LH-20 aus einem Leberextrakt (Kalb). Zusatz 1 mg je Substanz (freie Steroide). Schicht: Kieselgel$_{254}$ (Fertigplatte Merck, Darmstadt, B.R.D.), nicht aktiviert. Laufmittel: EM 1 und EM 2 (s. Text). Nachweis: Sprühen mit Anisaldehyd–Schwefelsäure–Eisessig (1:2:100); erst mit Föhn, dann im Trockenschrank 5 min bei 150° erwärmen und Farbentwicklung beobachten; Zuordnung nach Lit. 7. 1= Androstendion; 2= Progesteron; 3= Cholesterin; 4= Androsteron; 5= 17-α Hydroxyprogesteron; 6= Dihydrotestosteron; 7= Testosteron; 8= Östron; 9= Östradiol; 10= Diäthylstilböstrol; 11= Hexöstrol; 12= Östriol.

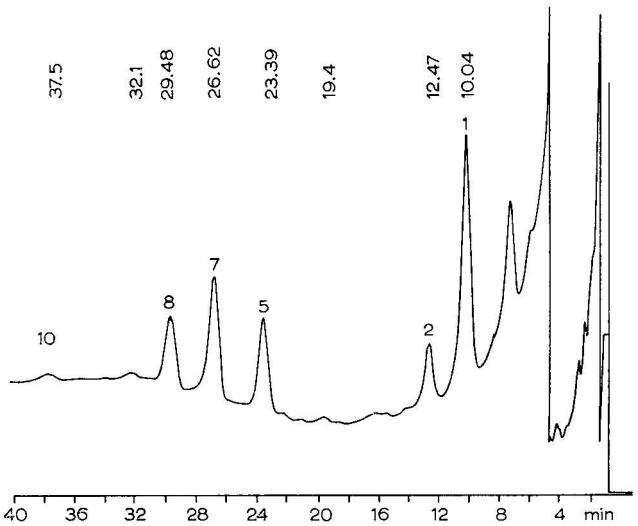

Fig. 2. Gaschromatographische Analyse der Silyläther der Östrogenfraktion aus der Gruppentrennung der Steroide mit Sephadex LH-20 (nach Vortrennung mit Merckogel 6000). Die Östrogene wurden einem Leberhomogenat vor der Extraktion zugesetzt. Trennbedingungen: Temperaturgradient 2.5°/min von 220–270°. Probe: 1 μl; Gasfluss: 40 ml N_2; Empfindlichkeit: 16; Säule: 5 m Glas 1%ig belegt mit Phase OV-17 auf Chromosorb G HP. Vorschub: 0.5 cm/min. Keine Nullinienkorrektur, ohne Integrator. Peakbezeichnung: 1 = Hexöstrol; 2 = Diäthylstilböstrol; 5 = Östron; 7 = Östradiol; 8 = Östriol.

gen und nach Einengen gaschromatographisch analysiert. In Fig. 2 is das Gaschromatogramm der Östrogenfraktion wiedergegeben. Soll die Methode an ein neuartiges Untersuchungsmaterial adaptiert werden, dann ist im ersten Trennungsgang das Eluat wie in Fig. 1 portionsweise dünnschichtchromatographisch zu kontrollieren.

Da die gaschromatographische Trennung der Steroide in Form ihrer Silyläther an Dünnfilmkapillaren aus Glas[4] aufwendig und kostspielig ist, empfehlen wir selbstgepackte Glassäulen (Länge 5 m, Durchmesser 1.5–2.0 mm) und 1–3% Belegung mit OV-17-Phase[5]. Werden Metallsäulen benutzt, dann ist bei sauerstoffhaltigen Steroiden mit Artefaktbildung und Verlusten zu rechnen[6].

Mit diesem Verfahren wurden an Schlachttiere verfütterte Steroide und Stilbene in deren Leber und Muskelfleisch nachgewiesen. Mit Zusätzen von freien und konjugierten Steroiden wurden die Ergebnisse kontrolliert und durch dünnschichtchromatographische Analyse in mehreren Systemen und mit verschiedenen Färbereaktionen[7] sowie im Falle von Diäthylstilböstrol auch durch Infrarotspektroskopie gesichert. Publikation in Vorbereitung.

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Journal of Chromatography, 95 (1974) 235–237

CHROM. 7475

Note

Thin-layer chromatographic examination of various seed oils

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(Received April 8th, 1974)

Stahl[1] outlined a relatively simple thin-layer chromatographic procedure for the characteristic identification of fatty oils. In the present work, it is believed that the procedure has been simplified by incorporating certain modifications. Also, the usefulness of the technique has been extended into investigations on linseed oil *per se* and materials containing linseed oil.

EXPERIMENTAL

Thin-layer chromatography (TLC) was carried out with pre-coated plastic sheets (20×20 cm) coated with a 0.1-mm layer of microcrystalline cellulose (Polygram Cel 400 UV_{254}; Macherey-Nagel & Co., Düren, G.F.R.). Prior to use, the sheets were coated with liquid paraffin by ascending chromatography for a distance of 15 cm using a solution of 5% liquid paraffin in light petroleum (b.p. 40–70°). The air-dried sheets were then pre-washed with glacial acetic acid by ascending chromatography. The sheets were air-dried thoroughly prior to the application of the samples to be examined. One microlitre samples of the oils (2–10 μg) were applied on a line 2 cm from the edge of the TLC sheet and the sheets were developed in glacial acetic acid to a distance of about 10–11 cm. The development time was 2–3 h at room temperature (20–25°). The sheets were removed from the developing tank, air-dried, and then transferred into a closed chamber containing iodine vapour. Immediately after the removal of the sheets from the iodine tank, after a period of contact of about 5 min, the sheets were sprayed with a 4% aqueous solution of soluble starch.

Plastic sheets pre-coated with a 0.1-mm layer of cellulose (Polygram Cel 300) and prepared for use as described above were also used in similar experiments. However, they were less efficient in the separation of the various components of the oil samples and less sensitive to detection by the iodine–starch reaction.

The following additional experiments were included in this study: (1) putty samples, exposed for at least 4 years to the local atmosphere in Rome were obtained from an exterior window sash; (2) linseed oil films of about 2 mm thickness were exposed to the atmosphere in the laboratory at room temperature (20–25°) for a period of 54 days; (3) a compositional mixture was prepared so as to simulate

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an artist's painting. A layer of a mixture of pigment, basic lead carbonate and linseed oil was superimposed upon a layer of a mixture of plaster of Paris and glue. The film was then exposed to ultraviolet light for a period of 144 h.

Samples of the putty and the paint mixture were pulverized by means of a glass mortar and pestle. The pulverized samples and samples of the exposed linseed oil were then leached with carbon tetrachloride at room temperature for 24 h and the extracts were examined by TLC as described above.

RESULTS AND DISCUSSION

A typical example of the results obtained with some oils is shown in Fig. 1. Repeated experiments indicated that the procedure was remarkably reproducible and the degree of sensitivity, or limit of detection, was about 2μg for each oil, and even lower for the individual triglyceride components observed.

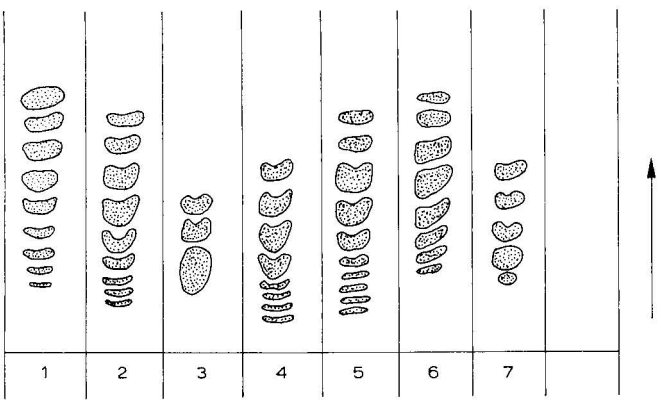

Fig. 1. TLC of various oils: (1) linseed, uncooked; (2) "mixed seeds"; (3) olive, freshly pressed; (4) groundnut; (5) poppy seed; (6) walnut; (7) oleomargarine. Amount of each oil used, 5 μg.

Drying oils, such as linseed oil, after a lengthy time of exposure to air and light, undergo chemical changes involving polymerization and some breakdown to a variety of small molecules[2]. This compositional change is illustrated markedly in Fig. 2. The spots (triglycerides) will decrease in intensity and additional spots will be observed near the origin and near the solvent front of the chromatogram. With increased time or accelerated exposure to ultraviolet radiation, the original spots will disappear completely with the formation of a new substance(s), which will appear in the area of the solvent front and the origin of the chromatogram.

The modification to the Stahl procedure included the elimination of the preparation and heat-treatment of the TLC plates prior to use. The use of commercially available plastic TLC sheets minimized the difficulty of preparing plates of uniform composition and constant performance characteristics. Some increase in the limit of detection was also observed. Washing the sheets with glacial acetic acid prior to their use eliminated the problem of iodine-sensitive contaminants at the solvent front and thereby eliminated the problems of interpreting any spots observed in the area of the solvent front.

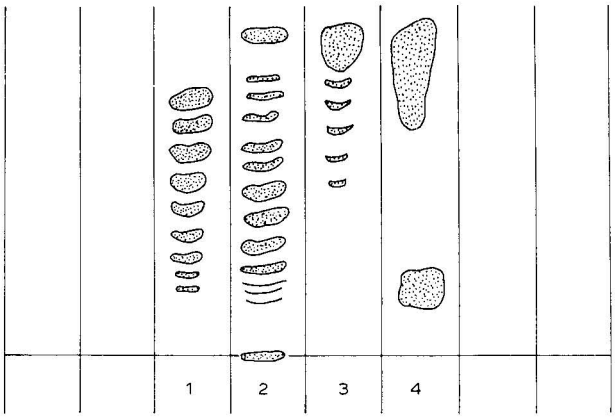

Fig. 2. TLC of "aged" linseed oil: (1) fresh, uncooked; (2) 2-mm oil film exposed to air and light at 20–25° for 54 days; (3) sample of putty exposed to the atmosphere for at least 4 years, obtained from exterior window sash; (4) simulated artist's painting composed of plaster of Paris, glue, pigment, basic lead carbonate and linseed oil; irradiated for 144 h under ultraviolet light (250–280 lux).

In addition to Stahl's original purpose to apply this procedure to the evaluation of "natural drugs and their mixtures and preparations," the technique is also useful for the preliminary screening of oils or materials that contain oils (such as works of art) for the purpose of identifying the oil and/or to determine the relative age of the oil or oil-bearing material.

ACKNOWLEDGEMENT

I am indebted to Prof. Paolo Mora, Istituto Centrale del Restauro, Rome, for the preparation of the artist's paint mixture.

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Journal of Chromatography, 95 (1974) 238–242

CHROM. 7476

Note

Chemisorptionschromatographie mit Dithiophosphinatokomplexen

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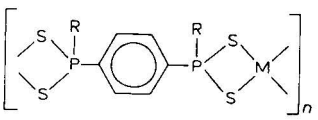
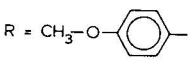
(Eingegangen am 4. Februar 1974)

Im Verlaufe unserer Untersuchungen über die Verwendung koordinativ ungesättigter Metalldithiophosphinato-Komplexe als stationäre Phase bei der gaschromatographischen Trennung von Lewis-Basen[1–3] zeigte es sich nunmehr, dass sowohl die polymeren Komplexe 1a und 1b

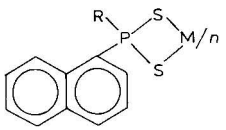


(1)

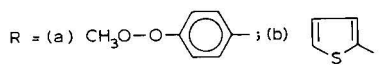


M: 1a = Ni(II)
1b = Co(II)
1c = Pd(II)

als auch die monomeren Komplexe



(2)



M: 2a = Ni(II)
2b = Ni(II), Pd(II), Pt(II)

zur Trennung einer Vielzahl von Aminen und Phosphiten geeignet sind. Wir fanden, dass darüber hinaus an 1a auch silylierte Aminosäuren[4] sowie an 1b Thiobasen (siehe Tabelle I) getrennt werden können.

Mit den Pd(II)- bzw. Pt(II)-Komplexen 1c und 2b lassen sich des weiteren sehr sauber auch Olefine, Ketone und Äther[4] trennen (siehe Tabellen II und III). Olefine lassen sich fernerhin vortrefflich auch an dem polymeren Ag(I)-Komplex der *p*-Phenylen-bis(*p*-methoxyphenyldithiophosphinsäure) trennen (siehe Tabelle IV).

Die Trennwirkung herkömmlicher Adsorbentien in der Gaschromatographie und damit die Elutionsdaten der verschiedenen Verbindungen hängt von unterschiedlichen, sich summierenden Kräften (z.B. London-, Keesom-, Debye-Kräften) ab. Verschiedene Autoren konnten zeigen, dass innerhalb homologer Verbindungs-

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TABELLE I

RELATIVE RETENTIONSVOLUMINA VON THIOBASEN IN RELATION ZUM MOLEKULARGEWICHT AM POLY[*p*-PHENYLEN-BIS(*p*-METHOXYPHENLYDITHIOPHOSPHINATO)–Co(II)] (1b)

Trägergasstrom: 35 ml He/min; Temperatur: 80° isotherm; Standard: *n*-Pentan.

Thiobase	*MG*	*Rel. Retentionsvolumen*
Thiophen	84.1	1.16
Isobutylmercaptan	90.2	1.16
tert.-Butylmercaptan	90.2	1.20
Diäthylsulfid	90.2	1.27
sek.-Butylmethylsulfid	104.2	1.36
2-Äthylthiophen	112.2	1.73
Diisopropylsulfid	118.2	1.29
Hexanthiol	118.2	2.18

TABELLE II

RELATIVE RETENTIONSVOLUMINA VON OLEFINEN IN RELATION ZUM MOLEKULARGEWICHT

A = Poly[*p*-phenylen-bis(*p*-methoxyphenyldithiophosphinato)–Pd(II)] (1c); B = Bis(2-thienyl-1-naphthyldithiophosphinato)–Pd(II) (2b); C = Bis(2-thienyl-1-naphthyldithiophosphinato)–Pt(II)-(2b). Trägergasstrom: A = 36 ml He/min; B = 36 ml He/min; C = 44 ml He/min; Temperatur: 100° isotherm; Standard: (a) = *cis*-2-Buten; (b) = 2,5-Dimethyl-2,4-hexadien.

Olefine	*MG*	*Rel. Retentionsvolumen*		
		A	*B*	*C*
1,3-Butadien	54.1	0.72 (b)	0.78 (b)	0.79 (b)
cis-2-Buten	56.1	0.72 (b)	0.78 (b)	0.79 (b)
2,3-Dimethyl-2-penten	99.2	1.09 (a)	1.09 (a)	1.06 (a)
2,5-Dimethyl-2,4-hexadien	110.2	1.40 (a)	1.28 (a)	1.22 (a)
2,3-Dimethyl-1-hexen	113.2	1.10 (a)	1.09 (a)	1.14 (a)

TABELLE III

RELATIVE RETENTIONSVOLUMINA VON KETONEN IN RELATION ZUM MOLEKULARGEWICHT

A = Poly[*p*-phenylen-bis(*p*-methoxyphenyldithiophosphinato)–Pd(II)] (1c); B = Bis(2-thienyl-1-naphthyldithiophosphinato)–Pd(II) (2b); C = Bis(2-thienyl-1-naphthyldithiophosphinato)–Pt(II) (2b). Trägergasstrom: A = 22 ml He/min; B = 35 ml He/min; C = 43 ml He/min; Temperatur: 100° isotherm; Standard: Petroleum ether (Kp. 30–50°).

Keton	*MG*	*Rel. Retentionsvolumen*		
		A	*B*	*C*
Aceton	57.9	1.10	–	–
Diisopropylketon	113.9	1.37	1.11	1.26
Di-*n*-propylketon	113.9	1.57	1.22	1.63

TABELLE IV

RELATIVE RETENTIONSVOLUMINA VON OLEFINEN IN RELATION ZUM MOLEKULARGEWICHT AM POLY [*p*-PHENYLEN-BIS(*p*-METHOXYPHENYLDITHIOPHOSPHINATO)-Ag(I)]

Trägergasstrom: 27 ml He/min; Temperatur: 100° isotherm; Standard: *n*-Pentan.

Olefin	*MG*	*Rel. Retentionsvolumen*
1,3-Butadien	54.1	1.48
2,5-Dimethyl-2-penten	99.2	1.15
2,5-Dimethyl-2,4-hexadien	110.2	1.65
2,3-Dimethyl-1-hexen	113.2	1.32

TABELLE V

RELATIVE RETENTIONSVOLUMINA VON AMINEN[1,2] IN RELATION ZUM MOLEKULARGEWICHT BZW. MOLVOLUMEN

A = Poly[*p*-phenylen-bis(*p*-methoxyphenyldithiophosphinato)-Ni(II) (1a); B = Poly[*p*-phenylen-bis(*p*-methoxyphenyldithiophosphinato)-Co(II)] (1b); C = Bis(*p*-methoxyphenyl-1-naphthyldithiophosphinato)-Ni(II) (2a); D = Bis(2-thienyl-1-naphthyldithiophosphinato)-Ni(II) (2b). Trägergasstrom: A = 40 ml He/min; B = 49 ml He/min; C = 48 ml He/min; D = 46 ml He/min. Temperatur: 100° isotherm; Standard: Diäthylamin.

Amin	*MG*	*Dichte*	*MV*	*Rel. Retentionsvolumen*			
				A	*B*	*C*	*D*
Pyridin	79.1	0.9819	80.56	1.57	1.36	1.49	1.50
Anilin	93.1	1.0216	91.13	5.20	5.29	10.15	7.16
Dimethyläthanolamin	89.1	0.8866	100.50	1.62	1.44	1.50	1.44
Monomethylanilin	108.1	0.9891	109.29	4.81	5.64	–	6.45
Dimethylanilin	123.1	0.9563	128.83	4.85	2.11	2.55	3.39
Dimethylcyclohexylamin	127.2	0.8680	146.54	1.84	1.72	1.33	1.40
N,N-Dimethylbenzylamin	135.2	0.9150	147.76	2.77	1.96	1.98	2.11
N,N-Dimethyl-2-äthylhexylamin	173.3	0.8260	209.81	2.07	1.72	1.68	1.75
Tributylamin	185.4	0.7782	238.24	3.02	2.66	2.56	3.00

reihen hierbei ein linearer Zusammenhang zwischen den Elutionsdaten und dem Molekulargewicht (MG) bzw. Molvolumen (MV) der verschiedenen Substanzen besteht[5–7].

Bemerkenswerterweise ist jedoch in den von uns bisher untersuchten Stoffreihen ein solcher Zusammenhang nicht gegeben, wie man aus den Tabellen I, II, IV–VI entnehmen kann.

Mithin scheinen Van der Waals-Kräfte (z.B. Polarisations- oder Dipol–Dipol-Kräfte) im vorliegenden Fall nicht ausschlaggebend für die Wechselwirkung zwischen Adsorbens und Adsorptum zu sein.

Vielmehr dürfte hier die aus einer Adduktbildung resultierende koordinative und damit wesentlich festere Bindung zwischen Komplex und Base das Retentionsverhalten der Substanzen bestimmen.

TABELLE VI

RELATIVE RETENTIONSVOLUMINA VON PHOSPHITEN[2] IN RELATION ZUM MOLEKULARGEWICHT BZW. MOLVOLUMEN

A = Poly[*p*-phenylen-bis(*p*-methoxyphenyldithiophosphinato)-Ni(II)] (1a); B = Poly[*p*-phenylen-bis(*p*-methoxyphenyldithiophosphinato)-Co(II)] (1b); C = Bis(*p*-methoxyphenyl-1-naphthyldithiophosphinato)-Ni(II) (2a); D = Bis(2-thienyl-1-naphthyldithiophosphinato)-Ni(II) (2b). Trägergasstrom: A = 50 ml He/min; B = 57 ml He/min; C = 50 ml He/min; D = 49 ml He/min. Temperatur: 120° isotherm; Standard: Diäthylamin.

Phosphit	*MG*	*Dichte*	*MV*	*Rel. Retentionsvolumen*			
				A	*B*	*C*	*D*
Dimethylphosphit	110.1	1.200	91.72	3.44	3.00	3.87	–
Trimethylphosphit	124.1	1.040	118.74	1.25	–	1.17	2.60
Diäthylphosphit	138.1	1.074	129.10	4.21	3.37	6.11	3.49
Triäthylphosphit	166.2	0.960	173.08	1.93	1.40	1.83	1.61
Diäthyltrimethylsilylphosphit	209.9	–	–	3.68	1.65	4.67	1.57

In einigen Fällen gelang es, wohldefinierte penta- bzw. hexakoordinierte Addukte von Komplexen des allgemeinen Typs 1 bzw. 2 mit verschiedenen Lewis-Basen in reiner Form zu isolieren und ihre Koordinationsgeometrie durch magnetische bzw. elektronenspektroskopische Untersuchungen zu erhärten[8].

Die Stärke einer koordinativen Bindung ist jedoch abhängig von einer Reihe von Faktoren. Bei gegebenem Akzeptor z.B. von der Basizität des Donators, von seinem Raumbedarf, von seiner Befähigung zur Rückkoordination etc., nicht aber von physikalischen Eigenschaften wie Molekulargewicht bzw. Molvolumen.

Da bei den hier beschriebenen chromatographischen Trennungen offensichtlich die Chemisorption von Adsorptum am Adsorbens dominiert, schlagen wir für diese Variante der Chromatographie, bei der die Trennung der Lewis-Basen an koordinativ ungesättigten Metallkomplexen erfolgt, die Bezeichnung "Chemisorptionschromatographie" vor.

EXPERIMENTELLER TEIL

Zur Verwendung als stationäre Phase belegten wir Chromosorb W AW-DMCS, 80–100 mesh, mit 4% (w/w) der Komplexe nach einem bereits früher beschriebenen Verfahren[1].

Bei den chromatographischen Untersuchungen gelten in allen Fällen folgende allgemeine Bedingungen: Gaschromatograph, Hewlett-Packard Modell 5750 D; Glassäulenlänge 3500 mm; lichte Weite, 1.5 mm; Detektor, W.L.D., Typ WXR filaments (320°); Injektionsblocktemperatur, 250°; Papiervorschub, 2.5 cm/min; Brückenstrom, 150 mA.

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Journal of Chromatography, 95 (1974) 243–246

CHROM. 7424

Note

Fluorescence of pesticides by treatment with heat, acid or base

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(Received February 26th, 1974)

A method for the detection of naturally fluorescent pesticides on silica gel layers was described in a preceding paper[1]. Benomyl, Coumatetralyl, Diphacinone, Fuberidazole, Propyl isome and Quinomethionate were investigated, the fluorescence spectra were measured and visual limits of detection were estimated. In most cases, as little as a few nanograms were detected. In addition, the effects of heat treatment on the fluorescence were observed.

In this study, a few more pesticides were investigated in addition to those already reported. It was intended to show how selectivity may be increased through the use of acid or base as spray reagent along with heat treatment of the chromatogram.

EXPERIMENTAL

For a more detailed description, see the preceding paper[1].

The Farrand VIS-UV Chromatogram Analyzer requires filters in addition to the monochromators. The combination found to be most useful was a No. 7-54 (250–390 nm, effective band pass) filter in the exciter drawer and a No. 3-73 filter (430–800 nm, effective band pass) in the analyzer leg (system A). This filter combination was found to be excellent for excitation in the region from 250 to 390 nm while emission is in the region of 435–800 nm. The second filter combination (system B) was obtained by placing two No. 7-54 filters (320–385 nm, overall effective band pass) in the exciter drawer with a No. 3-75 filter (370–800 nm, effective band pass) in the analyzer leg. This has the advantage of decreasing spectra overlap. To avoid excessive reflection peak interference, the No. 3-75 filter may also be doubled. The doubling of filters, however, cuts down on the percentage of light transmitted and consequently, may affect the detection limits. All of the above mentioned spectral filters are from Corning Glass Works (Corning, N.Y., U.S.A.).

General procedure

Aqueous solutions of H_2SO_4, HCl, HNO_3, NH_4OH, NaOH and KOH were prepared at the following concentrations: 0.1, 1.0 and 2.5 *N*. The chromatograms were sprayed until moist with the acidic or basic solution and heated at an optimum temperature for a definite time period.

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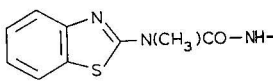
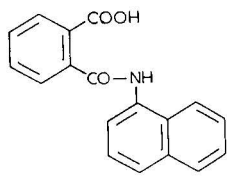
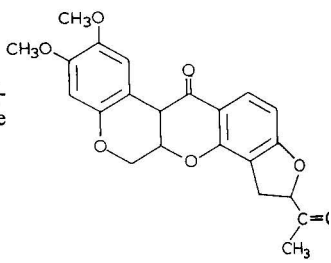
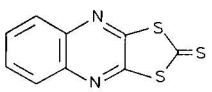
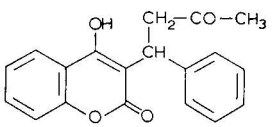
RESULTS AND DISCUSSION

The structures of the pesticides used in this study and not given earlier[1] are shown in Table I. To our knowledge, no reference is made in the literature on the *in situ* fluorimetric detection of the compounds Naptalam, Methabenzthiazuron, Rotenone, Thioquinox and Warfarin on silica gel chromatograms. However, Benomyl has been determined quantitatively by fluorescence measurements in solution[2]. Sensitivity was given as 0.1 ppm based on a 50 g sample. The other pesticides are analyzed either by UV-visible spectroscopy, *e.g.* Coumatetralyl[3], Fuberidazole[4], Rotenone[5], and Warfarin[6,7]; or by colorimetric methods, *e. g.* Naptalam[8–10], Methabenzthiazuron[11], Propyl isome[12], Quinomethionate[13–14], Thioquinox[13] and Rotenone[15]. Quinomethionate has been determined by polarography[16].

TABLE I

STRUCTURES OF SOME OF THE PESTICIDES USED

H = Herbicide; I = insecticide; F = fungicide; Ro = rodenticide.

Pesticide and manufacturer	*Chemical name*	*Structure*
Methabenzthiazuron (H) (Bayer)	1-(2′-Benzothiazolyl)-1,3-dimethylurea	
Naptalam (H) (Uniroyal)	N-1-Naphthylphthalamic acid	
Rotenone (I) (Niagara)	1,2,12,12a-Tetrahydro-2-isopropenyl-8,9-dimethoxy[1]benzopyrano[3,4-*b*]-furo[2,3-*b*][1]benzopyran-6(6a*H*)-one	
Thioquinox (F) (Chemagro)	2-Thio-1,3-dithiolo[4,5-*b*]quinoxaline	
Warfarin (Ro) (Penick)	3-(α-Acetonylbenzyl)-4-hydroxycoumarin	

Some of the results obtained in this study are given in Table II. The pesticides that were not previously studied, namely Methabenzthiazuron, Naptalam, Rotenone, Thioquinox and Warfarin, all give fluorescence upon heat treatment. With the exception of Rotenone, all are not (or very weakly) fluorescent naturally. An exceptionally good limit of detection is obtained with Methabenzthiazuron under such conditions.

TABLE II

FLUORIMETRIC RESPONSE OF SOME PESTICIDES

Pesticide	*Filter combination*	*Wavelength (nm)*		*Optimum conditions**	*I.L.D.***
		Excitation	*Emission*		
Coumatetralyl	B	358	450	a,c	0.01
Diphacinone	A	330	514	a,b	2.0
Fuberidazole	B	333	410	a,d	0.006
Methabenzthiazuron	B	353	439	a	0.002
Naptalam	A	361	455	a	0.08
	A	298	455	b,f	0.04
Propyl isome	A	352	472	a,d	0.008
Quinomethionate	B	337	458	a,e	0.04
	B	335	455	a,c	0.01
	B	362	417	a,d	0.06
Rotenone	A	362	453	F	0.8
	A	370	440	a	0.6
Thioquinox	B	329	441	a	0.04
	B	329	435	a,c	0.08
Warfarin	A	363	456	a	0.06

* F = Fluorescent naturally; a = heated at 200° for 45 min; b = sprayed with 2.5 *N* KOH; c = sprayed with 0.1 *N* NH_4OH; d = sprayed with 0.1 *N* HCl; e = sprayed with 0.1 *N* H_2SO_4; f = heated at 220° for 30 min.

** I.L.D. = Instrumental limit of detection.

In most cases, the spraying with a strong electrolyte, such as an acid or base, prior to the heat treatment does not increase the limit of detection markedly as compared to the heat treatment alone. However, there is sometimes a change in the spectra as shown with Naptalam and Thioquinox. The change is more drastic in other cases already mentioned[1]. For instance, Coumatetralyl has excitation and emission maxima at 330 and 415 nm naturally; Fuberidazole at 328 and 402 nm; and Propyl isome at 343 and 460 nm. It should be noticed that most of the compounds studied do not give the same combination of excitation and emission maxima.

Heat treatment has definitely an effect on organic compounds spotted on silica gel thin layers. The actual mechanisms involved are not as yet fully understood, although work in this area is presently being undertaken. It may be stipulated, however, that in some cases degradation of the initial substance takes place. It is known that Naptalam is unstable at elevated temperatures (200°), tending to form the imide[4]. Also, Propyl isome, Quinomethionate and Naptalam are hydrolysed by strong alkali[3]. Thioquinox, on the other hand, is resistant to hydrolysis[4] but susceptible to oxidation to sulfur oxides. Quinomethionate is closely related

to Thioquinox in chemical properties, but is more stable to oxidation[4]. Chemical or physical bonding of the compounds to the silica gel layers is still another factor which may very well affect fluorescence intensities. Thus, as solvent polarity and pH affect quantum efficiency in solution, the polarity, as well as the water content of the layer, are also important factors.

CONCLUSION

The ease with which TLC can be applied to pesticide residue analysis is by far the greatest asset of the technique. Coupled with the sensitivity of fluorimetric methods, a relatively inexpensive and time saving analytical method is possible. With detection limits well into the sub-microgram level, sensitivity is guaranteed. Work already done with Quinomethionate[17] gives limits of detection at the ppb level.

Alkali spray reagents were found to be most useful as fluorescence intensifiers. However, no marked difference was noted between NaOH or KOH as spray reagents. Acid causes shifts in the spectra, but, unfortunately, it is almost always followed by a decrease in fluorescence intensity. The most useful of the acidic sprays used was HCl. Nitric acid is useless since it tends to act as a quencher.

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Journal of Chromatography, 95 (1974) 247–249

CHROM. 7440

Note

Thin-layer chromatographic determination of simple phenols in microbial extracts

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(Received January 25th, 1974)

A study of the microbial metabolism of diverse chemical entities has commenced in our laboratories. The major purpose of this program is to define series of microorganisms that affect transformations identical to those occurring in mammals. It has been proposed that such systems could become a valuable tool in xenobiotic metabolism studies[1]. The hydroxylation of twelve simple aromatic compounds by eleven microorganisms has been studied[1] as an initial phase of this work. During these experiments, TLC systems were required for the qualitative determination of the potential phenolic metabolites of the substrates noted in Table I. More specifically, three solvent systems were sought for the metabolites of each substrate; for greatest possible discrimination[2], attempts were made to devise series containing acidic, basic and neutral solvent systems. The TLC systems were also required to distinguish between phenolic metabolites and co-extracted native components of the microorganisms employed. The literature describes numerous systems for the TLC analysis of simple phenols[3–10]. These reports were used as guides in developing TLC systems for determination of the compounds of interest in microbial extracts.

EXPERIMENTAL

TLC was carried out on 250-μm silica gel GF_{254} plates (Analtech, Newark, Del., U.S.A.) developed 10 cm in the following solvent systems: (A) benzene–methanol (95:5); (B) benzene–methanol (4:1); (C) benzene–ethyl acetate (9:1); (D) benzene–acetic acid (5:1); (E) benzene–acetic acid (5:2); (F) benzene–methanol–acetic acid (45:8:4); (G) chloroform–ethanol–acetic acid (18:1:1); (H) toluene–piperidine (5:2); (I) benzene–ethyl acetate–piperidine (6:3:1); (J) benzene–isopropanol–conc. ammonium hydroxide (5:4:1); (K) isopropanol–benzene–conc. ammonium hydroxide (3:1:1). Detection was via quenching of 254 nm-induced fluorescence and by diazotized sulfanilic acid[1].

RESULTS

Table I lists R_F values for the potential phenolic metabolites; in all systems, compounds developed as well consolidated spots, while developing times were usually

TABLE I

TLC OF PHENOLIC METABOLITES*

Compounds were chromatographed as such and as spiked ethyl acetate extracts (see ref. 1) of *Aspergillus niger* (ATCC 9142), *Penicillium chrysogenum* (ATCC 10002), *Cunninghamella blakesleeana* (ATCC 8688a), *Aspergillus ochraceous* (ATCC 1008), *Gliocladium deliquescens* (SP-WISC 1086), *Streptomyces* sp. (SP-WISC 1158w), *Rhizopus stolonifer* (NRRL 1477), *Curvularia lunata* (NRRL 2178), *Streptomyces rimosus* (ATCC 23955), *Cunninghamella bainieri* (ATCC 9244), and *Helicostylum piriforme* (QM 6945) cultures. ATCC= American Type Culture Collection, Rockville, Md.; NRRL= Northern Regional Research Laboratories, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Ill.; QM= Quartermaster Culture Collection, U.S. Army Laboratories, Natick, Mass.; SP-WISC= School of Pharmacy, University of Wisconsin, Madison, Wisc.

Substrate and phenolic metabolites	*Solvent systems and $R_F \times 100$ values*		
Acetanilide (AC)	*B*	*F*	*H*
2-Hydroxy AC	55	58	15
3-Hydroxy AC	47	47	34
4-Hydroxy AC	42	41	39
Aniline (AN)	*B*	*H*	*J*
2-Hydroxy AN	55	46	69
3-Hydroxy AN	49	40	61
4-Hydroxy AN	41	54	66
Anisole (AS)	*A*	*C*	*E*
2-Hydroxy AS	65	57	74
Phenol	48	48	68
4-Hydroxy AS	42	37	62
Hydroquinone	07	14	39
Benzoic acid (BA)	*D*	*G*	*K*
2-Hydroxy BA	60	65	63
3-Hydroxy BA	38	47	21
4-Hydroxy BA	38	40	34
3,4-Dihydroxy BA	21	26	05
Biphenyl (BP)	*A*	*D*	*I*
2-Hydroxy BP	69	64	52
4-Hydroxy BP	55	53	32
2,5-Dihydroxy BP	25	39	28
4,4′-Dihydroxy BP	15	27	18
Chlorobenzene (CB)	*A*	*D*	*H*
2-Hydroxy CB	59	63	21
3-Hydroxy CB	53	54	34
4-Hydroxy CB	48	52	41
Coumarin (CM)	*A*	*F*	*J*
4-Hydroxy CM	07	58	12
7-Hydroxy CM	18	55	30
o-Coumaric acid	03	51	04
Naphthalene (NA)	*A*	*D*	*H*
1-Hydroxy NA	50	56	55
2-Hydroxy NA	45	52	49

TABLE I (*continued*)

Substrate and phenolic metabolites	*Solvent systems and $R_F \times 100$ values*		
Nitrobenzene (NB)	*A*	*E*	*J*
2-Hydroxy NB	75	81	20
3-Hydroxy NB	43	60	52
4-Hydroxy NB	35	55	16
trans-Stilbene (ST)	*A*	*F*	*H*
4-Hydroxy ST	48	60	50
4,4'-Dihydroxy ST	16	53	38
Toluene (TL)	*A*	*D*	*I*
2-Hydroxy TL	51	58	49
3-Hydroxy TL	45	53	38
4-Hydroxy TL	45	50	40

* Reference materials were obtained commercially (see ref. 1) and used after homogeneity was established by TLC.

less than 20 min. Solvent systems A through K were also utilized to chromatograph ethyl acetate extracts of the aromatic hydroxylase-containing microorganisms (see footnote to Table I) spiked with the phenols of interest. In all instances, the TLC systems permitted differentiation of the phenolic metabolites from substrates and co-extracted microbial materials.

ACKNOWLEDGEMENT

Financial support from the Division of Undergraduate Education in Science, National Science Foundation (Grant GY 15075) is gratefully acknowledged.

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Journal of Chromatography, 95 (1974) 250–253

CHROM. 7450

Note

Simultaneous estimation of sterol and steroidal sapogenin in plant extracts by densitometric thin-layer chromatography

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Various methods have been published for the routine estimation of sterols including colorimetry[1], gravimetric digitonide precipitation[2], IR spectometry[3] and GLC[4]. Steroidal sapogenin has been estimated by direct gravimetry[5,6], colorimetry[7–9], IR spectrometry[10–12], GLC[13] and densitometric TLC[14–16]. The chromatographic methods have the advantages of high sensitivity and specificity and require only small amounts of sample. In the published densitometric methods[14–16] the amount of sapogenin is estimated by comparison with an external sapogenin standard. The present development involves the use of an internal standard to obviate the problems associated with accurate sample application[17] and the need for relatively complex apparatus. It also allows the simultaneous estimation of both sterol and steroidal sapogenin in a crude extract.

Lanosterol was selected as internal standard. It was adequately resolved from sterol and sapogenin in a 10-cm run on 250-μm silica gel G plates developed in *n*-hexane–acetone (4:1). The plates were air-dried for 10 min, sprayed with a saturated solution of antimony trichloride in chloroform, and heated in a forced air oven at

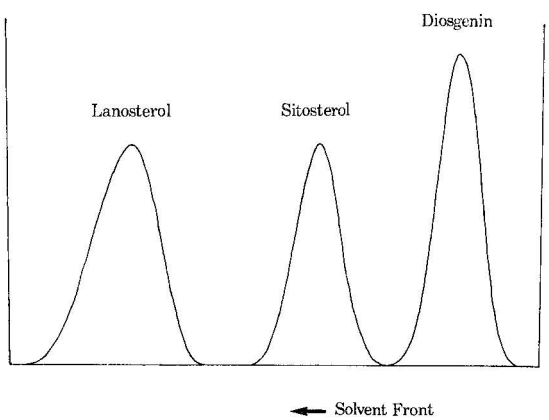

Fig. 1. A densitometric trace of TLC separation of lanosterol (internal standard), sitosterol, and diosgenin. Conditions: log-mode; filter, 477 nm; aperture, 0.25-mm circle; strike length, 10 mm; scan speed, 3 cm/min; chart speed, 20 cm/min; integrator rate, 7.

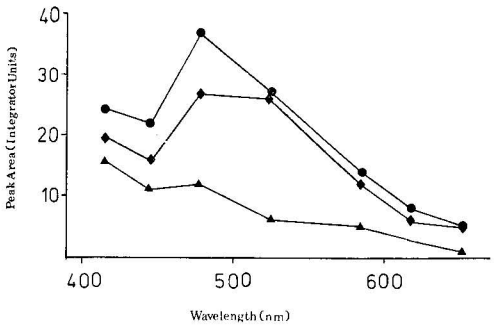

Fig. 2. The effect of filter wavelength on light absorption by sterol, sapogenin, and internal standard spots. ◆—◆, Sitosterol; ●—●, diosgenin; ▲—▲, lanosterol.

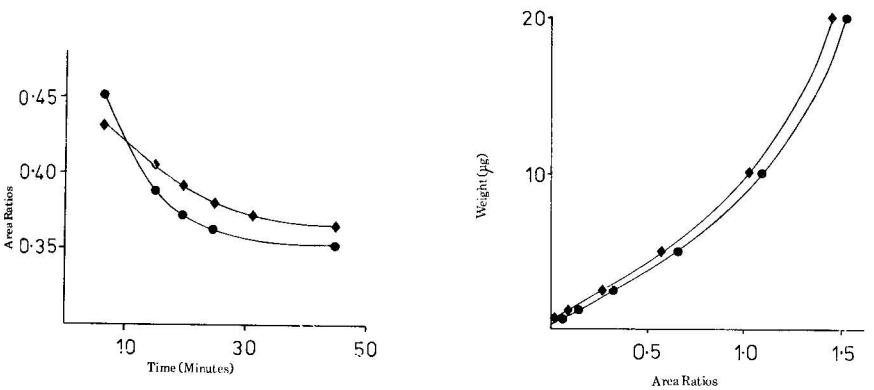

Fig. 3. The effect of time on the ratios of sterol and sapogenin response to that of the internal standard. ●—●, Diosgenin; ◆—◆, sitosterol.

Fig. 4. The relationship between the quantities of sterol and sapogenin and their absorption ratios to the internal standard. ●—●, Diosgenin; ◆—◆, sitosterol.

100° for 8 min (Table I). The plates were scanned in transmission on a Vitatron Model TLD 100 flying spot scanner and Fig. 1 shows a typical trace. The optimal wavelength for maximum absorption of the three compounds was 477 nm (Fig. 2). The spot intensity faded relatively rapidly at first but it was found that the ratio of the response of the sterol and sapogenin to that of the internal standard stabilised after about 30 min (Fig. 3). Measurements were therefore routinely made 40 min after removal of the plate from the oven. The relationships between sterol and sapogenin concentrations and their absorption ratios to the internal standard are shown in Fig. 4. As the response was not linear, and also varied to some extent from plate to plate, a separate calibration graph was used for each plate.

In the routine procedure adopted the test samples were taken up in 3 ml of chloroform containing 6.67 μg/10 μl lanosterol. Six test samples and six reference samples, each of 10 μl, containing varying ratios of sterol and sapogenin to internal

TABLE I

R_F AND COLOUR RESPONSE OF STANDARDS

Compound	R_F	*Colour response*
Lanosterol	0.53	Brown
Sitosterol	0.43	Purple
Diosgenin	0.35	Red

TABLE II

COMPOSITION OF REFERENCE SAMPLES APPLIED TO PLATES IN THE ROUTINE PROCEDURE

Concentration (μg/10 μl)		
Diosgenin	*Sitosterol*	*Internal standard*
0.5	0.5	6.67
1.0	1.0	6.67
2.5	2.5	6.67
5.0	5.0	6.67
10.0	10.0	6.67
20.0	20.0	6.67

TABLE III

PRECISION OF SIX REPLICATE ESTIMATIONS OF A TEST EXTRACT

Plate	*Standard deviation (%)*	
	Diosgenin	*Sitosterol*
1	1.4	1.5
2	0.9	0.5
3	1.4	1.2
Mean	1.2	1.1

standard (Table II) were applied to each 20 × 20 cm plate and the results were calculated in terms of sitosterol and diosgenin.

The precision of the procedure was calculated from six replicate estimations of a single test solution on each of three plates. Table III indicates that the total error involved was comparable to that found previously (0.95%) for sapogenin using the more lengthy and complex external standard method[16].

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Journal of Chromatography, 95 (1974) 254–256

CHROM. 7471

Note

Séparation de quelques dérivés de l'agmatine par électrophorèse et chromatographie sur papier

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(Reçu le 21 janvier 1974; manuscrit modifié reçu le 27 mars 1974)

Au cours de l'étude du métabolisme de l'agmatine dans les rameaux de *Limonium vulgare* Mill. et dans les jeunes plantes de *Soja hispida* Moench., il est apparu des composés dont la séparation et l'identification a présenté des difficultés. Il était donc nécessaire de mettre au point une technique qui permette d'isoler les produits pouvant se former au cours de l'utilisation de l'agmatine: urée, N-carbamylputrescine, N,N′-dicarbamylputrescine, γ-guanidinobutyraldéhyde, acide γ-guanidinobutyrique, arcaïne, putrescine, spermidine et spermine. Lopez-Gorge et Monteoliva[1] ont montré que la méthode de Biserte *et al.*[2] était applicable à la séparation des dérivés guanidiques. Cette méthode qui nous a permis aussi de séparer la N-carbamylputrescine, ne donne qu'une très mauvaise résolution de la N,N′-dicarbamylputrescine et de l'urée d'une part, de l'agmatine et des polyamines d'autre part. C'est pourquoi nous avons dû compléter la méthode en combinant à l'électrophorèse trois chromatographies parallèles.

PARTIE EXPÉRIMENTALE

Origine des composés utilisés

Les produits utilisés sont des produits commerciaux ou des produits obtenus au laboratoire. L'agmatine sulfate nous a été fournie par les établissements Sigma (St. Louis, Mo., É.U.), la putrescine, la spermidine et la spermine par Fluka (Buchs, Suisse) et l'urée par Merck (Darmstadt, R.F.A.). L'acide γ-guanidinobutyrique nous a été aimablement préparé par le Prof. A. Brunel du Centre de Physiologie Végétale de l'Université Paul Sabatier de Toulouse. La N-carbamylputrescine et la N,N′-dicarbamylputrescine ont été synthétisées, par action du cyanate de potassium sur le dichlorhydrate de putrescine[3], au Laboratoire de Physicochimie Structurale du Prof. R. Carrié, grâce à l'obligeance de J. Hamelin (U.E.R. Structure et Propriétés de la Matière, Université de Rennes). Nous avons préparé le γ-guanidinobutyraldéhyde par transamination de l'agmatine sur l'acide α-cétoglutarique[4], en présence d'une suspension de poudre de jeunes plantes de *Soja hispida*; le protocole expérimental retenu est celui décrit par Guitton[5] lors de l'étude des transaminases des plantules de *Pinus pinea* L.

Techniques de séparation

La technique utilisée associe l'électrophorèse en haute tension à la chromatographie descendante sur papier; elle met en oeuvre la méthode de couplage décrite par Efron[6] qui consiste à coudre, sur une feuille de papier Whatman 3 MM, la bande intéressante de l'électrophorégramme.

L'électrophorèse est effectuée à l'appareil Phérograph, sur papier Whatman 3 MM, 57×35 cm, imprégné d'une solution tamponnée de pH 3.9 (pyridine–acide acétique–eau, 30:100:3870)[2]; le champ électrique est fixé à 45 V/cm. Une migration de 1 h 35 min permet de séparer les composés étudiés en trois groupes suivant leurs vitesses décroissantes de migration: (I) les polyamines, l'agmatine et l'arcaïne; (II) la N-carbamylputrescine, le γ-guanidinobutyraldéhyde et l'acide γ-guanidinobutyrique; (III) l'urée et la N,N′-dicarbamylputrescine.

Le premier groupe est repris en chromatographie dans le mélange *n*-butanol–pyridine–acide acétique–eau (4:1:1:2) pendant 24 h. Dans ces conditions, on sépare de façon satisfaisante l'arcaïne, l'agmatine, la putrescine, la spermidine et la spermine. De même, les constituants du second groupe sont isolés par une chromatographie de 14 h dans le solvant *n*-butanol–acide acétique–eau (12:3:5). Enfin, pour le troisième groupe où l'urée et la N,N′-dicarbamylputrescine sont superposées, une chromatographie de 14 h dans le mélange phénol–eau–ammoniaque (150:50:1) permet leur séparation.

Réactifs de révélation

Les polyamines, l'agmatine et la N-carbamylputrescine sont révélées par une solution de ninhydrine à 0.4% dans le mélange *n*-butanol–acide acétique (100:4) et chauffage à 100°. L'arcaïne, le γ-guanidinobutyraldéhyde, l'acide γ-guanidinobuty-

TABLEAU I

MOBILITÉS ÉLECTROPHORÉTIQUES (R_P) ET R_F DES DÉRIVÉS DE L'AGMATINE

R_P: mobilité électrophorétique par rapport à la putrescine. Solvant 1: *n*-butanol–pyridine–acide acétique–eau (4:1:1:2); solvant 2: *n*-butanol–acide acétique–eau (12:3:5); solvant 3: phénol–eau–ammoniaque (150:50:1).

Composés	*Électrophorèse* (R_P)	*Chromatographie* (R_F)		
		Solvant 1	*Solvant 2*	*Solvant 3*
Groupe I				
Putrescine	1.00	0.25		
Spermidine	0.93	0.17		
Spermine	0.87	0.11		
Agmatine	0.85	0.33		
Arcaïne	0.71	0.46		
Groupe II				
N-carbamylputrescine	0.52		0.40	
γ-Guanidinobutyraldéhyde	0.51		0.61	
Acide γ-guanidinobutyrique	0.29		0.53	
Groupe III				
Urée	0.06			0.74
N,N′-dicarbamylputrescine	0.06			0.97

rique et de nouveau l'agmatine sont localisés par le réactif au diacétyle-α-naphtol[7]. Enfin, l'urée, la N,N'-dicarbamylputrescine, mais aussi la N-carbamylputrescine, donnent une couleur jaune vif sur fond blanc avec le réactif d'Ehrlich modifié[8]. Tous les révélateurs sont utilisés par pulvérisation.

RÉSULTATS

Les mobilités électrophorétiques, par rapport à celle de la putrescine R_P, et les R_F des différents composés étudiés figurent dans le Tableau I.

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2 G. Biserte, P. Boulanger et P. Paysant, *Bull. Soc. Chim. Biol.*, 40 (1958) 2067.
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6 M. L. Efron, dans I. Smith (Rédacteur), *Chromatographic and Electrophoretic Techniques*, Vol. II, Heinemann, Londres, 2 ème éd., 1968, Ch. 5, p. 173.
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8 R. Zimmermann, *Naturwissenschaften*, 43 (1956) 399.

Author Index

Subject Index

Errata

J. Chromatogr., 93 (1974) 7–15

Page 9, eqn. 11 should read "$\vec{F}_B(\vec{r},t)=q\cdot n(\vec{r},t)\cdot\vec{E}(\vec{r},t)-\zeta\vec{J}(\vec{r},t)+F_B^{\text{ext}}(\vec{r},t)$".
The first line after eqn. 12 should read "where P_i is the ionic partial pressure, $\vec{\sigma}$ the internal frictional force tensor among the".

Page 10, eqn. 20 should read "$n(\vec{r},0)=N\left(\frac{\alpha}{\pi}\right)^{3/2}\exp[-\alpha r^2]$".

eqn. 21 should read "$n(\vec{r},t)=N\left(\frac{\alpha}{\pi}\right)^{3/2}\cdot\frac{1}{(1+4D\cdot\alpha\cdot t)^{3/2}}\cdot\exp[-\alpha(\vec{r}\mp\mu\cdot\vec{E}\cdot t)^2/(1+4D\cdot\alpha\cdot t)]$".

J. Chromatogr., 93 (1974) 251–252

Page 251, the last equation should read "$4\int_{\delta_{\text{min.}}}^{\delta}(1/\delta)\mathrm{d}V_\varepsilon=4\omega\int_{\delta_{\text{min.}}}^{\delta}(1/\delta)\mathrm{d}\delta=4\omega\ln(\delta/\delta_{\text{min.}})$".

N.B.: Present address: R. N. Nikolov, Applied Physical Chemistry Department, University of Saarbrücken, 6600 Saarbrücken, G.F.R.

Vol. 95 No. 2

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APPARATUS

N-487

SYSTEM FOR ANALYSIS OF LINEAR FRACTION COLLECTIONS

A system enabling analysis of linear fraction collections from Shandon Southern is suitable for applications where serial fractions need to be collected and comprises a linear fraction collector with controls, detectors, racks etc., together with an absorbance monitor for analytical purposes.

The system enables collection and recording of quantitative results, covering, *e.g.*, column chromatography, gradient centrifugation, or collection of radioactive samples into counting vials. The system allows collecting into large tubes up to 25 mm diameter. Collection from up to 380 tubes or 133 scintillation vials is also possible. A delay timer ensures that accurate collation between absorbance trace and collected fractions can be achieved.

The linear fraction collector counts drops, multiple siphon or volumeter discharges, or time intervals for each tube. Removeable racks move continuously, and in a timed operation, can collect from 2 columns simultaneously, each column filling half the available test tubes. Accurate tube positioning is achieved by an IR sensor.

Absorbance recording is carried out by a dual-beam optical unit matched to an absorbance monitor which has 8 full-scale absorbance ranges. These cover 0.01 to 2.0 A with maximum sensitivity of 0.0001 A, typical noise level being 0.00005 A above or below the base line.

The unit can record %T when no information regarding solute mass is required. The highly compressed concentration scale at high concentration prevents large peaks from going off scale, while fairly small peaks still have adequate height. 8 Recorder speeds from 1.5 to 300 cm/h provide proper rates for slow or fast columns.

N-482

GC DETECTOR

Tracor's Model 310 Hall Electrolytic Conductivity Detector is designed for sensitive and specific detection of nitrogen, chloride and sulfur containing compounds. Providing sensitivities of 0.01 ng for N, Cl, or S, the Model 310 detector offers a linear range $> 10^5$.

Characteristics include small size and single package construction which includes all required electronics, miniature furnace and cell design of Teflon and stainless steel.

N-490

PORE GRADIENT GEL ELECTROPHORESIS

"Isolines", Vol. 3, No. 1, a periodical from Isolab describes their Gradipore® electrophoresis system using polyacrylamide gels with pores of continuously decreasing size for analysis of blood and serum proteins, lipoproteins, peptides, hemo–haptoglobin complexes, antigens, cellular extracts, nucleic acids and isoenzymes. The issue contains information on equipment and accessories and presents a related bibliography.

N-491

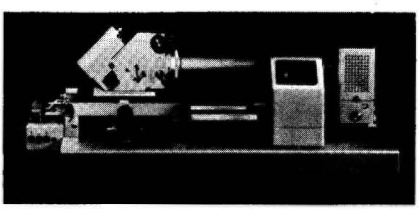
ROUTINE PHOTOMETERS

Carl Zeiss announce their PM-6 Spectrophotometers for routine purposes. Three types cover 3 different ranges, having the dual monochromator (200–800 nm, bandwidth 2 nm) and automatic zero-adjustment of the concentration in common. The apparatus measures 12 samples and prints out their concentration, date and sample number in 80 sec.

N-486

TLC SPECTROPHOTOMETER

A TLC Spectrophotometer equipped for routine work in laboratories for clinical and food chemistry from Carl Zeiss works either in the reflectance, or in the transmittance, the simultaneous reflectance/transmittance or the fluorescence mode. Spectral measurements down to 195 nm are possible. It takes less than 5 min to adjust the instrument and scan 10 spots (*e.g.* 4 standards and 6 samples). The photometric and mechanical reproducibility of the instrument is better than 0.3%. If the chromatographic conditions are good, a total reproducibility of 1.5–3% is obtainable, including application errors. The detection limit is 10^{-8} g in the reflectance mode, and 10^{-10} g to 10^{-11} g in the fluorescence mode. A comprehensive compendium of literature and an ample selection of applications in pharmacology, food chemistry, clinical chemistry, biochemistry, and environmental control has been compiled by Zeiss.



CHEMICALS

N-474

BIOMATERIAL SUPPORT

Electro-Nucleonics announces the availability of controlled-pore glass in a high flow rate, 20/80 mesh particle size for use as a biomaterial support. The product offers a large internal surface area of controlled access for attaching and immobilizing biologicals and catalysts. It is available in the following pore diameters: 170, 240, 500, 1400, 2000 and 3000 Å. Exclusion limits and surface areas are given in a 4-page product brochure.

N-481

GC KIT FOR AMINO ACID ANALYSIS

Gallard-Schlesinger introduce a kit for amino acid analysis by derivatization gas chromatography. N-acetyl amino acid *n*-propyl-esters are prepared in a two-step procedure. Reagents, solvents, standards and descriptions of procedures are supplied with the kit.

N-492

PIERCE PREVIEWS

Pierce Previews, April 1974, contains information on their following products:

PC-3210 Ultraphase™, a liquid silicone phase for GC columns, available on Chromosorb W(HP) 80/100 mesh and designed for drug screening and assay; several reagents for biochemical analysis; Reacti-Therm Heating Module, a reaction heater for vials.

PROCEDURES

N-496

AMINO ACID ANALYSIS

Vol. 1, No. 1 of 'Analysis', a publication from Hamilton, comprises an article entitled "Hydrolyzate *versus* Physiological Amino Acid Analysis" by J.V. Benson, Jr..

N-478

QUANTITATIVE TLC

A revised listing of a selection of technical papers that deals with quantitative thin layer chromatography is now available from Schoeffel. This collection of technical papers describes separation techniques with a spectrum of compounds and contains many unique, original insights. Drugs, steroids, amino acids, lipids and sugars are just a few of the topics covered. Some of the other techniques discussed include: spraying, impregnating TLC plates, correcting errors in TLC, separation methods simplified and rapid scanning methods.

NEW BOOKS

Choline and acetylcholine: handbook of chemical assay methods, edited by I. Hanin, Raven Press, New York and North-Holland, Amsterdam, 1974, xii + 234 pp., price Dfl. 48.00 (about US$ 17.50).

Amino acids, peptides and proteins, biochemical and immunochemical techniques in protein chemistry, by T. Dévényi and J. Gergely, Elsevier, Amsterdam, London, New York, 1974, 343 pp., price Dfl. 80.00 (about US$ 29.10).

The packed column in gas chromatography, by W.R. Supina, Supelco, Bellefonte, Pa., 1974, vi + 166 pp., price US$ 12.00 (50% discount on orders of 20 or more copies by educational institutions).

Modern methods of steroid analysis, edited by E. Heftmann, Academic Press, New York, xxii + 524 pp., price US$ 37.50.

Standardisation of gas chromatographic analysis of essential oils, by H. van den Dool, Thesis, State University of Groningen, 1974, 172 pp.

CALENDAR OF FORTHCOMING MEETINGS

August 26–31, 1974
Warsaw, Poland

IVth Polish Conference on Analytical Chemistry

Contact:
The Secretary of the Organizing Committee, Dr. hab. R. Dybczynski, Institute of Nuclear Research, ul. Dorodna 16, 03-195 Warsaw, Poland
(Further details published in Vol. 82, No. 2)

September 2–5, 1974
Milan, Italy

3rd International Symposium on Isoelectric Focusing and Isotachophoresis

Contact:
Prof. P.G. Righetti, Dept. of Biochemistry, University of Milan, Via Celoria 2, Milano 20133, Italy

September 2–7, 1974
Milan, Italy

12th International Congress for Fat Research

Contact:
12th ISF Congress, Secretarial Office, *c/o* Stazione Sperimentale, Oli e Grassi, Via Giuseppe Colombo 79, 20133 Milan, Italy

September 9–October 14, 1974, Basel, Switzerland

Internationale Fachmesse für Laboratoriums- und Verfahrenstechnik, Messtechnik und Automatik in der Chemie (ILMAC 74)

September 9–12, 1974 Prague, Czechoslovakia	**Conference on Analytical Chemistry of the Environment (Interan)** Contact: House of Technology SVTS, 01180-Zilina-Hliny, Czechoslovakia (Further details published in Vol. 88, No. 2)
September 24–27, 1974 Vienna, Austria	**Symposium on Computers in Analytical Chemistry** Contact: Prof. J.F.K. Huber, *c/o* Intercongress, Stadiongasse 6–8, A-1010 Vienna 1, Austria (Further details published in Vol. 98, No. 1)
September 30–October 4, 1974 Barcelona, Spain	**10th International Symposium on Chromatography** Contact: Director of the G.A.M.S., 10, rue du Delta, 75009 – Paris, France (Further details published in Vol. 81, No. 1)
October 27–November 1, 1974 Buenos Aires, Argentina	**8th Panamerican Congress on Endocrinology** Contact: Dr. C. Bergadá, Octavo Congreso Panamericana de Endocrinologia, Cas. Correo No. 2593 C. Central, Buenos Aires, Argentina
November 4–7, 1974 Houston, Texas, U.S.A.	**Ninth International Symposium on Advances in chromatography** Contact: Prof. A. Zlatkis, Chemistry Department, University of Houston, Houston, Texas 77004, U.S.A. (Complete program will be published in Vol. 96, No. 1)
November 18–22, 1974 Atlantic City, N.J., U.S.A.	**Federation of Analytical Chemistry and Spectroscopy Societies, 1st Annual Meeting** Contact: J.G. Grasselli, Standard Oil Co. (Ohio), 4440 Warrensville Ctr. Rd., Cleveland, Ohio 44128, U.S.A.
March 3–7, 1975 Cleveland, Ohio, U.S.A.	**26th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy**
June 1975 Knoxville, Tenn., U.S.A.	**28th Annual Summer Symposium on New Horizons in Analytical Spectroscopy** Contact: J. Winefordner, Dept. of Chemistry, University of Florida, Gainesville, Fla. 32601, U.S.A.
October 6–10, 1975 Indianapolis, Ind., U.S.A.	**Federation of Analytical Chemistry and Spectroscopy Societies, 2nd Annual Meeting** Contact: J.G. Grasselli, Standard Oil Co. (Ohio), 4440 Warrensville Ctr. Rd., Cleveland, Ohio 44128, U.S.A.

PUBLICATION SCHEDULE FOR 1974

Journal of Chromatography (incorporating *Chromatographic Reviews*)

MONTH	J	F	M	A	M	J	J	A	S	O	N	D
JOURNAL	88/1 88/2	89/1 89/2	90/1	90/2 91	92/1 92/2	93/1 93/2	94 95/1	95/2 96/1	96/2	97/1 97/2	99 100/2 100/1	
REVIEWS *			98/1						98/2	98/3		

* Volume 98 will consist of *Chromatographic Reviews*. The issues comprising this volume will not be published consecutively, but will appear at various times in the course of the year.

GENERAL INFORMATION

(A leaflet *Instructions to Authors* can be obtained by application to the publisher.)

Types of Contributions. (a) Original research work not previously published in a generally accessible language in other periodicals (Full-length papers). (b) Review articles. (c) Short communications and Notes. (d) Book reviews; News; Announcements. (e) Bibliography of Gas Chromatography, Column Chromatography, Paper Chromatography, Thin-Layer Chromatography and Electrophoretic Techniques. (f) Chromatographic Data.

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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 R. D. Marshall and A. Neuberger, in A. Gottschalk (Editor), *Glycoproteins*, Vol. 5, Part A, Elsevier, Amsterdam, 2nd ed., 1972, Ch. 3, p. 251.

Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts*.

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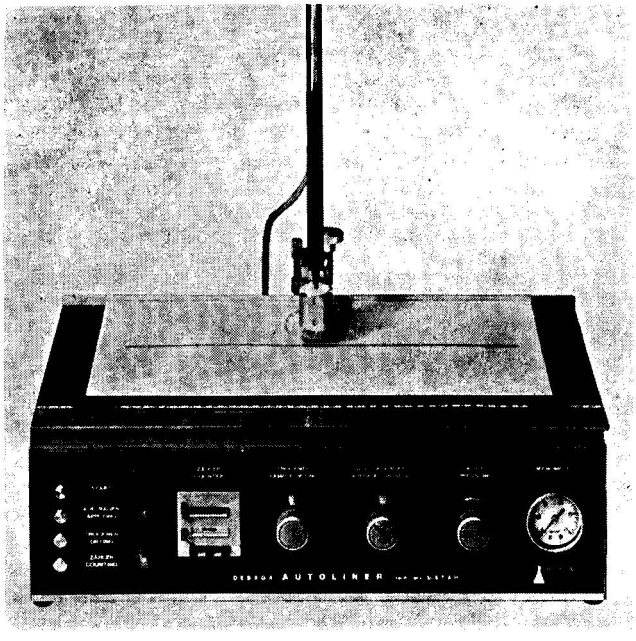

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