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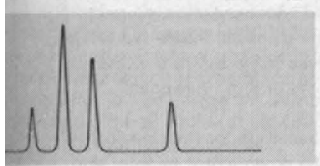

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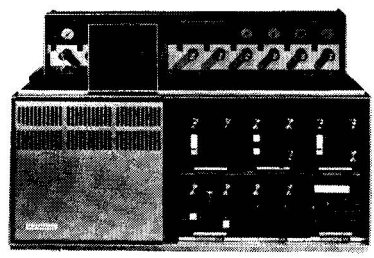

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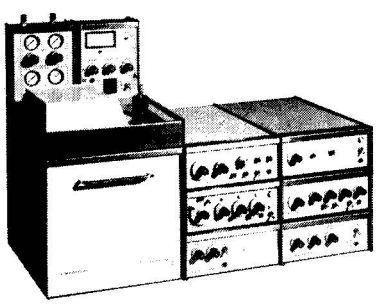

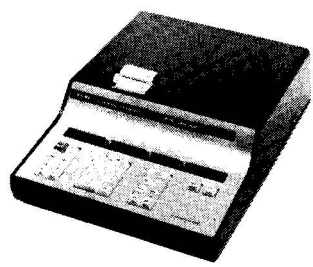

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Pharmaceutical applications of Thin-Layer and Paper chromatography

edited by **KAREL MACEK,** *Medical Faculty, Charles University, Prague*

1972, xvi + 744 pages, Dfl. 250.00
ISBN 0-444-40939-4

Chromatography is the most widely used modern procedure in analytical chemistry today. With an increasing awareness of the importance of the applications of paper and thin-layer chromatography in the fields of pharmaceutical research, production and control, it has become necessary to survey the possibilities of these methods with their associated literature, and to present this information in a useful form.
With this in mind, the editor provides an introduction to the techniques, the evaluation, and the applications of paper and thin-layer chromatography. Selected procedures such as the preparation of samples, detection methods, choice of solvent systems and sorbents, and the principles of quantitative analysis, are discussed generally and the book is richly tabulated. An appendix outlines the preparation of more than 150 detection reagents. On the basis of these data, readers with an understanding of the principles of the techniques described can solve any analytical problems they may encounter in drug analysis.

CONTENTS: Introduction. Techniques of paper and thin-layer chromatography. Radioactive compounds. Combination of TLC and PC with other chromatographic techniques. Combination of PC and TLC with some spectroscopic methods. Identification of organic compounds by PC and TLC. Documentation of chromatograms. Laboratory for PC and TLC. The tasks of paper and thin-layer chromatography. Synthetic drugs. Steroids. Cardiac glycosides and their genins. Saponins. Peptide and protein hormones. Alkaloids. Vitamins. Antibiotics. Plant extracts. Auxiliary compounds. Investigation of the fate of drugs. Detection reagents. Author index. Subject index. List of substances chromatographed.

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ELECTROPHORETIC THERMAL THEORY

I. TEMPERATURE GRADIENTS AND THEIR EFFECTS

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(Received April 22nd, 1974)

SUMMARY

The effects of radial temperature gradients in circular-section columns and central-peripheral gradients in rectangular and annular colums, and lateral temperature gradients in both, normal to the electric field, are considered, taking into account the synergistic effects of gradients of resistivity due to negative temperature coefficients of resistivity of electrolyte solutions. Longitudinal temperature gradients and their interaction with radial temperature gradients in the steady and unsteady states are discussed. Consequences for distortion of separand bands, and limitations on apparatus design and choice of conditions are stated. Two methods of unlimited preparative scale-up are explained.

INTRODUCTION

In electrophoretic columns the exterior of the column wall may be well cooled and the coolant temperature controlled by a good thermostat. But even if the column wall is very thin and of high thermal conductivity, the wall and column lumen have finite thickness, and there are temperature gradients both across the wall and from center to periphery of the lumen. Such temperature gradients in the lumen due to Joule heating have been calculated[1,2,22] as parabolic in the steady state. But that approach makes the simplifying assumption that the column lumen is a resistor of zero temperature coefficient of resistivity, which is not the case for aqueous electrolytes, whose resistance decreases by some 2%/°C. This means that the warmer center of the column will carry a higher current density than the periphery and the overall resistance will fall. For a given power dissipation, correction of temperature gradients to allow for this gives higher gradients, which are no longer parabolic[3,4].

While low wattages per unit length of column may involve a small correction, the error may approach 50% at higher levels with resultant increase of band distortion, convection, and denaturation of separand substances. Similar arguments apply to lateral and longitudinal temperature gradients. The interaction of the latter in steady and unsteady states becomes complex[5,6]. The effects of temperature gradients

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are different in zone methods, isoelectric focusing[7] and displacement methods[8,9] such as transphoresis[10] and isotachophoresis[10,11]. These considerations, and results of calculations in the following papers[12–14], were briefly reported in 1971[3] at a symposium.

Two methods of unlimited preparative scale-up emerge[6,15]. Lateral extension of flat-section and annular columns is possible. For transphoretic methods, an unsteady-state method is possible with any column section, particulary with counterflow.

TEMPERATURE GRADIENTS

Central–peripheral temperature gradients

Consider a uniform column of aqueous electrolyte perfectly cooled and thermostated at its exterior surface and imagine it to have an infinitely thin retaining wall. If the column is very long and communicates with large electrode vessels at either end, which pass a constant current, then in the steady state a temperature gradient will exist from center to periphery, longitudinally uniform at midpoints far from the ends. If we ignore or suppress thermal convection and other disturbing factors such as electro-osmosis and assume there are no internal solvent flows, this gradient will have bilateral symmetry for rectangular section columns and radial symmetry for columns of circular section. Annular section columns, where there is a large radius to radius-difference ratio, approximate to rectangular section columns where the shorter sides of the rectangle have been removed.

This steady-state gradient will be parabolic[1,2] for resistors of zero temperature coefficient of resistivity and it is conceivable than an electrolyte system of special dynamic properties could be devised to conform with this, or even have a positive coefficient to reduce the parabolic effect, which is the case with most metallic resistors. Most aqueous electrolyte solutions resemble certain solid resistors, such as graphite and thermistor materials, in having a negative coefficient, becoming less resistive when hotter, which is the case for electrophoretic media. In the case of buffer electrolytes whose pK is temperature-sensitive, and saturated solutions in the presence of undissolved solute, the effective temperature change will be further altered. A special case is that of colloidal electrolytes[16]. Treating the simple case of a dilute salt, such as potassium chloride, and ignoring differential change of hydration and transport number, we may assume a change of resistivity of arbitrarily 2%/°C. Thus, central equivalent shells of electrolyte coaxial with the column will be less resistive than peripheral cooler shells and may be regarded as parallel but different resistors between and normal to two planes of different potential which provide the electric field, these shells being in electrical contact with adjacent shells and adjacent parts of themselves. The column may therefore be considered as divisible into any number of equipotential planes normal to all lines of current intensity. The more central resistive shells will carry more of the current density following the temperature gradient, which also becomes a gradient of ion speed since lowered resistance is due to higher mobility. Since power dissipation is dependent on the product of current and potential gradient, the power dissipation gradient follows the current density gradient. Since the higher central dissipation is further from the peripheral heat-sink, there will be a further temperature rise in central regions, leading synergistically to further increase of the current density and power dissipation gradients. At the steady state this tendency is exactly opposed by the increased heat loss by conduction through outer shells of so-

lution due to the increased temperature gradient. The calculation of these steady-state gradients involves the use of Bessel functions[3,12–14] and is similar to Jakob's treatment of negative coefficient resistors[17] if a correction having the effect of Dusinberre's second correction[18] for current density gradient is written in. By rewriting the temperature coefficient of resistance, solutions containing the square root of -1 are avoided[19], and the equations become amenable to digital computation. Derivation of these equations and a computer program for same are given in the following papers[12–14]. Those calculations do not take into account the palliative effects of the gradient of thermal conductivity of the column solution (Dusinberre's first correction[18]), which could be written in without difficulty, though the error is small[20].

The computed results have been graphically plotted (Fig. 1), using a thermostat temperature of 4°, for circular and rectangular section columns whose side ratio is 10:1. Curves for various ratios of internal and external diameter, assuming walls of fused quartz, have also been plotted for use in practical cases. Since, in common with the simple parabolic case, the absolute dimensions are immaterial and only the wattage per unit length and geometry of section is important, the central temperature

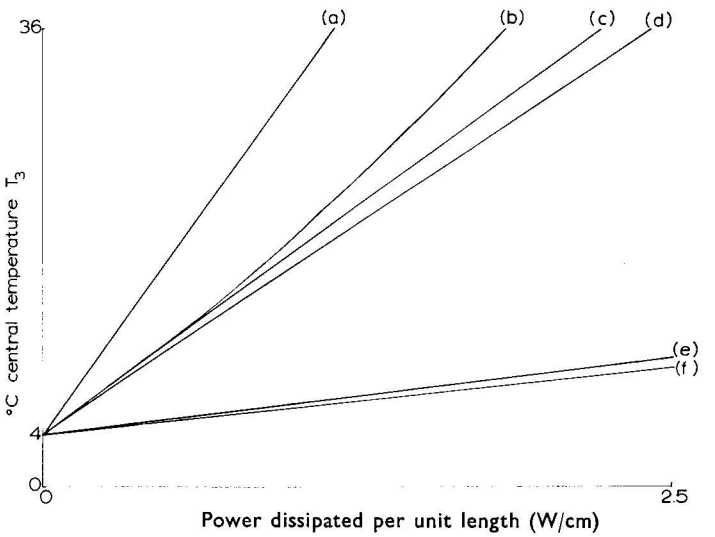

Fig. 1. The graph shows curves of temperature rise at the centre of an electrophoretic column, for a perfectly cooled and thermostatted exterior of 4° for various power dissipations per unit length of column from 0–2.5 W/cm. Curve (a) is for a filling of negative temperature coefficient of resistance electrolyte in a tube of outer to inner diameter ratio of 3.0. Curve (b) is similar, but the diameter ratio is 1.0, that is a wall-less tube. The wall is fused silica, the solvent water, and a 2% rise of mobility is assumed per °C, such as might be approximated by dilute potassium chloride. The tube section is circular. Curves (c) and (d) have the same different diameter ratios as (a) and (b), respectively, but are for an electrolyte of zero coefficient, which is the parabolic case. Curve (e) is for a flat section tube (rectangle side ratio of 10:1) with a negative coefficient as previously, and a similar wall material. The total thickness to lumen thickness ratio is 1.2. Curve (f) is for a wall-less lumen of similar rectangular section side ratio. At this scale, and at this wattage range, the zero and negative coefficient cases virtually coincide. These curves were computed for the steady state, and the above are a few of many such too numerous to reproduce usefully on this scale. The wattages are real wattages, after correction for temperature and current density gradients, *i.e.* those to be found from measurement of voltage and current in practical cases, not the nominal ones obtained by calculation from cold resistivities. T_3 is the central temperature, as listed and used in refs. 12–14.

increase is obtainable for any system in which the nominal or effective wattage is known or calculable. Thus, the temperature rise in a tube of similar geometry of section is the same if the sectional area is ten times larger and the electrolyte diluted appropriately (roughly ten times). The graph shows that the error in using the simple parabola method increases non-linearly with wattage at high powers for circular section tubes. Thick walls produce larger errors, and have a greater effect on rectangular section tubes, and therefore annular ones. The more favorable configuration is clearly rectangular or annular, as in the parabolic case.

The unsteady state, where there is progressive warm-up of the column resistor, or cooling on reduction of the electric field strength, may also be treated by Bessel functions[21], a requirement for the parabolic theory, and becomes more complex when the negative coefficient is written in. The general form of the rise in temperature and fall of resistance with time of the whole column on application of constant current may be obtained from a potentiometric detector in the column measuring the change of potential difference at two close points[5], or with a point thermal detector[8,9] or by measuring the overall voltage drop across the tube, and involves the thermal capacity of the column and lumen. The curves resemble those of Taylor for solid resistors[23], and those for passage of an ionic interface in displacement electrophoresis[8]. For negative coefficient resistors it is clearly prudent to use current-regulated power supplies, and voltage-regulated ones for positive resistors to avoid overheating if the cooling is inadequate.

The non-parabolic temperature-distance steady-state radial temperature gradients, which we have computed digitally for wall-less tubes, have further uses. If a smaller section of the tube is taken, by removal of a peripheral shell of lumen, this gives the radial gradient for a higher thermostat temperature at a lower wattage. For the zero coefficient case this wattage is directly proportional to the area of the section, and in negative coefficient cases is obtainable from integrals of temperature (current density) and area. Annular and rectangle-difference sections of the temperature profiles also apply to cases where the inner and outer lumen walls are at different temperatures, and inner walls supply heat at the rate of the (removed) lumen centre.

Longitudinal temperature gradients

These arise when there is relative movement of lumen contents and of a change of power dissipation and is a particular case of non-uniformity of column. Other non-uniformities of column may be due to cooling irregularities and construction of apparatus, including the case of a detector which interferes with the cooling locally. There are two types of relative movement of lumen and dissipation, which may also be combined, and may interact with other non-uniformities. First, if there is a flow of solution in the column, there is addition at one end of unsaturated thermal capacity. This leads to a linear longitudinal temperature gradient in the absence of cooling. If cooling of the lumen periphery is added, the longitudinal gradient is hybridised with unsteady-state radial gradients, and isothermal surfaces become a train of figures of revolution of different curves until the thermal capacity is saturated, when these surfaces become the coaxial cylindrical shells of the steady state. This applies to the use of counterflow of solvent in electrophoresis[24]. In the second case, there is a traveling change of resistivity of the column resistor. In the absence of cooling there will be a change of the rate of temperature rise proportional to the resistance change,

which will become more complex and change continuously if the resistance change varies along its length. If peripheral cooling is added, combination with radial gradients gives a similar train of isothermal surfaces as in the first case. Examples of a moving change of resistance are found in zone electrophoresis[25], and displacement methods such as transphoresis and isotachophoresis, and therefore in the steady-state stacking stage of disc electrophoresis[26,27]. In such cases, calculation of the unsteady-state radial temperature gradients at various depths gives the combined longitudinal and radial gradient if distance is substituted for time in the constant-current mode. Use of sheets of encapsulated cholesteric thermochromic liquid crystals across the lumen may render such gradients visible.

Lateral temperature gradients

If there is a difference of cooling or heat dissipation towards one wall of the column, bilateral and radial symmetry no longer apply, and the regions of higher temperature, power dissipation, and migration speed are shifted in the direction of the hotter side. This may arise at inflexions of an electrophoretic tube, where the inner part of the inflexion is a shorter and more used current path, for which Martin and Everaerts suggested thermal banking compensation[8], using a lateral externally imposed temperature gradient to counteract it. It may also occur in free solution electrophoresis where the separand is significantly different in density to adjacent solution and is relatively displaced to one wall of the tube by gravity[6]. Such displacement is seen in zone electrophoresis of proteins and particles and may be relieved by rotation of horizontal columns[2]. This has also been seen in transphoresis[6,28].

APPLICATION OF TEMPERATURE GRADIENTS TO ELECTROPHORESIS

In zone electrophoresis, isoelectric focusing, and displacement methods, radial temperature gradients, as calculated with correction for current-density gradients induced by Joule heating, indicate that previous calculations of central temperature rise underestimate its size. This means that zones will bow more than previously estimated and the bow will not be parabolic. If the resistance difference between separand and buffer electrolyte is negligible, previous theory of zone bowing[29] will require further refinement. If there is a resistance difference, theory probably becomes too complex to be useful, as unsteady longitudinal gradients will be superimposed. The graph in Fig. 1 shows that rectangular and annular section tubes should be even more favorable thermally than was previously thought to be the case[1], for high power dissipations, a conclusion particularly applicable to preparative-scale electrophoresis in wide columns.

In isoelectric focusing the radial temperature gradients will require more convective stabilization by density gradients or viscous colloids than simple theory would indicate, and progressive denaturation at the center would be more rapid by virtue of the higher temperature. Increased theoretical migration speeds would increase separand return to the isoelectric band as much as diffusion from the bands would increase, but if the generation and stability of ampholyte regions is not altered the system is largely self-stabilizing.

Inflected columns, causing spreading of separand over a greater length of column due to differential migration speeds, become less favorable still and should

be avoided, as should column constrictions and dilatations, being antiseparative in all cases. Similar arguments apply to local cooling imperfections. Use of one discontinuity to oppose another may be palliative,[8] but is difficult to arrange really well.

In transphoresis radial temperature gradients give rise to a stable bow of the ionic interface, rather than progressive bowing found in zone methods, and the three-dimensional interface structure must therefore have properties which are self-restorative[6] in ways other than its already stated control of diffusion[8], since a current density gradient would involve faster migration of central compartments. This bow limits the accuracy of analytical quantitation by a high-resolution detector since the volume of the bow is not easily ascertainable, except empirically[5]. The longitudinal temperature gradient in transphoresis would be unimportant if there was no change of the mobility ratio of co-ion and counter-ion with temperature. But since most analysand ions of interest are complex and require buffering counter-ions, there is indeed a temperature effect and therefore a progressive change of Kohlrausch-regulated[30] concentration of the following separand compartment, through the temperature gradient, impairing quantitation. This is complicated by interaction of longitudinal and radial temperature gradients, whose unsteady state treatment is complex.

In isotachophoresis, where a co-running train of continuous mobility spectrum spacer ampholytes migrate at the same speed as the separand[5], temperature gradient alteration of concentration will be added to already existing pH and concentration gradients and the effects of these ampholytes on quantitation and resolution can only be studied empirically.

Thermometric effects will also increase for unsteady states, such as beginning and end of an electrophoretic run, unless solutions are preheated to start with to a temperature matching the mean volume temperature of the column contents, with adjustment for differential wall expansion, and kept at the run temperature at the end, by substitution of a.c. for the electrophoretic d.c. current. Judicious choice of tube wall material, or composite walls may help. Suppression of thermometric effects, until quantitation or sample removal, is desirable for accuracy and controlled work, and to prevent remixing of regions due to relative fluid or gel movement. Working at about 4°, the expansion plateau of water, is desirable for thermometric and convective reasons, and the graph of Fig. 1 has therefore been based on that temperature.

If the pK temperature sensitivity of the system, the pH or the gel properties can be so chosen as to minimise the apparent effect of temperature on separand net mobility, or even reverse it, then thermal limitations are less severe, and bowing is not a problem.

Such reversal of bow for proteins in agar gels is discussed by Wieme[31], particularly for hemoglobin. Similar phenomena have also been seen in transphoresis and isotachophoresis[6,32,33] with some proteins and dyes, using agarose and polyacrylamide. It also occurs with Ampholines (LKB)[34], and whatever their mechanism such effects appear to require the presence of gel, and seem dependent on the way it is prepared and handled. Trailing effects of weak anions in agar gels was noted by Kendall *et al.*[35] in pioneer work on transphoresis.

UNLIMITED SCALE-UP FOR PREPARATIVE PURPOSES

For preparative purposes, the use of thermally favourable rectangular or

circular section columns suggests methods of almost unlimited scale-up of production capacity. Thus the rectangular section, whose width is far greater than its thickness, may be compacted into a corrugated or spiral section. The annulus may be of very large internal radius or may be likewise convoluted to render the coolant facility more manageable. A minor disadvantage may be the more elaborate introduction of sample in non-circular sections in zone methods. For transphoretic and focussing methods this is less troublesome as these systems are self-correcting, the former particulary with counterflow of leader electrolyte[15].

This leads to another concept of cooling which may allow major scale-up of column bore of any section, for preparative or other purposes. In transphoretic methods, the longitudinal temperature gradient due to separand compartments, considered in a frame moving with the interface, depends on the power dissipation per unit length and the rate of presentation of unsaturated thermal capacity of tube and contents. If the latter can be increased per watt at a given migration speed, by diluting the system and enlarging the bore in proportion, then it may be possible to run the separand train in that part of the column which suffers only the initial small portion of the temperature gradient. This use of unsteady-state gradients may be extended in the case of balancing counterflow (which is meaningful only in transphoretic methods) to the longitudinal gradient in the leader and separand, where the separand may occupy most of the column, with commensurate power saving per unit of product. Since this is now an internal form of cooling, the walls may be insulated, and radial gradients abolished, so there is no longer a thermal restriction of bore, but one of length. In practical apparatus this suggests use of transphoretic three-dimensional interface self-correction to maintain flat bands, and fine woven monofilament flow-smoothing meshes, single or duplex, at either end of a wide, short column, to induce a substantial element of plug flow and suppression of the Stokes parabolic flow profile. The system is self-stabilising for a given voltage and a wide range of constant counterflow: Increase of voltage merely shifts the position of the bands, as does a small change of counterflow. Using a voltage-stabilised power supply, a too low counterflow shifts the bands to the leader end of the column, current and potential gradient are reduced, and a new stable position is found. The convenience of such a system for intermittent introduction and removal of separands using stationary plumbing is attractive[15]. Apparatus has been constructed using these principles, and will be the subject of a future report. Methods using radial fields and counterflows with transverse flows, or radial transverse flows in annular section columns, have been described for continuous preparative use[15].

A third method, which is restricted to batch production, should be mentioned. Brownstone[36] did zone electrophoresis in a gel cylinder whose side walls were poorly cooled, the heat loss being more from large end-faces[37], and found reduced radial temperature gradient profiles. This principle is also implicit in the methods described above, where both electrode vessels and their flows may be cooled and thermostatted. In zone electrophoresis there is no self-correction of zone-bowing, unless a sieving or conductivity[19,40] gradient is introduced. Difficulties of removing sample from the gel remain.

ACKNOWLEDGEMENTS

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draws on prior work of Transphoresis Co., London, Great Britain. Thanks are due to D. H. Jones, who urged the use of digital computation, programmed and computed values, from which Fig. 1 was plotted with the help of J. F. Brown, who checked values manually. Thanks are due also to R. B. Hall for advice and arranging access to computing facilities through Brunel University.

NOTE ADDED IN PROOF

Temperature gradients were measured by Porath[22], using particulate columns for which correction factors were applied. Conditions and materials used make comparison with our results difficult. Close correspondence with calculated parabolas was found, though the difference of shape between those and our Bessel curves for circular sections may not be easily detectable thus. Indirect confirmation may be obtainable using say a thick thermistor rod. However, measurements in electrolytes require reduction of convection. Very uniform gels of known controlled properties are a possibility. Centrifugal stabilization by rotation about the column long axis might be tried. Another solution would be to use the effectively zero gravity of spacecraft, such as NASA's projected Shuttle, for which continuous transphoretic preparators have been proposed[15]. It is hoped to present experimental work later.

The work in this and the following papers[12–14] was done in London in 1971–1972, and briefly reported then[3] and later[4–6]. Its purpose was to help specify design and performance limits of transphoresis[4–6] (and isotachophoresis[4–6,11]), as opposed to zone separators[44], to aid calculation of stable and incipient three-dimensional shapes of transphoretic ionic interfaces[6], to be of use secondarily in direct method transport number determination[10], and generally for negative coefficient resistors and analogous systems. Such considerations also underlay the author's endorsement of transphoresis as the method of choice in space[15,43]. The work we now present was therefore circulated in 1973 for study and use to former colleagues, who endorsed and extended it, as outlined elsewhere[32], to comprehend the unsteady state[5,6], in two more recently submitted papers[38,39], likewise on both circular and rectangular section columns. While many basic features are common to the two treatments, readers may wish to study other significant apparent differences of inference revealed by their later work, in order to reconcile our and their approaches and tacit assumptions as best they may. Thus for the same central temperature rise, in circular and rectangular cases, we did not find that increased relative wall thickness gave higher power dissipations, nor that internal radius or dissipation depended on voltage gradient alone nor that there was a maximum lumen section (since we purposely left concentration and resistivity unspecified[3]), nor that dissipation was less in rectangular than in circular sections. We did not apply the field strength determinant[5,8,41,42] of unidimensional interface sharpness[8,41,42] directly to the real three-dimensional case[5,6,43], nor did we equate stable interface convexity with ionic velocity gradients of a uniform electrolyte column. Their use of the unsteady state for rapid separation is implicit in methods discussed herein and elsewhere[6,15], and relates to Hjertén's similar proposals for zone electrophoresis[2]. While thick walls may confer some useful gain in electrical efficiency, by acting as thermal insulators and thereby raising mean lumen temperature and conductivity, it would seem preferable to achieve this with thin walls and a higher coolant temperature, particularly in the thermally discontinuous systems of transphoresis and isotachophoresis.

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ELECTROPHORETIC THERMAL THEORY

II. STEADY-STATE RADIAL TEMPERATURE GRADIENTS IN CIRCULAR SECTION COLUMNS

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SUMMARY

Central-peripheral temperature gradients are calculated for circular section columns of uniform bore, filled with electrolyte, and carrying current, in the steady state. Wall thickness and material are allowed for. Cases of electrolytes or fillings having zero, positive, and negative temperature coefficients of resistivity are calculated, correction being made for non-uniform current density, using Bessel functions. For negative coefficients, the results differ substantially from simple parabolic theory, gradients being progressively higher than the latter at high heat dissipations.

INTRODUCTION

Central-peripheral temperature gradients have been calculated previously, assuming a zero temperature coefficient of resistivity, or uniform density of current through the lumen section. In this paper these gradients are recalculated without these simplifying assumptions, giving substantially different results, in which, for negative coefficients, gradients are progressively larger for higher power dissipations than simple parabolic theory indicates. This is particularly so for increased wall thickness. Similar calculations for rectangular section columns are dealt with in an accompanying paper[1]. A discussion of the anticipated effects of these gradients in electrophoresis, and a comparison of results of digitally computed gradients, in the context of factors determining ideal column shapes and thermal properties is given in a preceding paper[2]. A further paper[3] describes programming and digital computation of the gradients calculated here and in ref. 1. The results of this work were briefly

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reported at a symposium in 1971, by the second author[4], at whose instigation this project was undertaken.

This treatment of radial temperature gradients was undertaken in order to determine the thermal limitations of columns in transphoretic[5] and isotachophoretic[6,7] displacement[8] electrophoretic methods, both for analytical[9,10] and preparative applications. A second purpose was to lay a basis for calculation of hybrid radial and longitudinal temperature gradients[6] in the steady and unsteady states, and thus of the three-dimensional ionic interface bow in these methods, using current-line and field distortion stabilization theory[11]. These calculations have subsequently been extended to unsteady states by the second author's former colleagues[15], for the same purposes, under a NASA contract to investigate the displacement method for the preparative separation of cells in zero gravity, the subject of a recent experiment in Skylab[12]. A previous paper deals with the general application of these gradients to electrophoresis[2].

THEORY

For an electrolyte of zero temperature coefficient of resistivity[2]

Consider a circular section column filled with electrolyte. Assume it to be long enough to neglect end effects in a medial region. Assume heat to be uniformly generated in the electrolyte, and that there is no convective or other fluid movement. Assume a zero temperature coefficient of thermal conductivity and of resistivity. Assume the column exterior to be perfectly cooled and thermostatted, and that the system has reached a steady state. Using the listed symbols, which refer to the column section normal to the long axis,

$$\frac{1}{r}\frac{\mathrm{d}}{\mathrm{d}r}\left(r\frac{\mathrm{d}t}{\mathrm{d}r}\right)+\frac{W_0}{k_1}=0$$

for which the general solution is

$$t = A + B\ln r - \frac{W_0 r^2}{4k_1}$$

where A and B are integration constants, whence

$$T_2 = A - \frac{W_0 R_2^2}{4k_1} \tag{1}$$

which gives the temperature at any point in the column, the equation being a parabola. For the tube wall,

$$W_0 R_2^2 \ln\left(\frac{R_1}{R_2}\right) = -2k_2(T_1 - T_2)$$

So

$$T_2 = T_1 + \frac{W_0 R_2^2}{2k_2}\ln\left(\frac{R_1}{R_2}\right)$$

and

$$A = T_1 + \frac{W_0 R_2^2}{4 k_1} + \frac{W_0 R_2^2}{2 k_2} \ln \left(\frac{R_1}{R_2}\right)$$

Substituting in eqn. 1 above,

$$t = T_1 + \frac{W_0}{2} \left[\frac{R_2^2 - r^2}{2 k_1} + \frac{R_2^2}{k_2} \ln \left(\frac{R_1}{R_2}\right)\right] \tag{2}$$

For positive temperature coefficients[2]

$$\frac{d^2 t}{dr^2} + \frac{1}{r} \frac{dt}{dr} = - \frac{W_0}{k_1} (1 + \alpha t) \tag{3}$$

Let

$$\alpha \frac{W_0}{k_1} = \beta^2$$

then

$$\frac{d^2 t}{dr^2} + \frac{1}{r} \frac{dt}{dr} + \beta^2 t = - \frac{W_0}{k_1}$$

the general solution of which is

$$t = A J_0 (\beta r) + B Y_0 (\beta r) - \frac{W_0}{\beta^2 k_1}$$

But

$$\beta^2 = \alpha \frac{W_0}{k_1}$$

so

$$t = A J_0 (\beta r) + B Y_0 (\beta r) - \frac{1}{\alpha}$$

where $r = 0$, $Y_0 = -\infty$ and $J_0 = 1$, so

$$t = A J_0 (\beta r) - \frac{1}{\alpha} \tag{4}$$

When $r = R_2$

$$t = T_2 = T_1 + \frac{W_t}{2\pi k_2} \ln \left(\frac{R_1}{R_2}\right)$$

Substituting in eqn. 4 above

$$A\,J_0(\beta\,R_2) - \frac{1}{\alpha} = T_1 + \frac{W_t}{2\pi\,k_2}\ln\left(\frac{R_1}{R_2}\right)$$

therefore

$$A = \frac{1}{J_0(\beta\,R_2)}\left[T_1 + \frac{1}{\alpha} + \frac{W_t}{2\pi\,k_2}\ln\left(\frac{R_1}{R_2}\right)\right]$$

Substituting in eqn. 4 for A

$$t = \frac{J_0(\beta\,r)}{J_0(\beta R_2)}\left[T_1 + \frac{1}{\alpha} + \frac{W_t}{2\pi\,k_2}\ln\left(\frac{R_1}{R_2}\right)\right] - \frac{1}{\alpha} \tag{5}$$

For negative temperature coefficients[2]

This is the usual case for electrolytes in aqueous solution. Similarly to eqn. 5 above

$$t = \frac{I_0(\gamma r)}{I_0(\gamma R_2)}\left[T_1 + \frac{1}{\alpha} + \frac{W_t}{2\pi\,k_2}\ln\left(\frac{R_1}{R_2}\right)\right] - \frac{1}{\alpha} \tag{6}$$

where

$$\gamma^2 = -\,\alpha\,\frac{W_0}{k_1}$$

The relationship of W_t and W_0

Consider an annulus in the lumen section of radius r, of thickness δr and at a temperature of t °C.

$$W_t = 2\pi\,W_0\int_0^{R_2}(1 + \alpha\,t)\,r\,\mathrm{d}r$$

Substituting for t, from eqn. 4 above

$$W_t = 2\pi\,\alpha\,W_0\,A\int_0^{R_2} J_0(\beta\,r)\,r\,\mathrm{d}r$$

Let $\beta r = y$ and $\beta R_2 = Y$, then

$$W_t = \frac{2\pi\,\alpha\,W_0\,A}{\beta^2}\int_0^{Y} y\,J_0(y)\,\mathrm{d}y$$

But

$$\left[y\,\frac{\mathrm{d}\,J_0(y)}{\mathrm{d}y}\right]_0^{Y} = -\int_0^{Y} y\,J_0(y)\,\mathrm{d}y$$

Therefore

$$W_t = \frac{-2\pi\,\alpha\,W_0\,A\,R_2\,J_0'\,(\beta\,R_2)}{\beta} \tag{7}$$

When $r = R_2$

$$t = T_2 = T_1 + \frac{W_t}{2\pi k_2} \ln\left(\frac{R_1}{R_2}\right)$$

Substituting in eqn. 4

$$A = \frac{1}{J_0\,(\beta R_2)}\left[T_1 + \frac{1}{\alpha} + \frac{W_t}{2\pi k_2} \ln\left(\frac{R_1}{R_2}\right)\right]$$

Substituting in eqn. 7

$$W_t = \frac{-2\pi\,\alpha\,W_0\,R_2}{\beta} = \frac{J_0'\,(\beta R_2)}{J_0\,(\beta R_2)}\left[T_1 + \frac{1}{\alpha} + \frac{W_t}{2\pi k_2} \ln\left(\frac{R_1}{R_2}\right)\right]$$

But

$$J_0'\,(x) = -J_1\,(x)$$

So for positive α

$$W_t = \left[\frac{2\pi\,\alpha\,W_0\,R_2\,k_2\,J_1\,(\beta\,R_2)}{\beta\,k_2\,J_0\,(\beta\,R_2) - \alpha\,W_0\,R_2 \ln\left(\frac{R_1}{R_2}\right) J_1\,(\beta\,R_2)}\right]\left(T_1 + \frac{1}{\alpha}\right) \tag{8}$$

and for negative α

$$W_t = \left[\frac{2\pi\,\alpha\,W_0\,R_2\,k_2\,I_1\,(\gamma\,R_2)}{\gamma\,k_2\,I_0\,(\gamma\,R_2) - \alpha\,W_0\,R_2 \ln\left(\frac{R_1}{R_2}\right) I_1\,(\gamma\,R_2)}\right]\left(T_1 + \frac{1}{\alpha}\right) \tag{9}$$

Current density correction

The foregoing theory is valid only for uniform current density. But current density cannot be be uniform for temperature coefficients other than zero. To correct for this, modify eqn. 3

$$\frac{\mathrm{d}^2t}{\mathrm{d}r^2} + \frac{1}{r}\frac{\mathrm{d}t}{\mathrm{d}r} = -\frac{W_0}{k_1}\frac{1}{1+\alpha t}$$

But this is only numerically soluble. To render this more easily soluble, let

$$\frac{1}{1+\alpha t} = 1 + \mu t$$

then

$$\frac{\mathrm{d}^2t}{\mathrm{d}r^2} + \frac{1}{r}\frac{\mathrm{d}t}{\mathrm{d}r} = -\frac{W_0}{k_1}(1 + \mu t)$$

the solution of which has been derived for positive and negative values of α and thus for μ, in eqns. 5, 6, 8 and 9. For positive α and negative μ

$$t = \frac{I_0(\gamma r)}{I_0(\gamma R_2)}\left[T_1 + \frac{1}{\mu} + \frac{R_2^2 W_t}{2k_2}\ln\left(\frac{R_1}{R_2}\right)\right] - \frac{1}{\mu} \tag{10}$$

and

$$W_t = \left[\frac{2\pi\mu W_0 R_2 k_2 I_1(\gamma R_2)}{\gamma k_2 I_0(\gamma R_2) - \mu W_0 R_2 \ln\left(\frac{R_1}{R_2}\right) I_1(\gamma R_2)}\right]\left(T_1 + \frac{1}{\mu}\right) \tag{11}$$

and for negative α and positive μ

$$t = \frac{J_0(\beta r)}{J_0(\beta R_2)}\left[T_1 + \frac{1}{\mu} + \frac{R_2^2 W_t}{2k_2}\ln\left(\frac{R_1}{R_2}\right)\right] - \frac{1}{\mu} \tag{12}$$

and

$$W_t = \left[\frac{2\pi\mu W_0 R_2 k_2 J_1(\beta R_2)}{\beta k_2 J_0(\beta R_2) - \mu W_0 R_2 \ln\left(\frac{R_1}{R_2}\right) J_1(\beta R_2)}\right]\left(T_1 + \frac{1}{\mu}\right) \tag{13}$$

Calculation of μ

In practice $(1 + \alpha t)$ is an approximation of a series $(1 + \alpha t + \beta t^2)$. Plotting the reciprocal of the latter against t, an approximately straight line is obtained, of gradient $\mu = 0.033$, using values for 0.1 m*M* KCl[14]. This value was therefore used in digital computation[3]. Above 34°, for T_1 of 4°, the relation is less linear and therefore less reliable.

CONCLUSIONS

Comparison of equations for zero and negative temperature coefficients of resistivity, where current density changes have to be allowed for in the latter case, shows that for electrolyte solutions the temperature gradient in the steady state is not parabolic. It departs from the parabolic shape increasingly with higher real dissipated power per unit length of column, central temperatures likewise becoming increasingly greater than for zero coefficients. For positive coefficients, the gradients are more favourable. For electrolytes, Joule heating problems are therefore increasingly more severe than simple theory suggests. The general application to electrophoresis is dealt with in a previous paper of the series[2].

SYMBOLS AND UNITS

r = radius of tube section
R_1 = external radius of tube, cm
R_2 = internal radius of tube, cm
k_1 = thermal conductivity of electrolyte, cal·sec^{-1}·cm^{-1}·°C^{-1}

k_2 = thermal conductivity of tube material, cal·sec^{-1}·cm^{-1}·°C^{-1}
t = temperature
T_1 = temperature at wall exterior, °C
T_2 = temperature at lumen periphery, °C
T_3 = temperature at lumen section centre, °C
W_0 = nominal power dissipation at T_1, assuming a zero value of α, cal·cm^{-3}
W_t = actual power dissipation per unit length of column, cal·cm^{-1}
J_0 = Bessel function of zero order and first kind
J_1 = Bessel function of first order and first kind
I_0 = Bessel function of zero order and modified first kind
I_1 = Bessel function of first order and modified first kind
Y_0 = Bessel function of zero order and second kind
α = temperature coefficient of resistivity of electrolyte, °C^{-1}

$$\beta = \sqrt{\frac{\alpha W_0}{k_1}}$$

$$\gamma = \sqrt{-\frac{\alpha W_0}{k_1}}$$

μ = a function of α

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ELECTROPHORETIC THERMAL THEORY

III. STEADY-STATE TEMPERATURE GRADIENTS IN RECTANGULAR SECTION COLUMNS

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SUMMARY

Temperature gradients in the steady state are calculated for columns of electrolyte solution having rectangular section, and uniform bore, for various power dissipations. Wall thickness and material are allowed for. Fillings having zero, positive, and negative temperature coefficients of resistivity are calculated, correction being made for non-uniform current density. For negative coefficients, central-peripheral gradients are more severe than simple theory suggests, especially at higher powers, but this tendency is very much less than in circular section columns.

INTRODUCTION

Central-peripheral temperature gradients have been calculated previously, assuming a zero temperature coefficient of resistivity and uniform density of current through the lumen section. In this paper, these gradients are recalculated without these simplifying assumptions. For negative coefficients, gradients are more severe, increasingly so at high power dissipations. But the case is far more favourable than in circular section tubes, even more so than simple theory indicates. Wall thickness, however, has a larger influence for thick walls than is the case for cylindrical columns. Similar calculations, using Bessel functions, are given in a previous paper of this series[1]. A discussion of the anticipated effects of these gradients in electrophoresis, and a comparison of results of digitally computed gradients, in the context of factors determining the ideal column shapes and thermal properties, are given in a preceding paper[2]. A further paper[3] describes programming and digital computation of the

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gradients calculated here and in ref. 1. The results of this work were briefly reported at a symposium in 1971, by the second author[4], at whose instigation this project was undertaken for the reasons outlined in refs. 1 and 2. The treatment has subsequently been extended at the second author's suggestion to the unsteady state by Coxon and Binder[5].

THEORY

For an electrolyte of zero temperature coefficient of resistivity[2]

Consider a rectangular-section column filled with electrolyte. Assume it to be long enough to neglect end effects in a medial and uniform region. Assume the ratio of lengths of adjacent sides of the rectangle to be large enough to neglect edge effects. Assume that heat is uniformly generated in the electrolyte, and that there is no thermal convection or other internal fluid movement. Assume a zero temperature coefficient of resistivity and thermal conductivity[2]. Assume that a steady state pertains, and the column exterior is perfectly cooled and thermostatted. Using the listed symbols, which refer to the tube section normal to the long axis

$$\frac{\mathrm{d}^2 t}{\mathrm{d}x^2} = -\frac{W_0}{k_1}$$

Solving by Laplace transforms, the subsidiary equation is

$$p^2 \bar{t} = -\frac{W_0}{k_1 p}$$

so

$$\bar{t} = -\frac{W_0}{k_1 p^3}$$

and

$$t = -\frac{W_0}{k_1}\frac{x^2}{2} \tag{1}$$

For the column material

$$\frac{W_t}{2(2a+b)} = \frac{k_2 (T_2 - T_1)}{d}$$

so

$$T_2 = T_1 + \frac{W_t d}{2(2a+b) k_2} \tag{2}$$

The limits for eqn. 1 are

$$x = a,\ t = T_2$$

and

$$x = 0,\ \frac{\mathrm{d}t}{\mathrm{d}x} = 0$$

Using these and eqn. 2, we obtain

$$T_1 + \frac{W_t\, d}{2\,(2a + b)\,k_2} = A - \frac{W_t}{2\,a\,b\,k_1} \cdot \frac{a^2}{2}$$

so

$$A = T_1 + \frac{W_t}{2}\left[\frac{d}{(2a + b)\,k_2} + \frac{a^2}{2\,a\,b\,k_1}\right]$$

Substituting into the original equation

$$T = T_1 + \frac{W_t}{2}\left[\frac{d}{(2a + b)\,k_2} + \frac{a^2 - x^2}{2\,a\,b\,k_1}\right] \tag{3}$$

When $x = 0$

$$T_3 = T_1 + \frac{W_t}{2}\left[\frac{d}{(2a + b)\,k_2} + \frac{a}{2\,b\,k_1}\right] \tag{4}$$

For positive and negative coefficients

$$\frac{\mathrm{d}^2 t}{\mathrm{d}x^2} = -\frac{W_0\,(1 + \alpha\, t)}{k_1} \tag{5}$$

so

$$\frac{\mathrm{d}^2 t}{\mathrm{d}x^2} + \frac{W_0\,\alpha\, t}{k_1} = -\frac{W_0}{k_1}$$

Solving by Laplace transforms, the subsidiary equation is

$$\left(p^2 + \frac{W_0\,\alpha}{k_1}\right)\bar{t} = -\frac{W_0}{k_1\, p}$$

so

$$\bar{t} = -\frac{1}{\alpha\, p} + \frac{p}{\alpha\left(p^2 + \frac{W_0\,\alpha}{k_1}\right)}$$

Inverting

$$t = \frac{1}{\alpha}\left[\cos\left(x\,\Big/\frac{W_0\,\alpha}{k_1}\right) - 1\right] \tag{6}$$

For the tube material

$$\frac{W_t}{2\,(2a + b)} = \frac{k_2\,(T_2 - T_1)}{d}$$

so

$$T_2 = T_1 + \frac{W_t\, d}{2\,(2a + b)\,k_2} \tag{7}$$

For eqn. 5 the limits

$$x = a,\ t = T_2$$

and

$$x = 0,\ \frac{\mathrm{d}t}{\mathrm{d}x} = 0$$

apply. Using these and eqn. 7

$$T_2 = T_1 + \frac{W_t\, d}{2\,(2a + b)\,k_2} = \frac{1}{\alpha}\left[A \cos\left(a \sqrt{\frac{W_0\,\alpha}{k_1}}\right) - 1\right]$$

so

$$A = \left[\alpha\left(T_1 + \frac{W_t\, d}{2\,(2a + b)\,k_2}\right) + 1\right] \frac{1}{\cos\left(a \sqrt{\frac{W_0\,\alpha}{k_1}}\right)}$$

Substituting in the original equation, and using

$$\beta = \sqrt{\frac{W_0\,\alpha}{k_1}}$$

then

$$t = \frac{\cos(\beta x)}{\cos(\beta a)}\left[T_1 + \frac{1}{\alpha} + \frac{W_t\, d}{2\,(2a + b)\,k_2}\right] - \frac{1}{\alpha} \tag{8}$$

This expression is valid for both positive and negative values of the coefficient α.

For negative coefficients

Where α is negative, we have to evaluate the cosines of imaginary numbers

$$\cos x = 1 - \frac{x^2}{2!} + \frac{x^4}{4!} - \frac{x^6}{6!} + \cdots$$

so

$$\cos(ix) = 1 + \frac{x^2}{2!} + \frac{x^4}{4!} + \frac{x^6}{6!} + \cdots$$

which is also the value of cosh (x), which series we may use in digital computation. So for negative α, the temperature expression becomes

$$t = \frac{\cosh(\gamma x)}{\cosh(\gamma a)}\left\{T_1 + \frac{1}{\alpha} + \frac{W_t\, d}{2\,(2a + b)\,k_2}\right\} - \frac{1}{\alpha} \tag{9}$$

where

$$\gamma = \sqrt{-\frac{W_0\,\alpha}{k_1}}$$

The relationship of W_t and W_0

Consider an element in the column section parallel to b, of width b, and thickness dx, a distance x from the central line of the tube section

$$W_t = 2\,W_0 \int_0^a (1 + \alpha\, t)\, b\, dx$$

From eqn. 8

$$t = \frac{1}{\alpha}\left\{\left[\alpha\left(T_1 + \frac{W_t\, d}{2\,(2a + b)\,k_2}\right) + 1\right]\left[\frac{\cos(\beta\, x)}{\cos(\beta\, a)}\right] - 1\right\}$$

so

$$W_t = 2\,W_0 \int_0^a b\left[\alpha\, T_1 + \frac{W_t\, d\alpha}{2\,(2a + b)\,k_2} + 1\right]\left[\frac{\cos(\beta\, x)}{\cos(\beta\, a)}\right]dx$$

$$= \frac{2\,W_0\, b}{\cos(\beta\, a)}\left[\alpha\, T_1 + \frac{W_t\, d\alpha}{2\,(2a + b)\,k_2} + 1\right]\int_0^a \cos(\beta\, x)\, dx$$

$$= \left[\frac{2\,b\,W_0\,\alpha\,(2a + b)\,k_2 \tan(\beta\, a)}{(2a + b)\,k_2\,\beta - W_0\, b\,\alpha\, d \tan(\beta\, a)}\right]\left(T_1 + \frac{1}{\alpha}\right) \qquad (10)$$

which is valid for positive α. Similarly for negative α

$$W_t = \left[\frac{2\,b\,W_0\,\alpha\,(2a + b)\,k_2 \tanh(\gamma\, a)}{(2a + b)\,k_2\,\gamma - W_0\, b\,\alpha\, d \tanh(\gamma\, a)}\right]\left(T_1 + \frac{1}{\alpha}\right) \qquad (11)$$

Current density correction

The foregoing theory is valid only for uniform current density. But current density cannot be uniform for coefficients of resistivity other than zero[2,4]. To correct for this, modify eqn. 5 to

$$\frac{d^2t}{dx^2} = -\frac{W_0}{k_1}\cdot\frac{1}{1 + \alpha\, t}$$

To render this more easily soluble, let

$$\frac{1}{1 + \alpha\, t} = 1 + \mu\, t$$

and substitute negative μ for positive α and positive μ for negative α in eqns. 8–11. For positive α and negative μ

$$t = \frac{\cosh(\gamma\, x)}{\cosh(\gamma\, a)}\left[T_1 + \frac{1}{\mu} + \frac{W_t\, d}{2\,(2a + b)\,k_2}\right] - \frac{1}{\mu} \qquad (12)$$

and

$$W_t = \left[\frac{2b\,W_0\,\mu\,(2a + b)\,k_2 \tanh(\gamma\, a)}{(2a + b)\,k_2\,\gamma - W_0\, b\,\mu\, d \tanh(\gamma\, a)}\right]\left(T_1 + \frac{1}{\mu}\right) \qquad (13)$$

and for negative α and positive μ

$$t = \frac{\cos(\beta\, x)}{\cos(\beta\, a)}\left[T_1 + \frac{1}{\mu} + \frac{W_t\, d}{2\,(2a + b)\,k_2}\right] - \frac{1}{\mu} \qquad (14)$$

and

$$W_t = \left[\frac{2b\ W_0\,\mu\,(2a + b)\,k_2 \tan(\beta\, a)}{(2a + b)\,k_2\,\beta - W_0\,b\,\mu\,d \tan(\beta\, a)}\right]\left(T_1 + \frac{1}{\mu}\right) \tag{15}$$

Calculation of μ

In practice $(1 + \alpha t)$ is an approximation of a series $(1 + \alpha t + \beta t^2)$. Plotting the reciprocal of the latter against t, an approximately straight line is obtained, of gradient $\mu = 0.033$, using values for 0.1 mM KCl[6]. This value was therefore used in digital computation[3]. Above 34°, for T_1 of 4°, the relation is less linear and therefore less reliable.

CONCLUSIONS

Comparison of equations for zero and negative temperature coefficients of resistivity, in the latter case allowing for non-uniform current densities, shows that for electrolyte solutions there is an increase in central temperature not previously allowed for. This is less severe than in the case of circular column sections, dealt with in the previous paper. On the other hand, wall thickness has a greater influence. The comparison between circular and rectangular sections favours the latter more than was apparent from simple theory, particularly if the walls are thin compared to the lumen. As in circular sections, positive coefficients are palliative.

SYMBOLS AND UNITS

a = half-thickness of interior of column
x = values of a from zero to a
b = width of column
d = thickness of column wall
k_1 = thermal conductivity of electrolyte, cal·sec^{-1}·cm^{-1}·°C^{-1}
k_2 = thermal conductivity of wall material, cal·sec^{-1}·cm^{-1}·°C^{-1}
t, T = temperature
T_1 = temperature at wall exterior, °C
T_2 = temperature at lumen periphery, °C
T_3 = temperature at lumen section centre, °C
W_0 = nominal power dissipation assuming a zero value of α, or at switch-on at T_1, cal·cm^{-3}
W_t = actual power dissipation, per unit length of column, cal·cm^{-1}
$\bar{t}$, p = operators in Laplace transforms
α = temperature coefficient of resistivity of electrolyte, °C^{-1}

$$\beta = \sqrt{\frac{\alpha\, W_0}{k_1}}$$

$$\gamma = \sqrt{-\frac{\alpha\, W_0}{k_1}}$$

μ = a function of α

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CHROM. 8113

PECULIARITIES IN GEL PERMEATION CHROMATOGRAPHY OF FLEXIBLE-CHAIN POLYMERS ON MACROPOROUS SWELLING SORBENTS

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SUMMARY

In gel permeation chromatography on macroporous swelling sorbents, deviations from the Benoit principle of universal calibration were observed. It is suggested that these are caused by different degrees of thermodynamic compatibility of the eluted polymers with the sorbent matrix.

INTRODUCTION

The separation of macromolecular components by gel permeation chromatography (GPC) is due to several mechanisms, mainly the molecular sieve mechanism[1–4], the diffusion mechanism[5–9] and the exclusion mechanism (*i.e.*, the mechanism of volume exclusion)[10–12]. As a result of each of these mechanisms, larger macromolecules move along the chromatographic column faster than smaller macromolecules. The molecular sieve mechanism is based on the comparable sizes of the macromolecules and the sorbent pores. The diffusion mechanism is determined by the mobility of macromolecules in the stationary phase of the column and is responsible for the degree of non-equilibrium of the process. The exclusion mechanism is based on the effect of the mutual volume exclusion of polymer segments typical of macromolecules[13].

Usually, GPC should be carried out under conditions close to the equilibrium conditions, when the effect of the diffusion mechanism on the separation of macromolecules becomes negligible. Moreover, if non-swelling solvents such as porous glasses, silica gels and Styrogels are used as packing and the volume interaction of macromolecules with the sorbent matrix is virtually absent, the GPC process is based only on the molecular sieve effect. In this case, the chromatograms can be successfully interpreted in terms of the molecular weight distribution of polymers by using the principle of the Benoit universal calibration graph[14,15]. This principle maintains that the GPC separation of macromolecules occurs according to their hydrodynamic volume, $V \approx M[\eta]$, where M = molecular weight and $[\eta]$ = intrinsic viscosity. This principle is also widely used in GPC on swelling sorbent gels, although this is not always correct. Recently, we have described an important deviation from the universal calibration in GPC on Sephadexes[16].

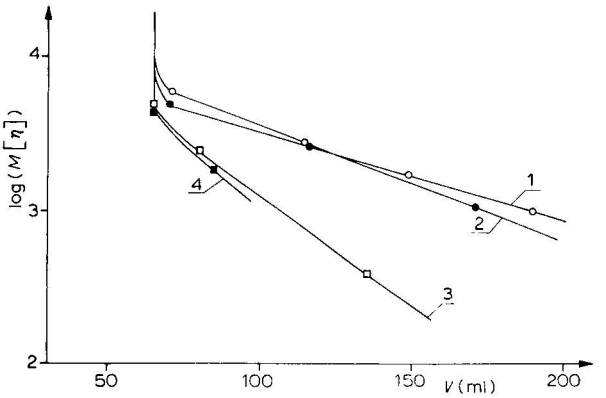

Fig. 1. Retention volumes *versus* logarithm of molecular weight multiplied by intrinsic viscosity of the polymer (obtained on a column packed with Sephadex G-100). 1, Dextran; 2, polyvinylpyrrolidone; 3, polyoxyethylene; 4, polyvinyl alcohol.

EXPERIMENTAL AND RESULTS

GPC for four types of polymers, *viz.*, dextran, polyvinylpyrrolidone (PVP), polyvinyl alcohol (PVA) and polyoxyethylene (POE), was carried out on columns 96 cm long with an I.D. of 20 mm packed with Sephadex G-75 and G-100 (particle diameter 40–120 μm). The flow-rate of the eluent (0.3% sodium chloride solution) was 50 ml/h. A differential flow refractometer with a cell volume of 50 μl and a sensitivity (ΔH) of 10^{-6} was used as a detector. The resulst are shown in Figs. 1 and 2. It is clear that the plots of the retention volume, V, *versus* log ($M[\eta]$) for PVP and dextrans differ

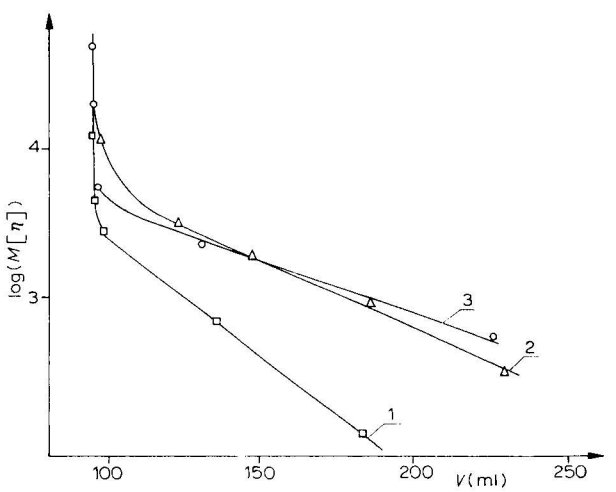

Fig. 2. Retention volumes *versus* logarithm of molecular weight multiplied by intrinsic viscosity of the polymer (obtained on a column packed with Sephadex G-75). 1, Experimental curve for polyoxyethylene; 2, curve for dextran calculated by using curve 1 and eqns. 6 and 7 in which χ^* is found from the data obtained with Sephadex G-100 and shown in Fig. 1; 3, experimental curve for dextran.

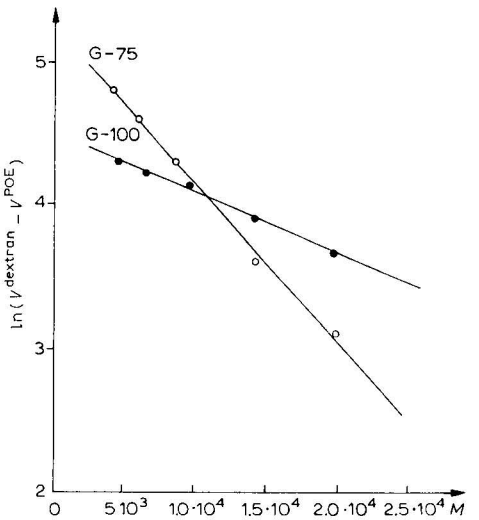

Fig. 3. Logarithm of difference between retention volumes of POE and dextran *versus* molecular weight of dextran (obtained on columns packed with Sephadex G-100 and G-75).

greatly from those for POE. Moreover, the logarithm of the difference in the V values is inversely proportional to the molecular weight of PVP or dextran (Fig. 3).

These results can be interpreted as follows. In GPC on swelling sorbents, macromolecules permeate into the macropores of the sorbent according to the molecular sieve mechanism with different probabilities determined by their hydrodynamic size. The value of the sorbent volume accessible to macromolecules, V^{acc}, and therefore the value of the retention volume, V, depends on the total pore volume. This dependence is universal, *i.e.*, it is general for all types of macromolecules. Nevertheless, the walls of macropores of the swelling sorbent are permeable to macromolecular units and, in accordance with the exclusion mechanism, there is a certain probability that these units may penetrate through them into dense sorbent regions, *i.e.*, into micropores, increasing the accessible volume of the sorbent by a certain value, ΔV^{acc}. This ability of macromolecular chains to diffuse into dense regions of the swelling sorbent is closely related to the thermodynamic compatibility of macromolecules with the sorbent matrix and can be treated on the basis of the concept of the excluded volume[13] as a property supplementary to the molecular sieve factor. Possible arrangements of macromolecules in pores of a swollen sorbent are shown schematically in Fig. 4.

The calculation of ΔV^{acc} for macromolecules compatible with the sorbent can be carried out as follows. We will consider the swollen sorbent gel in the solvent as a solution of macromolecules represented by dense regions in each gel grain. For this solution, the free energy of mixing of the polymer with the solvent can be calculated by a standard method[17]. Then, the free energy change related to the permeation of segments of macromolecules into dense gel regions can readily be estimated by using the analogy with the excluded volume of macromolecules[13]. If we assume that each dense gel region is a sphere of radius R, uniformly filled with units and of a much greater size than the macromolecule regarded as a Gaussian coil [$R \gg (\overline{r^2})^{\frac{1}{2}}$, where

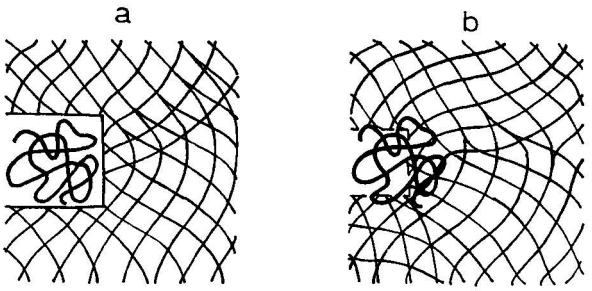

Fig. 4. Schematic arrangement of macromolecule in a pore of the swollen sorbent. (a) Macromolecule incompatible with the sorbent; (b) macromolecule compatible with the sorbent.

$(\overline{r^2})^{\frac{1}{2}}$ is the radius of gyration of macromolecules], then the expression for the total free energy change in the system gel–macromolecule–solvent when the macromolecule and the dense gel region approach and the distance between them becomes a, by analogy with theory[17], can be written as

$$\Delta F_a = kT \cdot \frac{V_g V_m}{V_1}\left[\int \varrho_g \varrho_m \mathrm{d}v + \sum_{k=2}^{\infty}\sum_{n=1}^{k} \frac{(k-1)!}{n!(k+1-n)!} \cdot V_g^{n-1} V_m^{k-n} \int \varrho_g^{n} \varrho_m^{k+1-n} \mathrm{d}v - (\chi_{1g} + \chi_{1m}) \int \varrho_m \varrho_g \mathrm{d}v + \chi_{gm} \cdot \frac{V_1}{V_g} \int \varrho_m \varrho_g \mathrm{d}v\right] \quad (1)$$

where V_1 is the volume of the solvent molecule, V_g and V_m are the volumes of the units of the gel and the macromolecule, ϱ_g and ϱ_m are the densities of these units in a volume element δV, a is the distance between the centres of the dense gel region and of the macromolecule, χ_{1g} and χ_{1m} are the constants of interaction of the units of the gel and of the macromolecule with the solvent, χ_{gm} is a constant characterizing the interaction of these units in a particular solvent and the value of ϱ_g outside the dense gel regions is reduced to zero.

Integrating eqn. 1 and, for simplicity, neglecting the series included in it (because of its low value), we obtain

$$\Delta F_a = \begin{cases} 0 \text{ if } a > R \\ kT \cdot \dfrac{V_g V_m}{V_1}\left[1 + \chi_{gm} \cdot \dfrac{V_1}{V_g} - (\chi_{1g} + \chi_{1m})\right] \varrho_g z \text{ if } a < R \end{cases} \quad (2)$$

where $z = M/M_0$ is the number of units in a macromolecule and M_0 is the weight of one unit.

The free energy change, ΔFa, determines the probability, $\exp(-\Delta F_a/kT)$, that the macromolecule is at a distance a from the dense gel region. Thus, the product $\exp(-\Delta F_a/kT)\, 4\pi a^2 \mathrm{d}a$ represents the part of the volume of the spherical gel layer that is accessible to the macromolecule, $4\pi a^2 \mathrm{d}a$. Extending the integration over a to the whole gel grain and taking into account the fraction of dense regions in each grain, we obtain

$$\Delta V^{\mathrm{acc}} = \frac{V_g - V_{\mathrm{sieve}}^{\mathrm{acc}}}{V_g} \int_0^{R_g} 4\pi a^2 \exp(-\Delta F_a/kT)\, \mathrm{d}a \quad (3)$$

where R_g is the radius of swollen gel grains, V_g is its total volume and V_{sieve}^{acc} is the gel volume that is accessible to macromolecules according to the molecular sieve mechanism. If the macromolecules undergoing chromatography are incompatible with the gel matrix under the conditions of a particular GPC experiment, then for these macromolecules the value of the retention volume, V^{incomp}, is completely determined by the value of V_{sieve}^{acc}:

$$V^{incomp} = V_0 + V_{sieve}^{acc} \tag{4}$$

For a polymer compatible with the gel, we have another dependence:

$$V^{comp} = V_0 + V_{sieve}^{acc} + \Delta V^{acc} = V^{incomp} + \Delta V^{acc} \tag{5}$$

where ΔV^{acc} is determined by eqn. 3 and V_0 is the mobile phase in the chromatographic system.

Eqns. 2–5 show that if we know the values of the Flory–Huggins constants, χ_{1m}, χ_{1g} and χ_{gm}, and the retention volumes for polymers that are definitely incompatible with the sorbent under the conditions used, it is easy to calculate the retention volumes for compatible polymers. We can adopt the opposite procedure and determine the χ_{ij} constants from the values of V^{comp} and V^{incomp}. In our experiment, POE and PVA were incompatible with the sorbent, whereas PVP and dextran were compatible with it. The choice of a dextran gel (Sephadex) as sorbent made it possible in calculating V^{acc} to put for dextran macromolecules $\chi_{1g} = \chi_{1m} \equiv \chi^*$, $\chi_{gm} = 0$. The calculations were carried out according to the equation

$$V^{comp} = V^{incomp} + (V_c - V^{incomp}) \exp(-k_c M_c) \tag{6}$$

which is obtained by substituting into eqn. 5 the value of ΔV^{acc} from eqn. 3; V_c is the total volume of the chromatographic system, M_c is the molecular weight of dextran and k_c is the parameter characterizing the system Sephadex–dextran–aqueous sodium chloride solution; $k_c = (1/M_c)\cdot(\Delta F_a/kT)$ for $a < R$.

Eqn. 2 gives

$$\chi^* = \frac{\lambda}{2}\left(1 - k_c \cdot \frac{M_0 V_1}{V_g V_m \varrho_g}\right) \tag{7}$$

The values of the retention volumes obtained in experiments on a column with Sephadex G-100 were substituted into eqn. 6. The k_c parameter was found and the χ^* constant was calculated by using eqn. 7. The results are shown in Table I. The average value of the Flory–Huggins constant found by this method for dextran, $\chi^*_{av} = 0.47$, was used for calculating the retention volume of dextran macromolecules during their elution through a column packed with Sephadex G-75, which differs from Sephadex G-100 in its density, porosity and degree of swelling. As in the preceding case, the values of the retention volumes for polymers incompatible with Sephadex were identified with the retention volumes of POE. The results of the calculations are shown in Fig. 2. The experimental results agree with the calculated values to within 15%.

TABLE I

CALCULATED χ^* VALUES

χ^* was calculated by using eqn. 7 and data from experiments on Sephadex G-100.

Molecular weight of dextran, M_c	χ^*
$5 \cdot 10^3$	0.45
$7 \cdot 10^3$	0.48
$1 \cdot 10^4$	0.47
$5 \cdot 10^4$	0.49
Average	0.47

These results, in combination with the dependence of ΔV^{acc} on the molecular weight of dextran shown in Fig. 3 and consistent with the dependence in eqn. 6 predicted by theory, permit us to assume the adequacy of our model of the GPC of flexible-chain polymers on swelling sorbents.

Results obtained in GPC on columns filled with Enzacryl K_1 and K_2 gels have been published recently[18]. In our opinion, the arguments used by the authors of that paper for the interpretation of the results should be supplemented by taking into account the difference in thermodynamic compatibility between POE on one hand and oligosaccharides on the other hand with the poly(acrylomorpholine) matrix of these gels in aqueous solutions and the difference in thermodynamic compatibility between POE with Enzacryl gels in aqueous solutions and in chloroform.

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CHROM. 8202

RAPID DETERMINATION OF SELENIUM IN VARIOUS SUBSTRATES BY ELECTRON CAPTURE GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

In the proposed procedure for the determination of selenium, 0.5–1 g of sample is wet ashed with concentrated nitric acid. After adding 1,2-diamino-4,5-dichlorobenzene to the digest at pH 1, the resulting dichloropiazselenol derivative is extracted with toluene. The extract is purified by column chromatography over Florisil and analyzed by gas–liquid chromatography with electron capture detection. Recoveries of selenium added to various substrates ranged from 72 to 102%. The limit of detection is approximately 0.01 ppm, but smaller amounts can be determined by increasing the sample size or by concentration of the final extract.

INTRODUCTION

Present methods for the determination of low levels of selenium in plant and animal tissues, foods and other materials are based on the reactions between selenious acid and aromatic *o*-diamines that yield piazselenol compounds which are determined by fluorimetry[1–3]. Other methods for the determination of trace amounts of selenium involve the use of neutron activation[4] and isotope dilution[5], which require equipment that is not available to many laboratories.

In this laboratory, there was a need for the analysis of many samples of the same nature with selenium contents that ranged from 0.01 to 20 ppm. The fluorimetric methods mentioned above were considered to be unsuitable for this purpose, because the average analyst needs considerable training before he is proficient in obtaining consistently high recoveries. For this reason, we considered the use of the gas chromatographic procedure described recently by Young and Christian[6], who treated selenium with 2,3-diaminonaphthalene at pH 2 and extracted the corresponding piazselenol compound with hexane. The latter compound could be readily determined by gas chromatography with electron capture detection, but the limit of detection was only 0.5 ng which was not sensitive enough for our purpose. It was decided, therefore, to develop an improved method, based on the determination of a derivative that has a much higher electron affinity.

Such a derivative was obtained by the reaction of tetravalent selenium with 1,2-diamino-4,5-dichlorobenzene:

$$NH_2C_6H_2(Cl_2)NH_2 + H_2SeO_3 \rightarrow SeN{:}C_6H_2(Cl_2){:}N + 3H_2O$$

This reaction was formerly used for the gravimetric determination of selenium[7]. The dichloropiazselenol compound was formed quantitatively in the pH range 1–1.5 and could be extracted completely from aqueous media with organic solvents such as toluene. Its response to the electron capture detector was found to be 50 times greater than that of the 4,5-benzopiazselenol employed by Young and Christian[6]. A detailed description of the improved method is given in this paper.

EXPERIMENTAL

Reagents

All chemicals used were of micro-analytical reagent grade. The solvents used were free from electron capturing impurities.

Florisil (synthetic magnesium silicate), 60–100 mesh, adsorbent was purified by heating overnight at 550°. After cooling, the adsorbent was standardized by adding 3% (w/w) of distilled water, vigorously mixing for at least 20 min and allowing the mixture to equilibrate for 10–12 h. The partly deactivated adsorbent thus obtained could be used only up to 5 days after its preparation, after which time it was heated and standardized again.

1,2-Diamino-4,5-dichlorobenzene was obtained from Ferak, Berlin, G.F.R. The reagent was recrystallized from hydrochloric acid solution by addition of sodium hydroxide. A 0.6% solution of the purified diamine in 1 *N* hydrochloric acid was used as a reagent for conversion of tetravalent selenium into the dichloropiazselenol derivative.

Dichloropiazselenol standard was synthesized as follows. A 240-ml volume of a 0.6% solution of the purified diamine in 1 *N* hydrochloric acid was added to 100 ml of distilled water containing 210 mg of selenium dioxide. Upon adjusting the pH to 1.5 by adding 4 *N* sodium hydroxide solution, a copious precipitate was obtained. After allowing the mixture to stand for 1 h so as to complete precipitation, the solid was separated from the liquid by centrifuging at 3000 rpm. The supernatant liquid was discarded and the precipitate washed successively with 0.1 *N* hydrochloric acid and distilled water until acid-free. After drying at 110°, the piazselenol was purified by recrystallization from a mixture of light petroleum–diethyl ether (7:3, v/v). The purified compound was found to contain 30.85% of selenium upon elemental analysis. The theoretical value is 31.34%. Reference solutions for gas chromatography containing 10, 20 and 40 pg/μl were prepared in toluene.

A standard solution of selenium was prepared by dissolving 50.0 mg of pure black selenium in 5 ml of nitric acid, sp. gr. 1.42. This solution was made up to 50 ml with water in a calibrated flask. For recovery experiments, dilutions were used containing 0.1, 1.0 and 10.0 μg/ml of selenium.

Field of application of the method

The method can be applied to vegetables, fruits, flours, mushrooms and soil samples. Among fatty substrates, only whole egg powder was tested, but the procedure will probably work equally well for other fatty foods.

Digestion of samples

Mineralise 0.50 g of sample with 3 ml of concentrated nitric acid in a PTFE and stainless-steel decomposition vessel according to Bernas[8] at 100° for 15–20 min. If these decomposition vessels are not available, perform the digestion in 170 × 20 mm tubes, made of 2-mm thick Pyrex glass, provided with a venting side-tube and a Quickfit Rotaflo TF 6/24 PTFE valve. When using these tubes, the sample size may be increased to 1 g, but in that case add 5 ml of concentrated nitric acid. In order to avoid too vigorous reaction upon heating, allow the mixture to digest overnight in open tubes at room temperature. Subsequently, evacuate air from the tubes with a water-jet pump, close it tightly with the valve and heat it in a boiling water-bath for 15 min. *Caution*: when handling tubes under pressure, wear safety goggles.

After cooling, slowly and cautiously vent the pressure and transfer the reaction mixture quantitatively with small portions of distilled water into a 100-ml conical flask. Add slowly about 2 g of pure urea and swirl the flask so as to expel nitrogen oxides. Adjust the pH of the mixture to approximately 1 by adding a few drops of 0.2% ethanolic thymol blue solution and sufficient concentrated ammonia solution until the red colour changes to orange-yellow. At this point, the pH is about 2.5. Make the final adjustment to pH 1 by adding dropwise 2 *N* hydrochloric acid and check after each addition with narrow-range pH paper.

Conversion of selenium into its dichloropiazselenol derivative

Extract any possibly present electron capturing impurities by shaking the reaction mixture with 10 ml of toluene in a 100-ml separating funnel for about 1 min. Allow the layers to separate. Drain the aqueous lower phase into a 100-ml conical flask and discard the toluene upper layer. Add 0.5 ml of 0.6% purified diamine solution in 1 *N* hydrochloric acid, close the flask tightly and heat it in a water-bath at 80° for 10 min. Allow the mixture to cool, then transfer it quantitatively into a 100-ml separating funnel, using a few millilitres of distilled water for rinsing. Extract the derivative by vigorous shaking with 20 ml of light petroleum–toluene (3:1, v/v) for 1 min. Allow the layers to separate completely and discard the aqueous phase.

Clean-up

Use a glass chromatography column, 8 × 200 mm, fitted with an outlet stopcock and having a 20-ml reservoir at the upper end. Tamp a small plug of glass-wool into this column and add 2.5 g of standardized Florisil. Ensure tight packing of the adsorbent by tapping the sides of the column with a glass rod.

Allow the light petroleum–toluene extract obtained above to pass through the column at a rate of 1–2 ml/min. Discard the liquid that has run through, then allow a further 15 ml of light petroleum–toluene (3:1, v/v) to pass through the column in order to wash out impurities. When the liquid has reached the top of the Florisil column, close the stopcock and discard the eluate. Elute the selenium derivative with

18 ml of toluene into a 20-ml calibrated flask, make the volume up to 20 ml and mix. The solution is now ready for gas–liquid chromatography.

Gas–liquid chromatography

The dichloropiazselenol can be chromatographed on all columns that are normally used in pesticide residue analysis, such as Dow 11, OV-17, QF-1 and DEGS. The conditions used for two of these columns are listed in Table I. It is not possible to give definite instructions concerning the parameters associated with optimal performance, because they are different for each instrument. The response for the selenium derivative is not only dependent on detector performance, but also on the state of the column.

TABLE I

GAS CHROMATOGRAPHIC CONDITIONS USED FOR THE DETERMINATION OF THE DICHLOROPIAZSELENOL DERIVATIVE

Parameter	*Stationary phase*	
	1.5% OV-17 + 1.95% QF-1 coated on Chromosorb W H.P., 100–120 mesh	*2% DEGS + 0.5% H_3PO_4 coated on Chromosorb W H.P., 100–120 mesh*
Instrument	Perkin-Elmer 3920	Perkin-Elmer F-11
Detector	Electron capture, nickel-63 source	Electron capture nickel-63 source
Detector operation	Pulse-modulated with extended linear range	Conventional negative d.c.
Attenuation	1 × 64	1 × 8
Carrier gas	Argon–methane (95:5, v/v), 40 ml/min	Nitrogen, 35 ml/min
Injection port temperature	250°	250°
Oven temperature	200°	170°
Detector temperature	275°	245°
Column	1.5 m × 3 mm, Pyrex	1.5 m × 3 mm, Pyrex
Sample volume injected	5 µl	5 µl
Approximate retention time of the dichloropiaz-selenol compound	3 min	2 min

Select a sensitivity at which 100 pg of the standard produce at least 50% full-scale deflection. Inject 5 µl of sample extract into the gas chromatograph with a micro-syringe. Compare the size of the peak of the selenium derivative with the size of the peak from a known amount of the standard. Check if the amount of the derivative in the injected sample aliquot falls within the linear range of the detector. If this is not so, prepare a suitable dilution and inject again. Sufficient accuracy is achieved when simply using the peak height (expressed in millimetres) for quantitation.

Calculate the selenium content of the sample aliquot by multiplying the dichloropiazselenol concentration by 0.3134.

Thin-layer chromatography for confirmation of identity

Concentrate the toluene sample solution to 0.5 ml by evaporation. Spot suitable aliquots of this concentrate, *i.e.*, volumes to give spots within the range 0.05–0.25 μg, on a neutral aluminium oxide thin-layer plate (Merck pre-coated No. 5550 aluminium sheets are suitable). Apply standard solutions to give spots of 0.05, 0.10, 0.15, 0.20, 0.25 and 0.30 μg. Develop the plate under conditions of saturation with light petroleum–acetone (98:2, v/v) as the mobile phase. After development, allow the adherent solvent to evaporate and spray abundantly with 0.5% ethanolic silver nitrate solution. Subsequently, irradiate the plate for 10 min with a high-power photochemical lamp. Remove the plate from the lamp and spray it lightly with distilled water until the plate is just moistened. Expose the plate again to the UV lamp. Spots should now become visible at R_F 0.50 in 1–2 min. The limit of detection is approximately 0.05 μg.

Alternatively, thin-layer chromatography can also be performed on silica gel layers using the same mobile phase. Reveal the spots of the selenium derivative by spraying the chromatogram with a 5% solution of tin(II) chloride in 6 *N* hydrochloric acid–ethanol (1:1, v/v) and subsequent UV irradiation as described above. The piazselenol is reduced to elemental selenium and red spots become visible at R_F 0.20. The limit of detection with this chromogenic reagent is only 0.5 μg. It is, therefore, only suitable for confirming parts per million levels of selenium.

RESULTS AND DISCUSSION

The digestion procedure in the PTFE and stainless-steel decomposition vessels or in the glass tubes equipped with a PTFE valve was chosen because it allowed the rapid mineralization of large series of samples*. It is possible, of course, to use the more conventional procedures in Kjeldahl flasks[2,3], provided that charring of the samples, which may result in loss of selenium, is avoided.

Initially, it was thought that it was necessary to base our calculations upon a standard curve obtained by taking different aliquots of selenium standard solution

TABLE II

RECOVERY OF SELENIUM FROM AQUEOUS SOLUTIONS

Amount of selenium added (μg)	*Recovery (%) (duplicate determinations)*
0.02	92, 94
0.10	92, 94
0.20	94, 95
0.50	89, 91
1.00	94, 96
10.0	89, 92
100.0	85, 87

Caution: After submission of this paper for publication, the authors experienced two explosions, one occurring during the mineralization of fish tissue in glass tubes and the other during digestion of dehydrated mushrooms in the PTFE and stainless-steel vessel. Although they performed about 400 such digestions without accidents, the authors consider that it may be safer to use conventional wet-ashing procedures.

through the whole procedure with each series of samples. However, recoveries obtained by adding the dichlorodiamine reagent to 10-ml aqueous solutions of different concentrations of selenious acid and treating the reaction mixture as described for the sample digests were excellent, as shown in Table II. Consequently, we decided to use directly standard solutions of the derivative for quantitation. Recoveries were checked from time to time by running a sample to which a known amount of selenium had been added. Table III clearly indicates that the recoveries obtained were satisfactory at all levels.

The reaction of selenium with 1,2-diamino-4,5-dichlorobenzene seems to be highly selective for this element. Although we analyzed several different substrates, ranging from flours to egg powder, we never observed supplementary peaks on our chromatograms. Starace *et al.*[7], who employed the reagent for the gravimetric determination of selenium, reported no interference from 27 other elements, but they obtained precipitates with osmium(VIII) and cerium(IV). We tested the reaction of these two ions under conditions as specified for selenium, but did not obtain a derivative that could be chromatographed, even at the microgram level.

TABLE III

RECOVERY OF SELENIUM ADDED TO SAMPLES

Sample	*Selenium added (ppb)*	*Selenium determined (ppb)*	*Mean value (ppb)*	*Recovery (%)*
Tomato flakes	0	61, 64, 65	63	–
	10	73, 75, 69	72	90
	50	103, 98, 96	99	72
	100	170, 158, 155	161	98
	200	250, 240, 252	247	92
	500	560, 550, 540	550	97
	1000	990, 980, 1020	997	93
	2000	1820, 1870, 1900	1863	90
Dehydrated leeks	0	78, 63, 59	67	–
	50	110, 120, 107	112	90
	200	240, 228, 220	229	81
	500	580, 590, 560	577	102
	600	590, 610, 575	592	88
	800	810, 785, 776	790	90
	1000	1050, 1080, 1110	1080	102
Dehydrated mushrooms (*Agaricus arvensis*)	0	5640, 5800, 5700	5713	–
	10000	15170, 15300, 14800	15090	94
Barley flour	0	30, 35, 40	35	–
	100	107, 110, 106	108	73
	400	390, 398, 375	388	88
Whole egg powder	0	450, 500, 475	475	–
	1000	1420, 1380, 1370	1390	92

In order to obtain quantitative conversion of selenium into the dichloropiazselenol derivative, it is essential to use the pH range 0–2. Outside this range, the recovery of selenium decreases sharply.

Considering the high selectivity of the reagent, gas chromatographic determination is usually adequate for routine analysis. We developed the thin-layer chromatographic confirmatory procedure mainly for ascertaining the high selenium levels that we encountered in some species of mushrooms.

The clean-up step on Florisil is necessary in order to eliminate excess of reagent and impurities. The dichlorodiamine itself is strongly retained on the Florisil column, but small amounts of the impurities also pass into the toluene eluate together with the selenium derivative. However, these compounds do not interfere in the determination, because they are eluted before and after the dichloropiazselenol. As the method requires only small amounts of reagents, the blank value is generally very low. In most instances, we did not observe a measurable derivative peak when running a blank. With our gas chromatographic equipment, we could still obtain a measurable peak upon injecting 5 μl containing 10 pg of the dichloropiazselenol. This means that the limit of detection, based on a 1-g sample and a final volume of 20 ml, was approximately 0.01 ppm of selenium. Lower levels can undoubtedly be determined by increasing the sample size or by concentrating the final extract.

Other gas chromatographic detectors were also tried in order to determine their response to the piazselenol compound. The flame photometric detector[9] responded to nanogram amounts of the derivative when operated with a sulphur filter (394 nm). Slightly better sensitivity was obtained with the Perkin-Elmer P/N detector[10], using a very cool flame, but both devices were obviously unsuitable for the determination of selenium at the parts per billion level.

Experience with this method over a period of 1 year indicates that it is especially suitable for the routine determination of selenium. One technician can analyze without difficulty twelve samples per day, including gas chromatography and calculation of the results. It is not costly, because it requires only small amounts of reagents and uses equipment that is normally available in any laboratory dealing with trace analysis.

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SIMULTANEOUS DETERMINATION OF ACETYLMETHADOL AND ITS ACTIVE BIOTRANSFORMATION PRODUCTS IN HUMAN BIOFLUIDS*

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SUMMARY

A method employing solvent extraction and gas–liquid chromatography has been developed for the quantitative determination of acetylmethadol simultaneously with its two major biotransformation products, noracetylmethadol and dinoracetylmethadol. Noracetylmethadol and dinoracetylmethadol are analyzed following their conversion to the corresponding amides. The amide structure is confirmed by the use of chemical ionization mass spectrometry and infrared spectroscopy.

The method can be used to determine the concentration of acetylmethadol and these compounds in plasma samples from acetylmethadol maintenance subjects. Methadol and normethadol do not attain measurable plasma levels.

Urine contains predominantly noracetylmethadol and dinoracetylmethadol. Evidence was also obtained for the urinary excretion of acetylmethadol, methadol and normethadol. A mean quantity equal to 28% of the administered dose was excreted in the urine of a 48-h dosing interval as acetylmethadol and metabolites.

INTRODUCTION

In 1952 Fraser and Isbell[1] reported that a single dose of acetylmethadol (AM) can suppress narcotic withdrawal symptoms for at least three days. Recent clinical studies[2–5] have found that the administration of three oral doses per week of AM is as effective as daily methadone in the treatment of opiate dependence. Studies in laboratory animals[6–8] have suggested that the biotransformation of AM to active metabolites is responsible for the time–action characteristics of certain of the pharmacologic effects of AM.

In a previous report[9] we identified AM, noracetylmethadol (NAM), methadol (MOL) and normethadol (NMOL) in the urine of AM maintenance subjects (Fig. 1). Billings *et al.*[10] initially identified dinoracetylmethadol (NNAM) in the biofluids of

* A preliminary report of these data appears in *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 33 (1974) 473.

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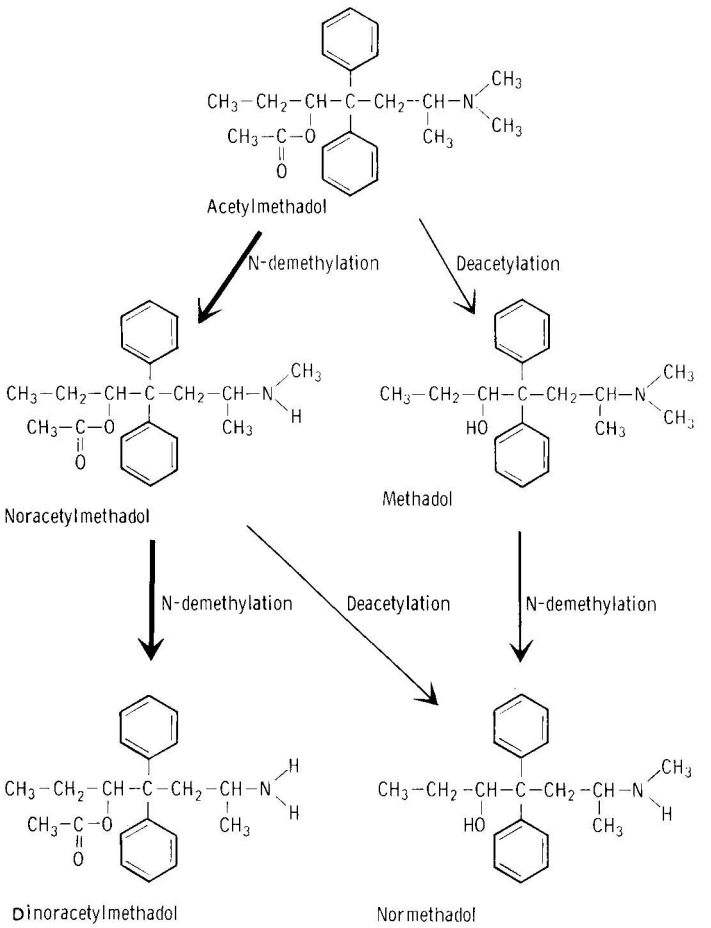

Fig. 1. Structural formulae of acetylmethadol, noracetylmethadol, dinoracetylmethadol, methadol and normethadol.

rats given AM. NNAM is found in the plasma and urine of AM maintenance subjects[11,12].

The purpose of this report is to describe a specific and sensitive method for the quantitation of AM, NAM and NNAM in a sample of human plasma or urine.

MATERIALS AND METHODS

Chemicals and reagents

The *l*-isomer of α-acetylmethadol was provided by Dr. M. Fink of New York Medical College. α-*d,l*-Noracetylmethadol hydrochloride, α-*d,l*-dinoracetylmethadol maleate, α-*l*-methadol hydrochloride and α-1-normethadol hydrochloride were complimentary research samples from Dr. A. Pohland of the Lilly Research Laboratories (Indianapolis, Ind., U.S.A.). Dr. E. L. May of the National Institutes of Health (Bethesda, Md., U.S.A.) also provided α-*l*-noracetylmethadol hydrochloride and

α-*l*-methadol hydrochloride (Fig. 1). SKF 525-A (β-diethylaminoethyldiphenylpropylacetate hydrochloride) was provided by Smith, Kline and French Labs. (Philadelphia, Pa., U.S.A.). Trifluoroacetylimidazole was obtained from Pierce (Rockford, Ill., U.S.A.). β-Glucuronidase, Type H-1, was obtained from Sigma (St. Louis, Mo., U.S.A.).

Hexane, *n*-butyl chloride and chloroform are Distilled in Glass® and obtained from Burdict and Jackson (Muskegon, Mich., U.S.A.).

Stock solutions

Aqueous solutions of AM and its biotransformation products each at a concentration of 4 μg/ml and SKF 525-A at a concentration of 20 μg/ml are prepared and stored in opaque plastic containers in a refrigerator.

Sample preparation from biofluids

The extraction procedure is adapted from that described by Inturrisi and Verebely[13] for the extraction of methadone from plasma and urine. A flow sheet outlining this procedure is given in Fig. 2.

To plasma (1–4 ml) in a siliconized 15-ml centrifuge tube with a Teflon®-lined screw cap are added 50 μl of the aqueous solution of SKF 525-A, the internal standard, 1.0 ml of 1 *M* phosphate buffer, pH 7.4, and one drop of 1-octanol. After thorough mixing the sample is extracted with 9.0 ml of *n*-butyl chloride by shaking for 10 min followed by centrifugation at 1500 rpm (500 *g*) for 5 min. The upper, organic, phase is transferred to a second tube and the contents of the initial tube are discarded. The compounds are extracted into 5.0 ml of 0.2 *N* hydrochloric acid by

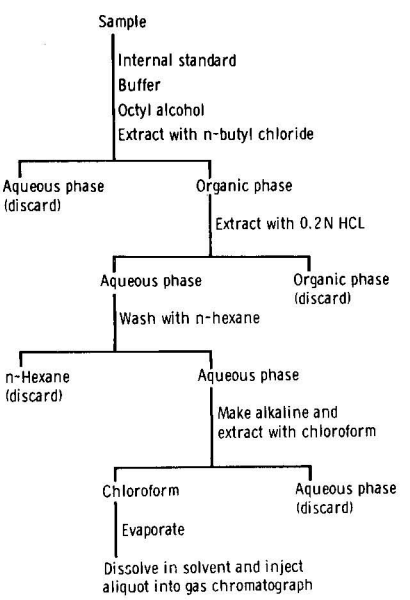

Fig. 2. A flow sheet describing the method for the isolation of acetylmethadol and metabolites from biofluids.

shaking for 7 min and centrifuging for 3 min under the said centrifugation conditions. The upper, organic, phase is removed by aspiration and discarded. The acid phase is washed by the addition of 5.0 ml of *n*-hexane and shaking for 5 min followed by centrifugation for 3 min. The hexane is removed by aspiration and discarded. The washed acid phase is adjusted to pH greater than 13 by the addition of four drops of 50% sodium hydroxide and incubated for 30 min in a heating block at 70° to effect the conversion of NAM and NNAM to their corresponding amides. The compounds are extracted into 8.0 ml of chloroform by shaking for 5 min followed by centrifugation for 3 min. The upper, aqueous, phase is removed by aspiration and discarded. The organic phase is transferred into a 12-ml siliconized centrifuge tube. The sample extract is concentrated by evaporating the organic phase to dryness with the use of a multiple flash evaporator with the bath at 50° (Evap-O-Mix®, Buchler, Fort Lee, N.J., U.S.A.). The sample extract is concentrated in the lower tip of the tube by rinsing the lower sides of the tube with 50 μl of chloroform and allowing this to evaporate. The sample extract is dissolved in 20 μl of chloroform and between 1 and 4 μl are injected into the gas chromatograph.

This extraction procedure is used for the isolation from plasma of AM as the free base and NAM and NNAM as their corresponding amides. NAM and NNAM are extracted from urine by the use of the above procedure. SKF 525-A is the internal standard at 250 μl per urine sample. AM is quantitated in urine according to the method of Kaiko and Inturrisi[9].

Recovery

The recovery of AM, NAM and NNAM from plasma was determined by adding 0.20 μg of each compound to 2.0 ml control human plasma and extracting as previously described. The amount of each compound recovered was compared to the amount obtained when 0.20 μg of each was added to 5.0 ml of 0.2 *N* hydrochloric acid and carried through the conversion and concentration procedures. Quantitation was achieved using gas–liquid chromatography (GLC) by the addition of internal standard to the acid phase in both cases.

The above procedure was also used to determine the recovery of 1.0 and 6.0 μg each of NAM and NNAM from urine.

Gas–liquid chromatography

The GLC analysis is performed on a Varian Model 1740 gas chromatograph equipped with a hydrogen flame ionization detector. The column is a 6-ft.-long spiral glass with an I.D. of 2 mm. The liquid phase is 3% SE-30 on Gas-Chrom Q, 80–100 mesh. The temperatures of the detector and injector port are 295 and 275°, respectively. Helium, at a flow-rate of 33 ml/min, is the carrier gas. The hydrogen and air flow-rates are adjusted to 17 and 273 ml/min, respectively, to give maximal detector response. A column oven temperature of 235° is used for the analysis. Detector sensitivity is varied from $4\text{–}64 \cdot 10^{-11}$ A/mV at full scale.

Calibration curves and quantitation

Standard calibration curves are generated by the addition of AM, NAM and NNAM in the selected amounts from 0.040–0.400 μg for AM and NNAM and from 0.100–0.400 μg for NAM to a 4.0-ml sample of control plasma and proceeding as de-

scribed above. Standard calibration curves for NAM and NNAM recovered from urine were established in the range from 1.00–6.00 μg per 4.0 ml urine. The peak height of the detector response to each compound is divided by the peak height of the internal standard to yield a ratio. Standard calibration curves are generated relating these peak height ratios to the amount of each compound added to the sample. Each calibration curve is constructed from duplicate or triplicate determinations of four to five different points. The amount of each compound in an unknown plasma or urine sample is determined by converting the peak height ratio obtained into the absolute amount of compound present in the sample. The linearity of the standard calibration curves within the range indicated allows the use of calculated slopes for these conversions.

Identification of compounds in biofluids

In addition to the identification of AM and its biotransformation products by the chromatographic systems on which they are separated for the purpose of quantitation, the presence of the compounds is confirmed by the use of other methods. A second GLC system is used for the identification of compounds present in plasma. Isobutane chemical ionization mass spectrometry is used similarly for urine.

Plasma samples from AM maintenance subjects are screened for AM, NAM, NNAM, MOL and NMOL in concentrations above 0.020 μg/ml. Standard calibration samples of each compound in concentrations ranging from 0.020–0.200 μg/ml are extracted through the plasma extraction procedure. The procedure is modified to prevent the conversion of NAM and NNAM to their corresponding amides. The final extraction is from 0.2 *N* hydrochloric acid adjusted to pH 7.4 by the addition of 1.0 ml of 10% ammonium hydroxide. The final extract is dissolved in 20 μl of trifluoroacetylimidazole in chloroform (1:4) to effect trifluoroacetyl derivatization of the biotransformation products of AM. The derivatized extract is analyzed on 3% SE-30. Each compound is separated from the others. The temperature of the column oven is increased 0.75 min after each injection at a rate of 10°/min from 210–260°. Other conditions are as in the "amide" method. The retention times are: MOL, 1.9 min; NMOL, 2.5 min; AM, 2.8 min; NNAM, 3.2 min; NAM, 3.5 min.

The identification of AM and its biotransformation products in urine from AM subjects is accomplished by use of chemical ionization mass spectrometry with isobutane as the reactant gas. Aliquots of 40 ml of urine are collected from a subject receiving 100 mg of AM three times per week. The urine is extracted and concentrated to 10-μl extracts.

These urine extracts are introduced directly into the mass spectrometer after removal of solvent. The spectra obtained from the urine extract are compared to that of a synthetic mixture of AM, NAM, MOL and NMOL. A characteristic of isobutane chemical ionization mass spectra is that relatively little fragmentation of compounds is observed and the spectra of most compounds consist of a protonated molecule [an ion at $m/e^{\star} = (M + 1)^{+}$] and one or two fragment ions[14]. With spectra of this degree of simplicity the components of a mixture can oftentimes be identified from a simple inspection of the mass spectrum of the mixture. The presence or absence

* *m/e* is the mass to charge ratio. Most ions have unit charge and thus, the value of *m/e* will be equal to the mass of the ion.

of the several peaks which are associated with each of the components may be taken as evidence for the presence or absence of the components.

Hydrolysis of conjugation products

For the determination of the presence of acid hydrolyzable conjugates of NAM and NNAM in urine from AM maintenance subjects, 0.2 ml of concentrated hydrochloric acid is added to 2.0 ml of urine and heated for 1 h at 121° under 18 p.s.i. in an autoclave according to the method of Mulé *et al.*[15]. Prior to the extraction of the bases, the pH is adjusted to 7.4 by the addition of ammonium hydroxide. Other samples are prepared as described above except that immediately after the addition of the acid the pH is readjusted to 7.4 and, thus, the samples are not hydrolyzed. A third set of samples is extracted according to the usual procedure and the concentrations of free NAM and NNAM obtained are compared to the concentrations obtained in the previous two sets of samples. Determinations are done in triplicate.

For the determination of glucuronide conjugates of NAM and NNAM according to the method of Mulé *et al.*[15], duplicate 1.0-ml urine samples are incubated with 1.0 ml of 0.5 *M* acetate buffer, pH 5.0, and 1.0 ml of a solution of β-glucuronidase (5000 Fishman Units per ml of acetate buffer) for 48 h at 37° with continuous gentle shaking. Duplicate 1.0-samples of the same urine are treated as above without enzyme added. After incubation and just prior to extraction, the pH is adjusted to 7.4 by the addition of 0.2 ml of 10% ammonium hydroxide.

Further analytical procedure

Infrared spectra in carbon tetrachloride solution were obtained on a Perkin-Elmer 257 grating infrared spectrophotometer.

Sample handling

Blood samples from an AM maintenance subject were drawn 4 and 48 h after an oral dose of 50 mg of AM. The heparinized blood was centrifuged and the plasma recovered. The excretion of AM and active biotransformation products was determined in urine collected from four subjects under treatment for opiate dependence during the 48-h period after an oral dose of AM. Plasma and urine samples were frozen at −20° until the day of analysis.

RESULTS AND DISCUSSION

During the course of the development of an extraction procedure it was observed that when NNAM is extracted from an aqueous solution at pH 9.8 or greater, less compound is recovered as measured by GLC analysis. At a pH of 13 (using 50% sodium hydroxide) the recovery of NNAM is negligible. The decreased recovery concurrent with an increased extraction pH coincided with an increased recovery of an unknown compound. This observation led to the suggestion that NNAM was being converted to another compound under highly alkaline conditions. Subsequently, NAM was also extracted under highly alkaline conditions and found to react similarly. At a pH of 13 with heating at 70° for 30 min NAM is quantitatively converted to another unknown compound. The alkaline conversion products of NAM and NNAM are of interest because of their potential use as alternative forms for the

separation and quantitation of NAM and NNAM. The chromatographic liquid phase, SE-30, does not separate NAM and NNAM from AM. However, AM and the alkaline conversion products of NAM and NNAM are all separable on SE-30.

Isobutane chemical ionization mass spectral analysis of NAM and its conversion product demonstrates that they are of equal mass ($m/e = 340$) but have slightly different fragmentation patterns. Infrared spectroscopic analysis of the alkaline conversion product of NAM indicates the absence of an ester and the presence of an alcohol and an amide function as would be expected of the proposed structure. There is no absorbance corresponding to that of the ester function of NAM, which exhibits characteristic bands at 1730 and 1240 cm^{-1}. A broad peak, attributable to a hydroxyl function, appears at 3400 cm^{-1}. An intense band at 1630 cm^{-1} provides evidence for the presence of an amide function. The conversion products of both NAM and NNAM are not extractable into 0.2 *N* hydrochloric acid from *n*-butyl chloride. The chemical nature of the newly formed compounds and the spectral features enumerated lead to the assignment of the amide structure. The amides would result from an intramolecular acyl shift as shown in Fig. 3. Two compounds of similar structure to NAM and NNAM, norpropoxyphene and dinorpropoxyphene, undergo such a rearrangement under highly alkaline conditions. McMahon *et al.*[16] showed that at pH 11 or above norpropoxyphene undergoes an intramolecular acyl shift.

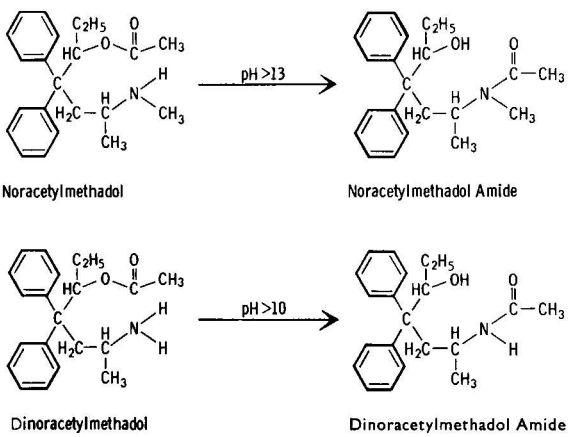

Fig. 3. The alkaline conversion of noracetylmethadol and dinoracetylmethadol to their corresponding amides.

The conditions of the extraction procedure developed allow the amines to be recovered from plasma or urine and then converted to amides by the addition of strong base at the last step in the extraction procedure. This is similar to the method for the determination of norpropoxyphene as described by Verebely and Inturrisi[17]. AM itself is unaffected by this procedure.

After correcting for aliquot losses, the mean recovery from control plasma based on the determinations was 105.0% ± 6.0 S.D. for AM, 86.0% ± 8.2 S.D. for NAM and 91.3% ± 4.6 S.D. for NNAM. After correcting for aliquot losses, the

mean recovery from control urine based on six determinations was 88.2% ± 6.8 S.D. for NAM and 93.0% ± 3.9 S.D. for NNAM. The recovery of the compounds was independent of concentration in the above range.

The method can be used to quantitate as little as 0.010 μg of AM and NNAM and 0.025 μg of NAM per ml of biofluid. The mean precision is 5% relative S.D. for triplicate determinations for each concentration of compound added to plasma and used as the standard calibration curves. The mean linear correlation coefficient for the standard calibration curves is 0.997. The mean precision is 1.7% relative S.D. for duplicate determinations for each concentration of NAM and NNAM added to urine. The mean linear correlation coefficient for the standard calibration curves is 0.995.

Trifluoroacetyl derivatization of extracts of plasma from AM maintenance subjects provided a preliminary screen for the compounds that achieve measurable plasma levels. Most plasma samples contained AM, NAM and NNAM in concentrations above 0.020 μg/ml. Concentrations of MOL and NMOL above this were not observed in any samples obtained from eight subjects drawn at 4, 8, 24, 48 and 72 h following a mean dose of 50 mg of AM. Combined gas chromatography–mass spectrometry analysis of extracts of plasma from AM subjects confirms the absence of methadol and any demethylated biotransformation products of methadol[18]. The trifluoroacetyl derivatization procedure is not used for the routine quantitation of any compound because of the nonlinearity of the standard calibration curves and the relative inconsistency between triplicate determinations compared to the system used. In addition, the need for an extra step, derivatization, is obviated.

TABLE I

PARTIAL CHEMICAL IONIZATION MASS SPECTRUM (ISOBUTANE) OF AN EXTRACT OF URINE FROM AN ACETYLMETHADOL MAINTENANCE SUBJECT

*m/e**	*Associated compound*	*Absolute intensity***
354	Acetylmethadol	676
340	Noracetylmethadol	2925
326	Dinoracetylmethadol	4600
312	Methadol	384
298	Normethadol	134

* Molecular weight of protonated nolecule.
** Arbitrary intensity units.

Table I lists the absolute intensity of the protonated molecular ion for each compound in the isobutane chemical ionization mass spectrum of the urine extract. All five bases were identified. The presence of an intense ion at *m/e* 326 corresponds to NNAM*. This is 14 mass units lower than the ion at *m/e* 340 observed for NAM. Fourteen corresponds to the mass of a methylene group. The two demethylated products of AM are, apparently, present in the urine in a high concentration relative to the other compounds.

* At the time of the mass spectral analysis a synthetic chemical standard of NNAM was not available. Subsequently, a standard was obtained, the mass spectral analysis of which revealed the protonated molecular ion at *m/e* 326.

The acid hydrolysis of urine samples obtained from AM maintenance subjects results in a decrease in the measurable concentrations of free NAM and NNAM compared to the non-hydrolyzed control samples. One would expect an increase in these concentrations if NAM and NNAM are excreted in the urine as acid-hydrolyzable conjugates (*e.g.*, glucuronide or sulfate). No change in concentration would have been observed had the compounds not been present as conjugates. The decrease in free base of both compounds might be explained on the basis that they are acid-labile under the rigorous conditions employed for the hydrolysis. β-Glucuronidase hydrolysis results in no change in the concentrations of free NAM and NNAM. This preliminary observation suggests that glucuronidation is not a major route of biotransformation for NAM and NNAM in the human. An alternate explanation is that the conjugates are excreted principally via the gastrointestinal tract.

Examples of chromatograms obtained under the conditions described in Materials and methods are given in Fig. 4. The multi-step solvent extraction procedure

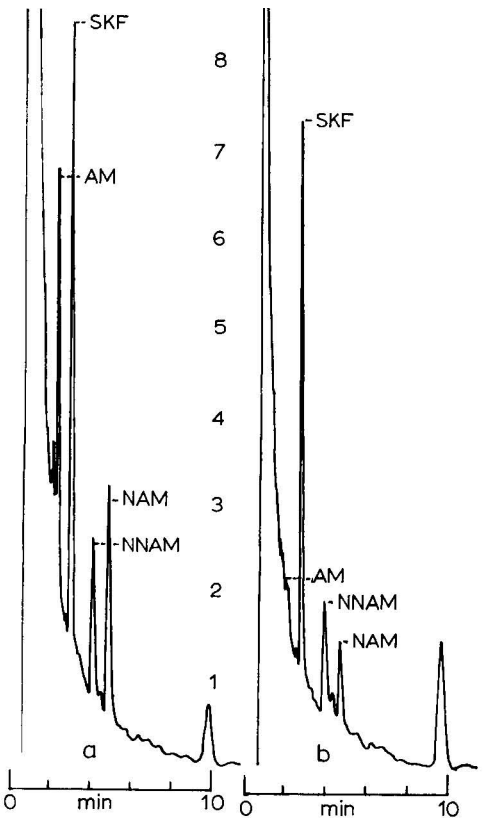

Fig. 4. Chromatograms of human plasma extracts from a patient who received an oral dose of 50 mg of acetylmethadol (AM). The internal standard, SKF 525-A, was added directly to the plasma and the extract prepared. Noracetylmethadol (NAM) and dinoracetylmethadol (NNAM) present in the extract were quantitatively converted to their corresponding amides just prior to the final step of the extraction procedure. (a) 4-h Post-drug plasma. (b) 48-h post-drug plasma. Retention times are: AM, 1.8 min; SKF, 2.5 min; NNAM, 3.6 min; NAM, 4.2 min.

results in an extract that is free of interfering peaks. In most cases it was possible to introduce samples into the gas chromatograph every 12 min. Fig. 4a shows a chromatogram of an extract of plasma collected 4 h after an oral dose of 50 mg of AM. The extract contains peaks that correspond in retention time to AM, NAM amide and NNAM amide in addition to the added internal standard, SKF 525-A. Fig. 4b shows the chromatogram of an extract of plasma collected 48 h after AM administration from the same subject. Still, NAM and NNAM are present while AM is not. The principle advantage of this method over that of Billings *et al.*[10] is that it allows the simultaneous determination of the parent compound, AM, along with its two major active biotransformation products, NAM and NNAM, in plasma. The method of Billings *et al.* is not applicable to the quantitation of the parent compound. In addition, the method presented here obviates the need for derivatization. However, the method of Billings *et al.* is more sensitive and thus may be more appropriately used for the determination of NAM and NNAM in plasma following initial AM doses.

Table II shows the percentage of administered dose recovered in the total 48-h post-drug urine as AM and biotransformation products. A mean of 28% with a range of 16.7–37.7% was recovered for four subjects. This included approximately 2% as AM, 8% as NAM, 5% as MOL (using the method of Kaiko and Inturrisi[9] for the quantitation of AM and MOL in urine), and 13% as NNAM. The pattern of excretion for each of the subjects is nearly the same despite a fivefold difference in administered dose.

TABLE II

PERCENTAGE OF ADMINISTERED ACETYLMETHADOL DOSE EXCRETED IN TOTAL 48-h URINE AS ACETYLMETHADOL AND BIOTRANSFORMATION PRODUCTS

Subject	*Dose (mg)*	*Acetyl-methadol*	*Noracetyl-methadol*	*Methadol*	*Dinoracetyl-methadol*	*Total*
L.M.	20	1.5	2.9	6.0	6.3	16.7
R.G.	50	1.4	15.0	2.9	18.4	37.7
G.E.	100	1.3	5.0	3.5	12.5	22.3
I.R.	100	2.8	10.1	6.8	15.5	35.2
Mean		1.8	8.2	4.8	13.2	28.0

Twenty-eight per cent of the administered dose of AM is excreted in the total 48-h dosing interval urine. It is likely that there are other routes of elimination for AM and that there are biotransformation products in addition to those discussed. Elimination via the gastrointestinal tract should be investigated. NAM and NNAM may be excreted as N-acetylated products[18]. These amides would not be extracted through the procedure described in this report. There is as yet no evidence that these amides or any possible biotransformation products in addition to those discussed here possess narcotic activity.

An incomplete recovery of the administered dose within a single dosing interval would be consistent with the suggestion that an equilibrium had not yet been attained between drug absorption and elimination in these subjects. Clearly, one would expect that the recovery of initial maintenance doses within a single dosing interval would

necessarily be quite low if these doses are to protect the subjects from abstinence symptoms for the complete duration of the dosing interval. There is evidence for a very slow rate of attainment of an equilibrium between absorption and elimination for acetylmethadol in the human[18]. The concentration of basic drugs by extravascular tissue is well documented[19]. It is likely that these tissues sequester the compounds to an extent capable of dramatically reducing their excretion. Once the tissues become "saturated" excretion increases. Dole and Kreek[20] have suggested that narcotic drugs which have high tissue-binding affinities would become longer acting in the suppression of abstinence once the reservoir of drug in tissues had been established upon repeated drug administration. Likewise, one might speculate that such drugs could be administered in considerably lower doses once the tissue reservoir had been adequately "saturated".

The results of these preliminary studies indicate that biotransformation is a prerequisite for the elimination of AM and that N-demethylation is quantitatively more important than deacetylation for AM in the human. Plasma contains measurable levels of only AM, NAM and NNAM (Fig. 5). Studies in animals[6–8] strongly suggest that NAM and NNAM are important in determining the time–action characteristics of certain effects of AM.

The methods we have described should prove valuable in the elucidation of the pharmacokinetics of AM in the human. We are currently completing the analysis of pharmacokinetic data in relation to the long duration of action of AM in maintenance subjects.

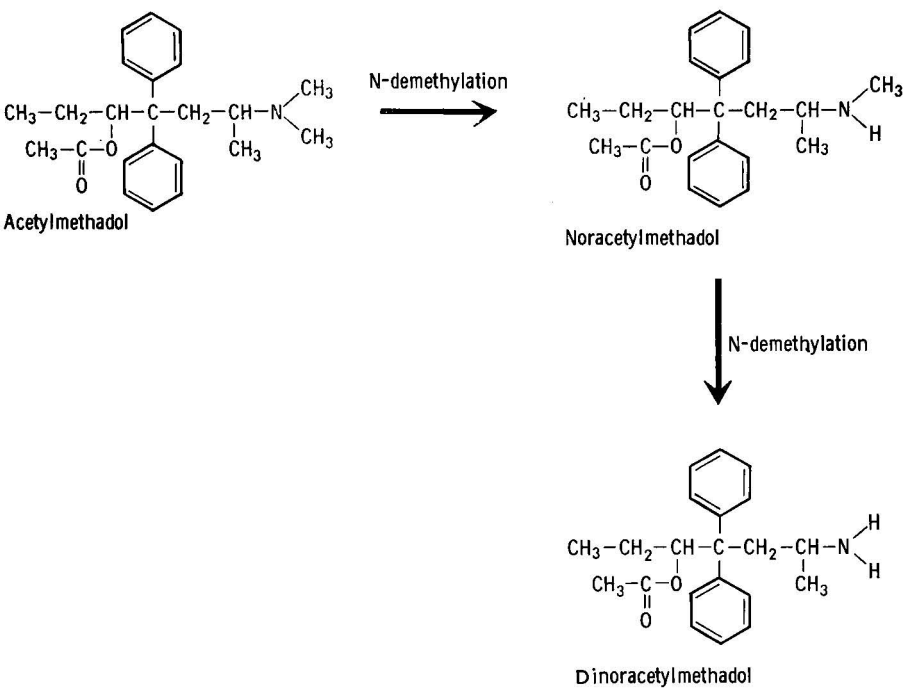

Fig. 5. Acetylmethadol and active biotransformation products, noracetylmethadol and dinoracetylmethadol, predominant in plasma from acetylmethadol maintenance subjects.

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CHROM. 8230

DETERMINATION OF PERPHENAZINE AND ITSSULPHOXIDE METABOLITE IN HUMAN PLASMA AFTER THERAPEUTIC DOSES BY GAS CHROMATOGRAPHY

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(Received January 23rd, 1975)

SUMMARY

A gas chromatographic method for the determination of perphenazine (Trilafon) and its main metabolite in human plasma, perphenazine sulphoxide, has been developed. The procedure involves the use of an electron capture detector and permits the determination of the drug and its metabolite at concentrations down to 0.2 μg/l. This is sufficient for analyzing plasma from patients on ordinary treatment with perphenazine. Tests for specificity revealed no interference by nortriptyline or biperidine.

INTRODUCTION

Until recently, the determination of neuroleptic drugs in human plasma has been possible only after high doses. Several methods have been tried in order to quantitate phenothiazines, but gas chromatography seems to be the most suitable[1–4].

Quantitation of perphenazine (PPZ, Trilafon) after normal doses requires an analytical method sensitive enough to measure concentrations in the range 0.2–5.0 μg/l in plasma. In order to achieve this sensitivity, some modifications were made in the method previously reported by us[3] and used in clinical investigations[5]. However, the analytical procedure did not permit the determination of the main metabolite in plasma, perphenazine sulphoxide (PPZSO).

Therapeutic control of plasma concentrations of the parent compound (PPZ) and of the main metabolite (PPZSO) might be important from a clinical point of view if a correlation between these concentrations and the effect could be established.

This paper describes a gas chromatographic method with a sensitivity sufficient to determine the concentrations of PPZ and PPZSO that occur after conventional single doses.

EXPERIMENTAL

Reagents and glassware

Toluene of analytical-reagent grade from E. Merck (Darmstadt, G.F.R.) was distilled once before use. Undiluted borate buffer (Titrisol buffer) of pH 10 (Merck) was used to buffer the solutions. For derivatization, N,O-bis-(trimethylsilyl)acetamide (BTSA) of specially purified grade from Pierce (Rockford, Ill., U.S.A.) was used. Glassware was cleaned with detergent in an ultrasonic bath and rinsed twice with redistilled water and once with methanol.

Reference substances

Structural formulae are shown in Fig. 1.

Stock solutions (1 g/l) in ethanol were prepared of PPZ, PPZSO and of the internal standard, 4-[3-(2,8-dichlorophenothiazin-10-yl)propyl]-1-piperazinethanol (8-chlorinated PPZ, CPPZ). The solutions should be kept in a refrigerator and protected from light. When stored in this way, the solutions are stable for 1 year.

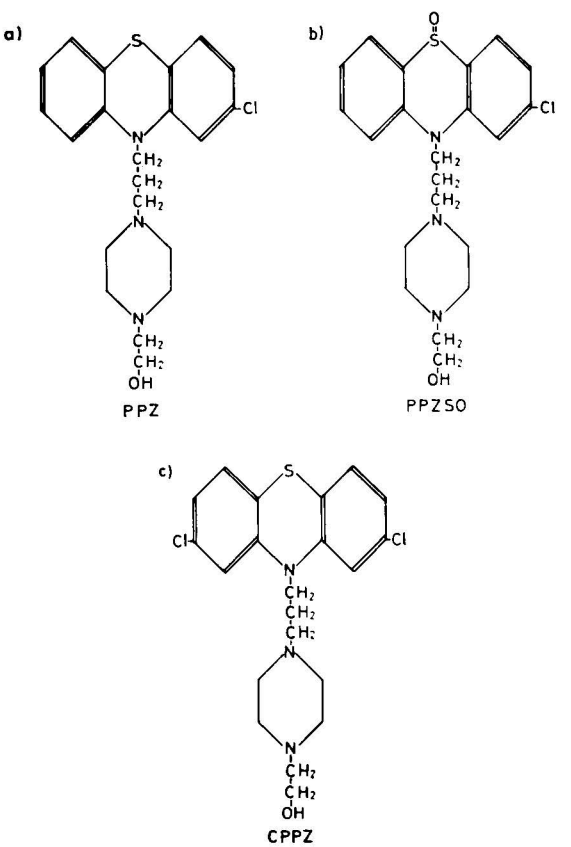

Fig. 1. Structural formulae of a, perphenazine (PPZ), b, perphenazine sulphoxide (PPZSO) and c, the internal standard (CPPZ).

Extraction procedure

To a centrifuge tube containing 2.5 ml of plasma, 5 ng of the internal standard (CPPZ) are added. The sample is buffered to pH 10 by adding 250 μl of borate buffer and extracted with 6 ml of toluene by mixing for 10 min in a rotary mixer (20–30 rpm). After centrifugation for 5 min, the organic phase is transferred into a 10-ml glass-stoppered tube containing 1 ml of 0.1 *N* sulphuric acid. The compounds are extracted into the aqueous phase by mixing for 10 min. After centrifugation, the organic phase is discarded. The buffered plasma sample is extracted once more with 6 ml of toluene, which is washed with the same portion of sulphuric acid. After centrifugation, this organic phase is also discarded. The aqueous phase is washed with 6 ml of fresh toluene. After centrifugation, the organic phase is discarded. The aqueous phase is made alkaline by adding 100 μl of 4 *N* sodium hydroxide solution. The compounds are extracted into 3 ml of toluene by mixing for 10 min in a rotary mixer. After centrifugation, the organic phase is transferred into a tapered tube and evaporated to dryness at 70° under a stream of nitrogen. The alkaline aqueous phase is extracted once more with 3 ml of toluene. This phase is transferred into the same tapered tube and likewise evaporated to dryness. The residue is dissolved in 1.5 ml of toluene and derivatized by adding 50 μl of a mixture consisting of 100 μl of BTSA in 10 ml of toluene. This mixture is made to react at 70° for 10 min. After reaction, the solvents are evaporated at 70° under a stream of nitrogen and the residue is dissolved in 30 μl of toluene. A volume of 1.5 μl of this solution is injected into the gas chromatograph.

Gas chromatography

A Pye Series 104, Model 74, gas chromatograph equipped with an electron capture detector was used. The pre-heater temperature was 310°, the column temperature 305° and the detector temperature 350°. A glass column, 1.5 m $\times$ 4 mm I.D. packed with 1% (w/w) OV-17 on Celite JJ CQ, 100–120 mesh, was used. The amount of column material was 12 g. The column was conditioned at 350° for 65 h. The carrier gas (argon–methane, 90:10) flow-rate was 60 ml/min. The pulse interval was 150 μsec and attenuation 5×10^2. The column was deactivated with hexamethyldisilazane and injections of ethyl acetate extracts of blank plasma.

Calculations

The plasma concentrations are read from standard curves constructed from chromatograms of plasma samples containing varying but known amounts of PPZ and of PPZSO (Fig. 2). Both compounds are added in amounts from 2.5 to 12.5 ng, corresponding to concentrations from 1.00 to 5.00 μg/l. The peak height ratios between PPZ and CPPZ, and between PPZSO and CPPZ, are plotted against the concentrations (Fig. 3).

RESULTS AND DISCUSSION

The presence of PPZSO in human urine after long-term treatment with PPZ was demonstrated about 10 years ago[6]. If this metabolite is active, which some preliminary results seem to indicate (unpublished work), it must be of importance to correlate the plasma levels of both this metabolite and the parent compound with the efficacy and side-effects. This is the reason for the development of the present method.

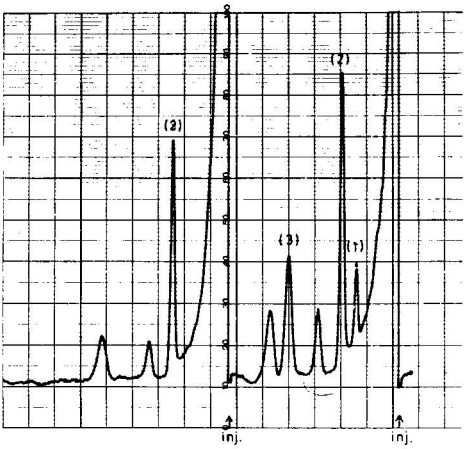

Fig. 2. Chromatograms of two plasma samples. The sample in the right-hand chromatogram contained PPZ (1), PPZSO (3) and the internal standard CPPZ (2). The left-hand chromatogram illustrates a blank plasma with added CPPZ (2).

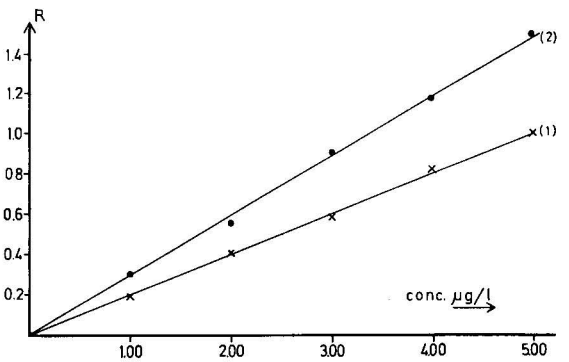

Fig. 3. Calibration graph constructed on the basis of chromatograms from plasma samples containing varying but known amounts of PPZ (1) and of PPZSO (2).

At first, we tried to apply the procedure described by Hansen and Larsen[5] to plasma samples drawn from patients receiving ordinary doses of PPZ. However, this method involves derivatization with acetic anhydride, and the procedure was evidently not suitable for the determination of PPZSO. It subsequently proved impossible to acetylate S-oxidised phenothiazines because of a displacement of the unsaturated electron bonds resulting from degradation of the aromatic ring system (unpublished work). By introducing N,O-bis-(trimethylsilyl)acetamide (BTSA) instead, stable trimethylsilyl derivatives of PPZ and of PPZSO were produced.

PPZ was previously shown to be quantitatively extracted with toluene from aqueous alkaline solutions[5]. Extraction recoveries with toluene carried out as described[5] of PPZ and of PPZSO were found to be 100% only for perphenazine. In order to obtain 100% recovery of all three compounds, it is necessary to repeat all toluene extractions. No loss was recorded on washing the sulphuric acid phases with the organic solvents.

Accuracy tests for PPZ and PPZSO when present in varying concentrations were performed on twenty plasma samples within the therapeutic concentration range and found to be ± 10% for both substances. Details are given in Table I. The lower limit for quantitation (sensitivity) was found to be 0.2 μg/l when a plasma volume of 2.5 ml was used.

TABLE I

ACCURACY TEST FOR PERPHENAZINE (PPZ) AND PERPHENAZINE SULPHOXIDE (PPZSO) FROM PLASMA

The plasma volume extracted was 2.5 ml in each instance.

Concentration added (μg/l)		*No. of samples, n*	*Calculated concentration (μg/l)**	
PPZ	*PPZSO*		*PPZ*	*PPZSO*
1.00	1.00	10	0.98 ± 0.05	1.04 ± 0.10
5.00	5.00	10	5.05 ± 0.07	5.01 ± 0.02

* Mean ± standard deviation.

Schizophrenic patients are often treated with other drugs in addition to PPZ [*e.g.*, nortriptyline and biperidine (Akinetone)]. The specificity of the method was therefore examined in the presence of these drugs added to plasma samples with known therapeutic PPZ concentrations. Details are given in Table II. As clearly demonstrated, none of these drugs obviously interferes with the determination of PPZ or PPZSO.

TABLE II

SPECIFICITY OF THE METHOD DEMONSTRATED IN MIXTURES OF DRUGS IN PLASMA SAMPLES

Results are given as concentrations (μg/l).

Perphenazine		*Perphenazine sulphoxide*		*Nortriptyline*		*Biperidine*	
Added	*Recovered*	*Added*	*Recovered*	*Added*	*Recovered*	*Added*	*Recovered*
1.00	1.05	1.00	1.05	100	—	20	—
2.00	1.90	2.00	2.05	100	—	20	—
3.00	3.10	3.00	2.90	100	—	20	—
4.00	4.05	4.00	3.95	100	—	20	—
5.00	4.90	5.00	5.05	100	—	20	—

In order to ensure that unknown metabolites probably formed *in vivo* are not co-determined, an attempt to check the specificity was made by means of mass fragmentography. However, the sensitivity turned out to be grossly insufficient, as 2.5 ng had to be injected in order to produce a reliable response. Consequently, specificity tests had to be carried out on the gas chromatograph with the use of various derivatives. It is difficult to produce stable compounds related to PPZSO except with BTSA and, furthermore, underivatized PPZSO is unsuitable for gas chromatography. It was therefore necessary to limit the specificity tests to PPZ. In pooled plasma samples

drawn from patients treated with PPZ the concentrations were determined in the following ways: (1) underivatized, (2) acetylated and (3) silylated (BTSA). The determinations turned out to give identical results indicating a high probability for specific PPZ determinations.

To illustrate the application of the method, the concentrations of PPZ and PPZSO were determined in plasma samples from a patient treated with perphenazine enanthate. The concentration curves are shown in Fig. 4.

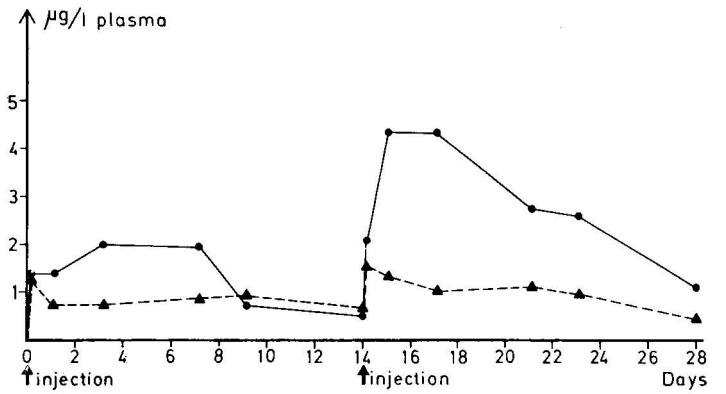

Fig. 4. Fluctuations in PPZ (●) and PPZSO (▲) concentrations in plasma. On each of the days 0 and 14, 100 mg of perphenazine enanthate (Trilafon enanthate) were given intramuscularly.

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CHROM. 8241

DUAL COLUMN GAS CHROMATOGRAPHIC SYSTEM FOR USE IN MASS SPECTRAL DETERMINATION OF NITROSAMINES

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(Received January 21st, 1975)

SUMMARY

A packed gas chromatographic column and a support coated open tubular (SCOT) column are connected in series. Between the columns are two micro-volume switching valves, one enabling solvent to be vented. Short retention nitrosamines are passed through both columns, whereas longer retention nitrosamines by-pass the SCOT column by means of the other switching valve.

INTRODUCTION

The presence of traces of volatile nitrosamines in cured meat and related products is now established[1–5] and techniques for their detection on a routine basis are available[6–12]. It is generally accepted that confirmation of the presence of nitrosamines must be carried out using combined gas chromatography and mass spectrometry (GC–MS) and papers describing suitable procedures have been published[9,11–13]. In view of the high capital and running costs of a mass spectrometer, it is essential that the analysis time for nitrosamine determination is kept to a minimum. In addition, the spectrometer must not be contaminated by extraneous eluted material from food extracts, which would preclude the immediate use of the spectrometer for other work. In a previous communication from this laboratory[13], rapid analysis time and minimal contamination was achieved using a combined pressure-programming and GC peak venting system. An alternative and equally effective means of satisfying these two criteria, without the need to build ancillary GC apparatus, is described below.

EXPERIMENTAL

A Pye Model 104 gas chromatograph, interfaced to an AEI MS 902 mass spectrometer with a silicone membrane separator[9] is used. The chromatograph is fitted with two flame ionisation detectors (D1 and D2). One is connected to the separator to monitor material entering the mass spectrometer, and the other although not mandatory is useful for monitoring vented material. It also offers a convenient means of destroying potentially harmful compounds which could otherwise enter the labora-

tory air. Two stainless-steel columns (C1 and C2) are connected in series, between which are placed two micro-volume four-port switching valves (V1 and V2). Details of the arrangement are shown in Fig. 1. Column C1 is 1.8 mm I.D. × 1.6 m, packed with 15% Carbowax 20M on 80–100 BS mesh Chromosorb W AW DMCS. Column C2 is a SCOT column, 0.5 mm I.D. × 30 m, containing Carbowax 20M. Each switching valve can be operated in two modes, one shown by the full line and the other by the pecked line, on Fig. 1. In the parallel reference circuit, restrictors R1 and R2 are short lengths of compressed steel tubing exerting back pressures equal to C1 and C2, respectively. This ensures that in whatever mode the valves are operated, the flow-rates within the GC columns and reaching the separator and flame detectors are unchanged. Equal flow-rates of helium are introduced at positions I and II. Position I is the sample injection port. Helium is also introduced at positions III and IV, as a make-up gas to optimise flame detector performance. Note that the make-up gas to detector D1 must be introduced after the separator to maintain a high transfer efficiency to the mass spectrometer.

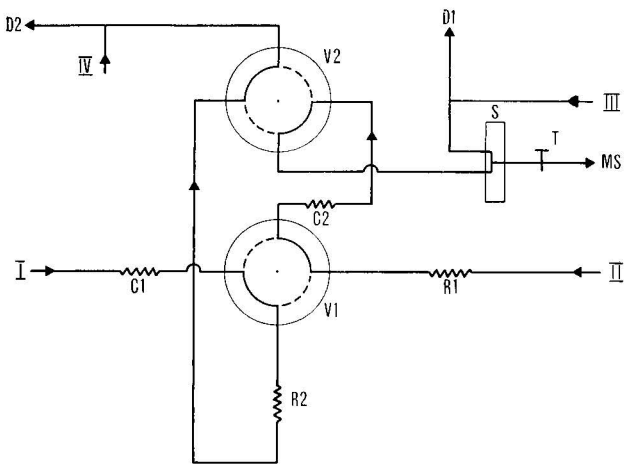

Fig. 1. Diagram of flow system. I–IV = Helium inlets; C1, C2 = columns; V1, V2 = switching valves; D1, D2 = flame ionization detectors; S = GC–MS interface; MS = mass spectrometer; T = GC–MS isolating tap; R1, R2 = restrictors.

It has previously been shown that with a single-column system connected to the separator, the mass spectrometer source pressure is typically 2×10^{-6} torr. Incorporating the micro-valves results in an unacceptably high pressure which is attributed to leakage from the atmosphere across the valve faces. Since there is little chance of eliminating such leaks, the valves were built into sealed chambers, pressurized with helium. Thus only helium is able to leak into the GC–MS system, giving rise to an acceptable pressure. Valves with this facility are available commercially (Carle, Fullerton, Calif., U.S.A.; Switching Valve type 2011P; distributed in Great Britain by Techmation, Edgware).

There are four possible modes of operation of the system, the appropriate mode being manually selected outside the GC oven. These are summarized below: Mode A, Column C1 to vent (detector D2); Mode B, Columns C1 and C2 to detector

TABLE I

GC OPERATING CONDITIONS

Chromatograph	Pye Model 104
Detectors	flame ionization
Carrier gas	helium
Carrier gas flow-rate, ml/min.	4
Make-up gas flow-rate, ml/min.	15
Temperatures, °C	
Injection port	160
Columns	140
Detectors	200
Transfer line	160
Separator	140
Sample size, μl	5
Detection limit (mass spectrometer), μg/ml	1

D1 and mass spectrometer; Mode C, Column C1 to detector D1 and mass spectrometer; Mode D, Columns C1 and C2 to vent (detector D2). Fig. 1 shows the system in Mode A.

In a typical run the valves are set to Mode A so that no solvent or other extraneous material reaches the mass spectrometer, but the flow is monitored by flame detector D2. After solvent elution, Mode B is selected and the compounds are resolved on columns C1 and C2 prior to reaching the flame detector D1 and the mass spectrometer. For long retention materials or for large amounts of a single compound which would overload the SCOT column (C2), Mode C may be used, in which material passes only through column C1 before reaching the mass spectrometer. Mode D is used for GC runs in which the mass spectrometer is not required, leaving it free for other work.

GC operating conditions are given in Table I, and retention and mode changing data in Table II. Mass spectrometer operating conditions and details of the detection procedure for mass spectrometry have previously been published[9].

TABLE II

ANALYSIS TIME DATA

Event	*Time after injection*
Mode A	zero
Solvent elution	0 min 30 sec
Select Mode B	2 min 0 sec
N-Nitrosodimethylamine elution	7 min 0 sec
N-Nitrosodiethylamine elution	9 min 0 sec
N-Nitrosomethyl-*n*-propylamine elution	11 min 0 sec
N-Nitrosodipropylamine elution	15 min 0 sec
Select Mode C	18 min 0 sec
N-Nitrosopiperidine elution	21 min 30 sec
N-Nitrosopyrrolidine elution	26 min 30 sec
Select Mode A	28 min 0 sec

DISCUSSION

Nitrosamines have been found in cured meat products up to the μg/kg level and all current techniques for their detection require a substantial concentration and clean-up of the substrate extract prior to analysis. The procedure used in this laboratory involves steam distillation of an aqueous suspension of the comminuted foodstuff followed by solvent extraction and evaporation[14]. This results in a thousandfold increase in the concentration of the nitrosamines. Chromatography of such an extract shows many compounds, some of which are present in amounts greatly exceeding those of the nitrosamines, and in many instances only partially resolved from them. Such interferants can result in undesirable contamination of the mass spectrometer, although they do not adversely affect the ability to confirm the presence of nitrosamines in a mass spectrometer operating under high resolution.

The use of capillary columns to obtain better separation of nitrosamines from extraneous material appears attractive, but has the disadvantage that the lower sample capacity of these columns results in a correspondingly poorer detection limit. This may be as much as two orders of magnitude higher than that obtained using a packed column. Support coated open tubular (SCOT) columns offer a superior performance to packed columns, but with a less restrictive sample capacity than capillary columns. Column overloading is caused predominantly by the solvent, rather than by material co-eluted with the nitrosamines. By injecting on to a packed column, venting the solvent, and allowing the remaining constituents of the extract to pass on to a SCOT column, good resolution of the nitrosamines from other co-extracted compounds may be achieved, without any adverse effect on the detection limit. An additional advantage accrues from the use of a two-column system. Retention times of the lower nitrosodialkylamines are somewhat shorter than those of the volatile heterocyclic nitrosamines, which thus dictate overall GC–MS analysis time. Temperature programming, which is used by some workers[15], is excluded on the grounds that there is a significant equilibration time between successive runs. Pressure programming, favoured by this laboratory[13], virtually eliminates the inter-run equilibration period but requires specially built ancillary equipment. Using a two-column system, N-nitrosodimethylamine and other low retention nitrosodialkylamines are passed through both columns, whereas long retention nitrosamines are allowed to pass only through the first column.

Most work has been centred on N-nitrosodimethylamine, N-nitrosodiethylamine, N-nitrosodipropylamine, N-nitrosopiperidine, and N-nitrosopyrrolidine. The apparatus described herein is designed predominantly for the determination of these compounds, but has general applicability in the volatile organic trace analysis field.

In the standard procedure used in this laboratory for nitrosamine determination[16] N-nitrosodipropylamine is incorporated as an internal standard to check recoveries from the clean-up process. The nitrosamines are detected in the mass spectrometer by parent ion monitoring as previously described[9], necessitating a time difference of at least 2 min between the elution of each nitrosamine for resetting the mass spectrometer. The GC conditions used in the present work satisfy these criteria.

It has become apparent during the examination of a wide range of foodstuffs that by far the most frequently occurring nitrosamines are N-nitrosodimethylamine and N-nitrosopyrrolidine. The use of N-nitrosomethyl-*n*-propylamine as an alter-

native internal standard enables the same reference fragment to be used in the mass spectrometer for peak matching purposes for the detection of the standard itself, the N-nitrosopyrrolidine and if necessary N-nitrosodiethylamine. Thus the mass spectrometer requires less frequent resetting during such a run.

CONCLUSIONS

The dual column system described herein enables volatile nitrosamines to be resolved and rapidly eluted from a gas chromatograph, prior to on-line mass spectrometric detection. The GC peak switching facility enables large volumes of extracts of foodstuffs to be injected without adversely affecting the performance of the SCOT column and mass spectrometer.

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TRACE ANALYSIS OF VOLATILE N-NITROSO COMPOUNDS BY COMBINED GAS CHROMATOGRAPHY AND THERMAL ENERGY ANALYSIS

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SUMMARY

Thermal energy analysis (TEA) has been combined with gas chromatography (GC). The new GC–TEA technique is highly specific to compounds which contain heat labile nitrosyl groups. Because of the specificity of the technique, full use may be made of the TEA sensitivity. Analysis by direct injection of solutions containing less than 1 ng/ml N-nitroso compound is demonstrated.

INTRODUCTION

Quantitative and selective gas chromatographic (GC) analysis procedures for volatile nitrosamines at the sub-μg/ml level, using either the alkali flame ionization detector or the Coulson electrolytic conductivity detector have been described[1–3]. When combined with adequate clean-up techniques, including concentration by factors of over 1000, these procedures can be used for μg/kg analysis of certain nitrosamines in foodstuffs. Elaborate clean-up and concentration procedures, apart from being tedious and costly, greatly limit the number of compounds which may be screened. In addition, it is never certain whether the nitrosamine concentrations which are found are due to artifacts of the clean-up procedures themselves. For these reasons, an N-nitroso compound specific, ultra sensitive GC detector would be of major value in evaluating the importance to human cancer of N-nitroso compounds in the environment.

Thermal energy analysis (TEA) has been shown[4–6] to be selective to N-nitroso compounds at the sub-ng/ml level. This paper describes the interfacing of a thermal energy analyzer with a gas chromatograph, the combined GC–TEA system retaining all the sensitivity and selectivity characteristics inherent in both GC and TEA.

APPARATUS

The principle of operation[6] and the detailed design parameters[7,8] of the TEA detector are described elsewhere.

The GC–TEA interface is shown schematically in Fig. 1. The GC effluent is introduced directly into the TEA catalytic pyrolyzer. In order to prevent condensa-

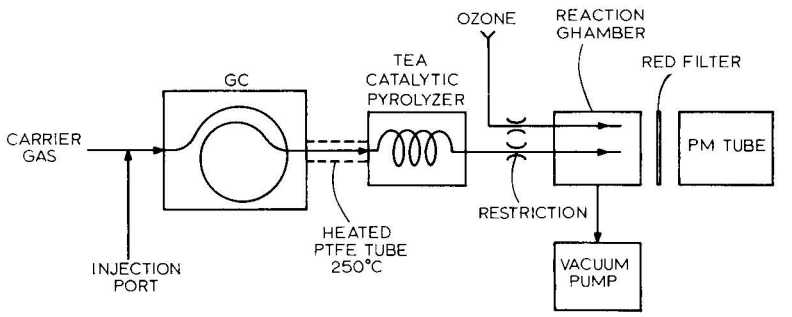
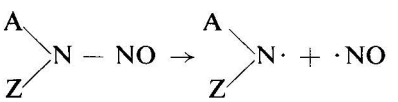


Fig. 1. Scheme of GC–TEA interface.

i on or adsorption of non-volatile components, it is preferable to have the TEA pyrolyzer joined directly to the GC column. If this is not possible, a heated PTFE line maintained above 200° may be used. Stainless-steel interconnecting tubing is not suitable unless the inside walls have been chemically polished so as to minimize sites for radical adsorption. Using a selective catalyst, the TEA pyrolyzer selectively pyrolyzes N-nitroso compounds, splitting off the nitrosyl radical:



where A and Z may be any organic radical. After the TEA pyrolyzer, the effluent is expanded through a narrow constriction into an evacuated reaction chamber, where the nitrosyl radical is allowed to react with ozone, giving electronically excited nitrogen dioxide:

$$\cdot NO + O_3 \rightarrow NO_2^* + O_2$$

The excited nitrogen dioxide rapidly relaxes to its ground state with the emission of light in the near infrared region of the spectrum:

$$NO_2^* \rightarrow NO_2 + h\nu$$

The emitted light is monitored by means of an infrared sensitive photomultiplier tube, the intensity of the emission being proportional to the number of nitrosyl radicals present.

Following Palframan[2] *et al.*, a chromatographic column was prepared from 6.5 m × 2 mm I.D. stainless-steel tube packed with 15% free fatty acid phase (FFAP) (15 g FFAP on 100 g Chromosorb W AW DMCS, 80–100 mesh) and conditioned for 36 h at 250° with carrier gas flowing prior to use. The column was operated isothermally at 185°, with a carrier gas flow-rate in the range of 10–30 ml/min. Commercial grade argon or helium was used as carrier gas with equal success. Although the gas bottles do contain traces of nitric oxide (at ppb* levels), the TEA response due to the

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

nitric oxide in the carrier gas is readily accounted for by adjusting the instrument zero. Purification of the carrier gas was not required, even for direct sensitivity at the sub-ng/ml level.

EXPERIMENTAL

Mixtures containing seven N-nitroso compounds, *viz.*, dimethyl nitrosamine (DMN), diethyl nitrosamine (DEN), dipropyl nitrosamine (DPN), dibutyl nitrosamine (DBN), N-nitroso piperidine (PIP), N-nitrosopyrrolidine (PYRN), and N-nitroso sarcosinate (SARCOSN), in dichloromethane (DCM) solvent were made up gravimetrically. Confirmation of the structure of all the N-nitroso compounds used was carried out by GC–mass spectrometry in the laboratories which supplied the chemicals. The solvents were used as received without further purification. In addition, for ease of comparing the data presented here with other techniques used in other laboratories, dilute solutions were made up from a standard mixture distributed for collaborative purposes by the International Agency for Research in Cancer (IARC) of the World Health Organization in Lyon, France. The IARC mixture contained 23.9 μg DMN/ml, 25.3 μg DEN/ml, 29.0 μg DBN/ml and 30.0 μg PYRN/ml in DCM.

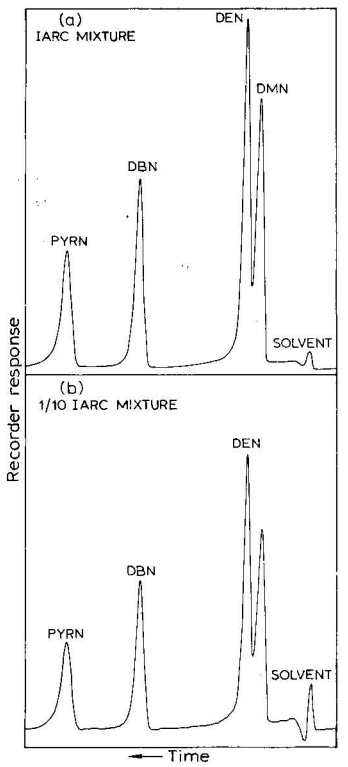
RESULTS AND DISCUSSION

Linearity

A chromatogram of 10 μl of the IARC standard solution is shown in Fig. 2a. Figs. 2b and 3a are chromatograms of 10 μl of DCM solutions obtained by 10-fold and 100-fold dilution of the IARC standard solutions. The chromatogram of 100 μl of a 1000-fold dilution of the IARC standard is shown in Fig. 3b, and a chromatogram of 500 μl of a 10,000-fold dilution is shown in Fig. 3c. Except for DMN and DEN in the 500 μl injection of the 10,000-fold dilution, all the chromatograms are clearly discernible. Even for the 10,000-fold dilution, in which each N-nitroso compound is present at less than the 3-ng/ml concentration level, the DBN and PYRN GC peaks are clearly visible above the noise level. A calibration plot for DMN, DEN, DBN and PYRN, obtained from the peak heights in Figs. 2 and 3 is shown in Fig. 4. The calibration is linear over five orders of magnitude, indicating that with the GC–TEA technique, quantitative analysis at the sub-3-ng/ml N-nitroso compound concentration level (30×10^{-12} moles/ml N-nitroso compound) is clearly feasible.

Sensivity

A chromatogram of 5 μl of the DCM standard solution containing each of the seven N-nitroso compounds at the 1 μg/ml concentration level (approximately, 1 ppm) is shown in Fig. 5. The chromatogram of 100 μl of the standard DCM solution diluted 200-fold, with each N-nitroso compound at the 5 ng/ml level (approximately 5 ppb) is shown in Fig. 6a. All seven GC peaks are seen to be clearly discernible. Fig. 6b is the chromatogram of 200 μl of the standard solution diluted 2000-fold, with each N-nitroso compound at the 500-pg/ml level (approximately 500 parts per trillion). Due to the extraordinary large amount of material injected onto the column, the baseline is seen to tail badly; nevertheless, the GC peaks of DMN, DEN, DPN



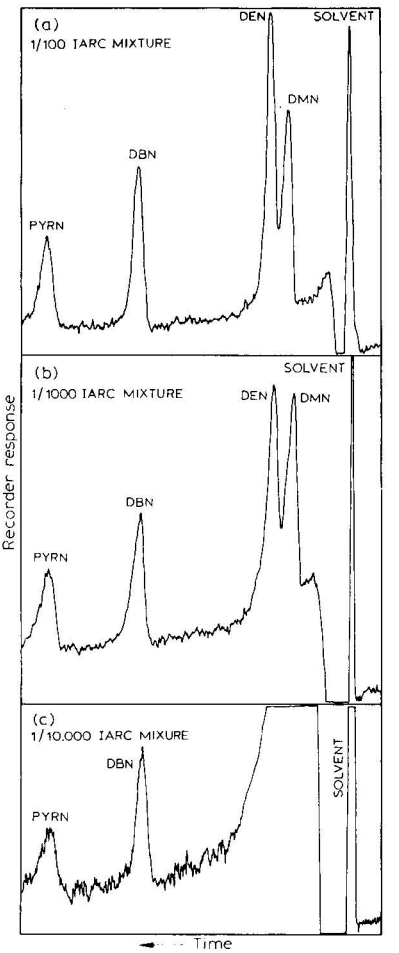


Fig. 2. (a) Chromatogram of 10 μl of IARC mixture containing 23.9 μg DMN/ml, 25.3 μg DEN/ml, 29.0 μg DBN/ml and 30.0 μg PYRN/ml in DCM. (b) Chromatogram of 10 μl of IARC mixture diluted 10-fold.

Fig. 3. Chromatograms of (a) 10 μl of IARC mixture diluted 100-fold; (b) 100 μl of IARC mixture diluted 1000-fold; (c) 500 μl of IARC mixture diluted 10,000-fold.

and SARCOSN are clearly identified above the noise level. The GC peaks of DBN, NIP and PYRN are not ambiguously displayed. A concentration level of 500 pg DMN/ml, corresponding to less than 1×10^{-12} moles N-nitroso compound introduced into the gas chromatograph, must therefore represent the practical detection limit of the GC–TEA technique as described here. Detection at levels below 1×10^{-12} moles N-nitroso compound is possible if steps are taken to remove the solvent, either by conventional temperature programming techniques or by freezing out the solvent in a cold trap placed between the TEA catalytic furnace and the TEA reaction chamber.

Because N-nitroso compound concentrations determined by direct injection at the 500-pg/ml level are so small as to be probably irrelevant (below the so-called

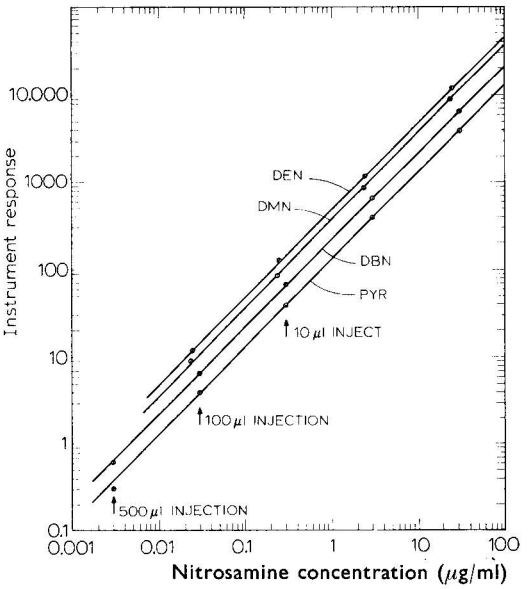


Fig. 4. GC–TEA calibration for DMN, DEN, DBN and PYRN calibration data taken from Figs. 2 and 3.

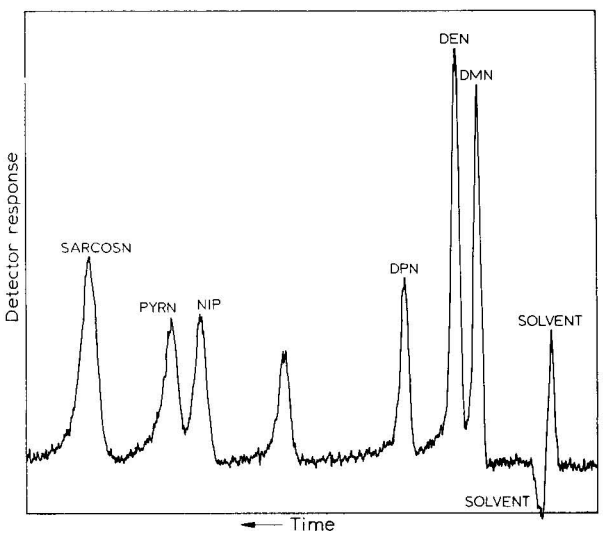


Fig. 5. Chromatogram of 5 μl of DCM solution containing 1 μg/ml of each of the following N-nitroso compounds: DMN, DEN, DPN, DBN, NIP, PYRN and SARCOSN.

"no effect" level) in terms of potential carcinogenic activity[9], we believe that efforts to enhance the direct sensitivity beyond what is reported here would be of limited practical value. Furthermore, if existing clean-up and concentrating procedures were used in conjunction with the GC–TEA system as reported here, detection by direct injection at the 500 fg/ml (5 parts per 10^{13}) concentration level would be possible.

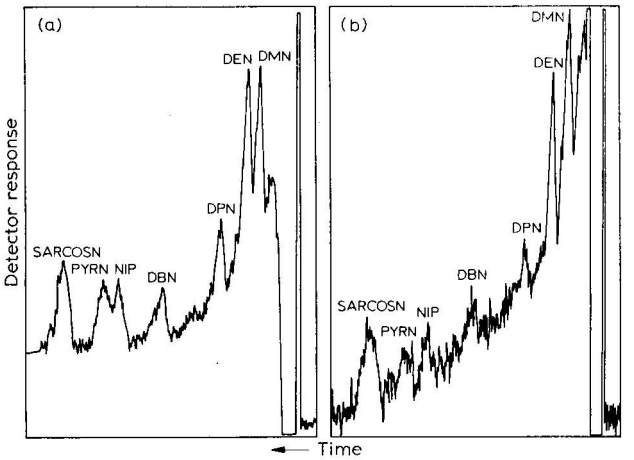


Fig. 6. Chromatograms of (a) 100 μl of DCM solution containing 5 μg/ml of DMN, DEN, DPN, DBN, NIP, PYRN and SARCOSN; (b) 200 μl of DCM solution containing 500 pg/ml of DMN, DEN, DPN, DBN, NIP, PYRN and SARCOSN.

Selectivity

The TEA detector is selective because it requires that the compounds be catalytically pyrolyzed at a low temperature to give a nitrosyl radical. The nitrosyl radical then reacts with ozone to produce light in the near infrared region of the spectrum Many other compounds also react with ozone to produce a luminescence, but the wavelength of the luminescence is in the blue or visible region of the spectrum. Thus, although a compound like carbon monoxide or ethylene produces an intense blue glow with ozone, the "blue" photons are screened out by the red optical filter. Many organic compounds with different functional groups have been evaluated for possible positive and negative interference[8], but to date none has been found.

At temperatures below $-150°$, the vapor pressure[10] of all but the lowest molecular weight species are substantially less than 1 torr. The nitrosyl radical, on the other hand, has a vapor pressure in excess of 760 torr at $-150°$. Thus, if compounds which are not N-nitroso are found which react with ozone to produce a luminescence in the near infrared, they may be removed by freezing them out in a cold trap placed between the TEA catalytic pyrolyzer and the TEA luminescent reaction chamber. Organic nitrites are extremely heat labile, and are readily distinguished on the GC–TEA because they decompose in the hot GC injection port (200°) or in the hot (185°) GC column, resulting in a large initial peak coincident with the solvent front. A similar behavior is observed with compounds such as N-nitrosodiphenylamine, N-nitroso urethane, and all the N-nitroso ureas.

Solvent front

As may be seen from Figs. 2, 3, 5 and 6, the GC–TEA solvent response becomes increasingly important as the injection volume is increased. Figs. 7a, b, c and d show the GC–TEA response for the injection of 25 μl, 15 μl, 10 μl and 5 μl of pure DCM, respectively. Three distinct effects are observed: There is an initial sharp positive peak, followed by a broader negative peak, and lastly, the negative peak is seen to overshoot

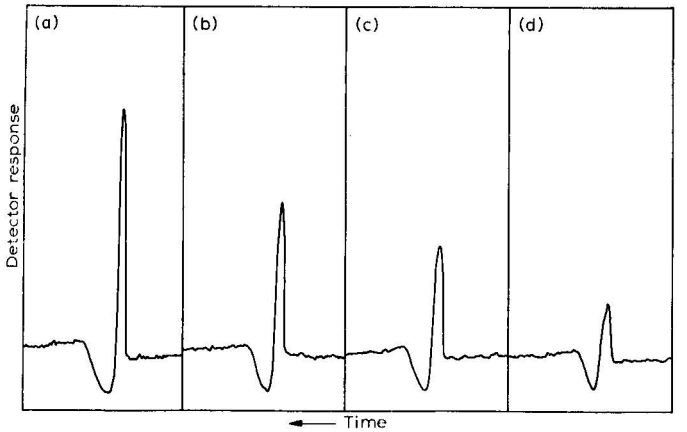


Fig. 7. Chromatograms of (a) 25 μl, (b) 15 μl, (c) 10 μl, and (d) 5 μl of pure DCM.

the baseline before slowly decaying back to the baseline. The behavior for other solvents is similar. All show a positive and then a negative peak, but the recovery of the negative peak may or may not rise above the baseline. The height of the initial positive peak is proportional to the volume of solvent injected. The proportionality is demonstrated in Fig. 8, which is a plot of the solvent peak height in Fig. 7 *versus* the injection volume. Because of the close relationship of peak height to the volume injected, and because the positive peak is sharp and well defined, even for a 500-μl injection (Fig. 3c), the peak must correspond either to a highly volatile impurity, such as dissolved nitric oxide, or more likely to an impurity such as an organic nitrite, which decomposes in the hot injection port to give nitric oxide.

As may be seen from Fig. 7, the negative peak falls to the same level whatever the volume of solvent which is injected. The lowest point corresponds to a zero level of nitric oxide impurity in the carrier gas. We believe that there is a negative peak

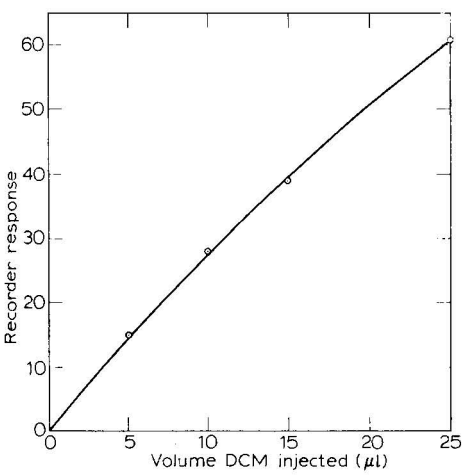


Fig. 8. Plot of solvent peak height versus volume injected for pure DCM.

because the solvent has displaced the carrier gas, with its nitric oxide impurity, from the reaction chamber. As the solvent vapor is in turn swept out of the chamber, the carrier gas with its nitric oxide impurity returns, returning the instrument response to the baseline. The relatively slow decay of the negative peak back to the baseline which may overshoot the baseline, as in the case of DCM, may be explained by a variation in the collisional deactivation of the excited nitrogen dioxide by solvent molecules.

The excited nitrogen dioxide can relax by one of two paths:

$$NO_2^* \rightarrow NO_2 + h\nu \quad (1)$$

$$NO_2^* \rightarrow NO_2 + M \quad (2)$$

The collisional deactivation by diluent species (eqn. 2) is always present but usually not important, unless the nature of M changes drastically, as may happen when the carrier gas is displaced by the solvent vapor. Further evidence for this explanation was obtained by observing the simultaneous thermal conductivity and TEA detector response. The solvent front, as seen by the thermal conductivity detector, extended far beyond the time that the TEA detector takes to return to its baseline.

In order to test these hypotheses further, a cold trap (20 × 3/16 in. I.D. stainless-steel tubing filled with steel wool and bent into a U-shape) was placed between the TEA catalytic pyrolyzer and the TEA luminescent reaction chamber. With the trap at a temperature of −150°, all the DCM is removed and prevented from entering the reaction chamber. A chromatogram of 10 μl of a DCM solution containing 1 μg DMN/ml is shown in Fig. 9, with and without the cold trap. The cold trap is seen to have eliminated the negative peak.

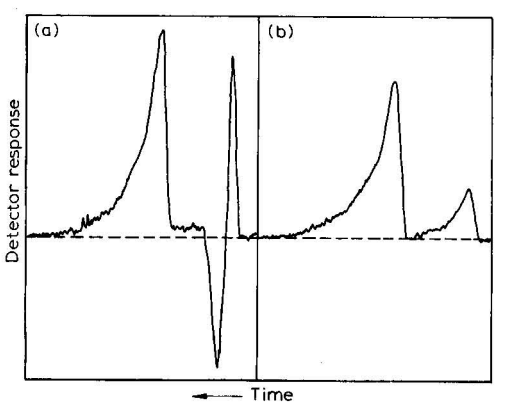


Fig. 9. Chromatograms of 1 μg DMN/ml in DCM: (a) without cold trap; (b) with cold trap at −150°.

CONCLUSION

The advantage of the GC–TEA system for the analysis of N-nitroso compounds over the existing GC–Coulson electrolytic conductivity detector and the GC alkali flame ionization detector techniques is that the GC–TEA is sensitive to sub-ng/ml

concentration levels and is at the same time selective to only N-nitroso compounds. Because of the selectivity, there is little need for extravagant clean-up or concentration procedures. Indeed, concentration is usually unnecessary and clean-up is only required so as to ensure that the extract is compatible with the GC column itself. No clean-up whatsoever is required for the TEA detector.

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CHROM. 8245

SEPARATION OF RESIN ACIDS FROM FATTY ACIDS IN RELATION TO ENVIRONMENTAL STUDIES

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SUMMARY

Kraft mills pulping coniferous wood species discharge to their receiving waters effluents containing mixed resin and fatty acids. To study the fish-toxic properties of these fractions it is desirable to be able to separate them without causing double bond isomerizations in the acid-labile resin acid fraction. A method is described using toluenesulphonic acid as catalyst under very mild conditions and four examples of its application are given. It was not possible to separate tall oil samples without a low degree of resin acid isomerization.

INTRODUCTION

The common resin acids are tricyclic diterpenes which possess abietane or pimarane skeletons. These co-occur with fatty acids in parenchyma cells of commercially important coniferous wood species. Resin and fatty acids are the main components of tall oil, which is a by-product of the kraft pulping of pinewood. Wood resin is also comprised of these substances and it may be recovered either by tapping living pine trees or by solvent extraction of weathered pine stumps[1,2]. Resin and fatty acids are separated from each other and from various neutral or unsaponifiable substances by fractional distillation *in vacuo* at tall oil or naval stores processing plants. Both acid fractions are valuable feedstocks to the chemical industry.

It has long been known that resin acids are toxic to fish[3]. Canadian research in recent years has established that two major contributors to the toxicity of whole kraft effluent are resin acids and polyunsaturated fatty acids[4–6]. In such studies it obviously is desirable to be able to isolate these fractions with minimum chemical change in composition of the reactive resin acids. This is not easily achieved because of the presence of the conjugated diene function in members of the abietane family. The standard method in wood chemistry relies on partial esterification of the mixed acids with methanol–sulphuric acid as catalyst[7]. This depends on steric hindrance around the tertiary carboxyl group thereby blocking attack by the esterifying agent. Such a method is satisfactory for the quantitative determination of total resin acids and also for the recovery of the fatty acid fraction. However, the use of strong mineral

acid causes double bond isomerization in the abietane-type resin acids and can give rise to misleading results in subsequent fish toxicity bioassays.

Two previous analytical procedures, based on gel permeation chromatography, successfully avoided the isomerization of double bonds. Zinkel and Zank[8] used diethyl ether as solvent and a polystyrene matrix, whereas Chang[9] preferred to use tetrahydrofuran with a different grade of polystyrene. In both methods differential refractometers were utilised and very small amounts only of mixed acids were applied to the columns. For fish toxicity bioassay work these methods have disadvantages including the use of low boiling solvents, inability to process more than one sample at a time, and difficulty in scaling up the sample size for preparative use.

For the past several years in this laboratory the separation of resin acids from fatty acids has been accomplished through an adaptation of the selective esterification procedure in which toluenesulphonic acid (TSA) replaces mineral acid as catalyst. A brief description of this technique is the purpose of this paper and some examples of its practical application are given. Previous investigators have used TSA for the titrimetric estimation of resin acids in tall oil fractions[10,11] but this work was performed without physical separation of the resin acids from the fatty acids. While the method was in use in this laboratory, a patent was granted for use of TSA in the commercial separation of tall oil resin and fatty acids[12].

EXPERIMENTAL

Preparation of various samples of mixed acids

Whole kraft effluent samples from a British Columbia Interior mill were processed through Amberlite XAD-7 resin beds as described elsewhere[6]. The ether-soluble portions of the methanol eluates from the resin beds comprised substantially the fish-toxic fraction and included the mixed fatty and resin acids.

Coarse woodmeal (passing a 3-mm screen) was prepared by passing air-dried Douglas fir wood [*Pseudotsuga menziesii* var. *glauca* (Beissn.) Franco] through a Wiley mill. The woodmeal was extracted with petroleum ether (b.p. 65–110°) in an all-glass Soxhlet extractor for 24 h and the extract recovered.

Ten sockeye salmon (*Oncorhynchus nerka*) fingerlings, which had died during a static bioassay test with dehydroabietic acid as toxicant, were freeze-dried, ground in a tissue homogenizer with chloroform and the slurry extracted three times with chloroform in a separatory funnel. The combined chloroform extract was washed with water and dried.

A sample of tall oil from a Western Canadian supplier was used to study optimization of the separation. A fresh tall oil sample was obtained from Longview Fibre Company at Longview, Washington, and used in one of the examples.

Separation of resin acids from fatty acids

Optimum conditions for separation of mixed acids from tall oil or wood extractives were as follows. A sample of tall oil or wood extractives (1–2 g) was dissolved in methanol (50 ml) containing 1 ml of a 10% methanol solution of TSA. The flask was flushed with nitrogen and stoppered, then stored in a refrigerator for 16 h at 36°F. The reaction product was transferred to a separatory funnel with the addition of isooctane (100 ml) and was extracted three times with 5% aqueous KOH

solution. The isooctane layer was washed successively with 5% KOH and water and dried. The combined alkaline extract was washed with isooctane, acidified with acetic acid, and the resin acid fraction recovered by extraction with benzene. The fatty acid methyl ester fraction was recovered from the isooctane phase.

Various experiments were conducted to explore the effect of changes in reaction time, temperature, TSA concentration and the substitution of naphthalene sulphonic acid for TSA or mineral acid for acetic acid in acidification of the resin acid soaps.

Gas chromatography

Control samples of mixed acids were methylated with an ethereal solution of diazomethane. Separated resin acid fractions were methylated similarly. Fatty acid methyl ester fractions also were methylated to ensure the absence of traces of free resin acids.

The gas chromatograph used in most of this work was a Hewlett-Packard Model 5711 A fitted with dual hydrogen flame ionization detectors and coupled to a Hewlett-Packard Model 3370 B digital electronic integrater. Choice of column and operating conditions are listed under Fig. 2. Earlier work on the separation of resin and fatty acid fractions from the fish-toxic portions of kraft effluent samples was performed using a Hewlett-Packard Model 7620 A gas chromatograph equipped with dual hydrogen flame ionization detectors. Operating conditions are listed under Fig. 1.

RESULTS AND DISCUSSION

In some preliminary experiments, a synthetic mixture was prepared using the resin acid fraction of a sample of Douglas fir wood and various C_{18} fatty acids which commonly are present in wood extracts. A clean separation of fatty acids from resin acids with quantitative recovery resulted when a methanolic solution of this mixture containing 1.7% TSA was shaken at room temperature for 2 h or for 16 h. No resin acid isomerization occurred and no emulsification problems were encountered.

An application of the method to the separation of a fish-toxic kraft effluent sample is shown in Fig. 1. The unsaponifiable fraction of this extract was first removed via column chromatography on DEAE-Sephadex A-25 according to the procedure of Zinkel and Rowe[13]. The separation was very clean and it can be seen that no significant degree of isomerization occurred. It was subsequently shown by fish bioassay tests that both the fatty acid and resin acid fractions of this sample were toxic.

Extension of the method to the separation of tall oil samples was not straightforward. Considerable problems were encountered with emulsions. These were alleviated by using isooctane as the preferred solvent for the fatty acid fraction, by using 5% KOH solutions rather than 5% NaOH solutions for removal of the non-esterified resin acid fraction, and by employing benzene for the final recovery of the resin acids. Additions of small amounts of saturated brine or methanol were also beneficial in overcoming the emulsion problem.

Difficulty was also encountered in preventing double bond isomerization of the resin acid fraction when separating tall oil. If reaction times were too short or the temperature was too low, incomplete separation without isomerization was observed. On the other hand, if reaction times were prolonged, or the temperature

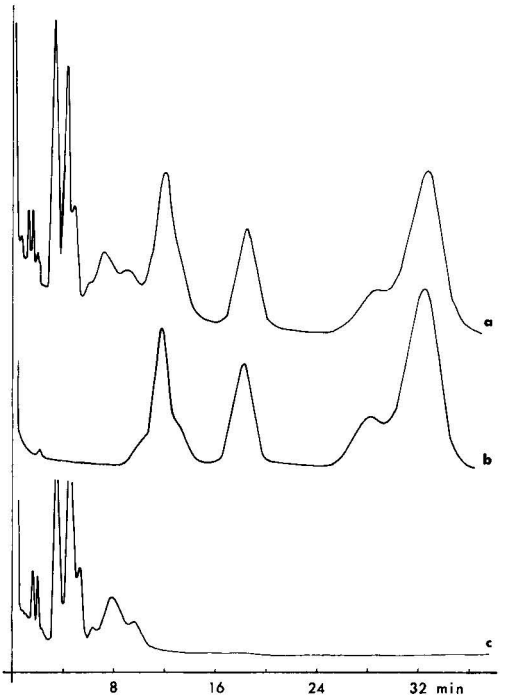


Fig. 1. (a) Methyl esters of a mixture of fatty and resin acids recovered from whole kraft effluent. (b) Methyl esters of resin acids after separation. (c) Methyl esters of fatty acids after separation. Column, 6 ft. × 1/8 in. O.D. stainless steel packed with 10% EGSS-X on 100–120 mesh Gas-Chrom P. Hewlett-Packard Model 7620 A gas chromatograph; detector, flame ionization; range, × 10^3; attenuation, 100; column temperature, 175° isothermal; injector temperature, 200°; detector temperature, 250°; carrier gas, nitrogen at a flow-rate of 70 ml/min; solvent, diethyl ether.

elevated, complete separation was achieved but always at the expense of some isomerization. This situation is illustrated in Fig. 2, which was conducted on a fresh sample of tall oil. Some isomerization has taken place, as is seen by comparing the relative heights and widths of peak 2 (abietate) with peaks 1 and 3 (palustrate and unresolved dehydroabietate with neoabietate) in the original sample with the corresponding peaks in the recovered resin acid fraction.

The problems with tall oil may relate to its past history. Tall oil is recovered from the evaporators in a kraft mill in the form of the sodium soap. This is subsequently acidulated with sulphuric acid to free the resin and fatty acids and no doubt some isomerization takes place during this operation. It appears that some of this mineral acid is trapped in the commercial tall oil and this may have a bearing on the experimental difficulty in the resin acid separations. It is apparent that palustrate and neoabietate, which are very sensitive to mineral acids and are difficult to distinguish from isopimarate and dehydroabietate, respectively, by gas chromatography, are being converted into abietate. Another reason why tall oil acids are difficult to separate without double bond isomerization is that the total content of resin acids of the abietane type is very high compared to that seen in Douglas fir wood extracts or whole kraft effluent samples from Western Canadian mills. In the latter case the

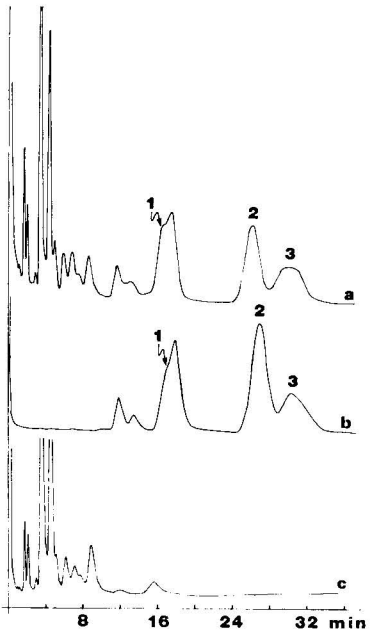
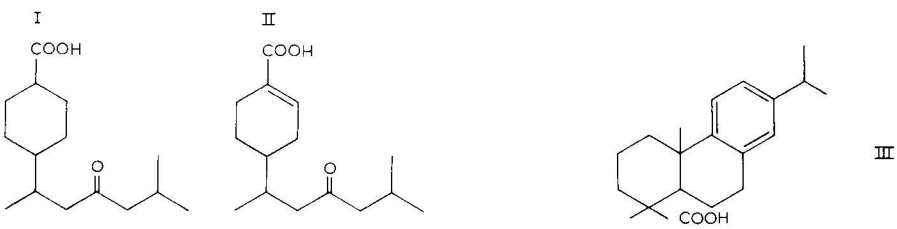


Fig. 2. (a) Mixed tall oil fatty and resin acid methyl esters. (b) Separated resin acid methyl esters. (c) Separated fatty acid methyl esters. Column, as in Fig. 1. Hewlett-Packard Model 5711 A gas chromatograph; detector, flame ionization, range × 10; attenuation, 100; column temperature, 185°; injector temperature, 200°; detector temperature, 250°; carrier gas, nitrogen at a flow-rate of 51 ml/min; solvent, diethyl ether. 1 = Palustrate; 2 = abietate; 3 = dehydroabietate and neoabietate.

main components are the chemically stable isopimarate and dehydroabietate. This reflects the pulping of spruce, Douglas fir and other coniferous species besides pine.

In a third example the technique has been used as part of the isolation scheme for the recovery of insect juvenile hormone analogs of the juvabione type from Douglas fir wood extracts. In this case the biologically active compounds I and II are readily esterified and remain in the fatty acid ester fraction from which they may in turn be removed by extraction with Girard T reagent[14]. In this example, partial esterification with conventional mineral acid catalysts is equally satisfactory since the resin acid fraction is of no subsequent interest.



III

A fourth and final example concerns the post mortem examination of fish which have died as a result of bioassay exposure to dilute solutions of resin acids. Analysis of internal organs for the presence of the resin acids or their metabolites

may yield valuable information as to the cause of death of the experimental animals. The problem here is that fish contain large amounts of lipids, including polyunsaturated fatty acids. The methyl esters of some of these acids unfortunately have retention times similar to the corresponding esters of the resin acids and thereby obscure the presence of the latter. The mixed acid fraction was recovered via solvent extraction of the homogenized total body tissues of dead sockeye salmon fingerlings. After saponification to destroy glyceride esters, the extract was partially esterified with TSA in methanol followed by methylation of the resin acid fraction with diazomethane. In this case the fish had been exposed to dehydroabietic acid (III) and the results of analysis of the resin acid fraction unequivocally showed the presence of this acid in the bodies of the fish. In this simple example the resin acid does not contain acid-labile double bonds. However, the method may equally well be applied to other resin acids which do possess this structural feature. For work on *post mortem* analysis of internal organs it would be preferable to use radioactively labelled acids because of the small amounts of tissue involved and the high dilution factor. Nevertheless, the method does have value in the investigation of fish kills in lakes and rivers which constitute the receiving waters for kraft pulping effluents.

In summary, the method is useful in the preparative-scale separation into fatty and resin acid fractions of the fish-toxic lipid fraction of kraft mill effluent or of tall oil. Small changes in composition of tall oil resin acids cannot be avoided but the procedure is very much less drastic than mineral acid catalysed methods in current use. In special cases the method may also be used to advantage to determine the reason for fish kills where contact with kraft pulping effluents is suspected.

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CHROM. 8254

ANALYTICAL RESPONSE OF POLYCHLORINATED BIPHENYL HOMOLOGUES AND ISOMERS IN THIN-LAYER AND GAS CHROMATOGRAPHY

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SUMMARY

Except for pure synthetic polychlorinated biphenyl (PCB), estimation methods of PCB by thin-layer and gas chromatography with electron capture detection give comparable results. Both give a good estimate of the true mass of a biologically modified residue, but where mostly hexachlorobiphenyls and above make up the residue, the estimate will be up to 50% too high. The Coulson detector does not, in our hands, yield comparable results with modified residues; the reason for this difference is not clear at present.

INTRODUCTION

To determine the mass of polychlorinated biphenyl (PCB) present in a given matrix, standard samples of each PCB homologue or isomer must be available to calibrate the response of the detection method employed. In spite of intensive efforts, relatively few of the 70–80 compounds present in commercial mixtures have yet been synthesized because the methods available to the synthesizing chemist do not easily yield the chlorine substitution patterns produced by the commercial process of catalytic chlorination over iron[1–3]. Therefore the two methods most widely used for quantitation of PCB have depended on empirical calibration. These methods are gas chromatography with electron capture detection (GC–ECD) and thin-layer chromatography (TLC) on either silver nitrate-impregnated silica gel or alumina plates[4–7], in each case using deposition of metallic silver under ultraviolet (UV) light for chromogenesis.

For GC–ECD the most common calibration procedure is to calculate the ratio of the total height or area of the most prominent chromatogram peaks of a known mass of a commercial PCB mixture (frequently Aroclor 1254) to the peak height or area of a known mass of a pure compound such as DDE. Using the response coefficient obtained, the total mass of PCB in an unknown sample can be estimated from the measurements of the chromatogram peaks which correspond in retention time with those in the standard chromatogram. Another method is to dispense with the use of a pure standard and simply measure the total area given by a known mass of a com-

mercial mixture and relate it to the total area given by the sample after employing a clean-up procedure which removes the common interfering chlorinated hydrocarbon pesticides. A slight variation on this procedure is used in our laboratory: individual peaks given by a known mass of a commercial mixture, using Apiezon L as the stationary phase, are compared with the corresponding peak in the sample, and a value with dimensions $\mu g \cdot g^{-1}$ (notional* $\mu g/g$) is calculated. The mean of the notional $\mu g/g$ values gives an estimate of the total mass of PCB in the sample[8]; and if the values for notional $\mu g/g$ are displayed on a bar graph, the degree of modification of the original mixture brought about by passage through a biological system, or by the clean-up process, is immediately obvious.

Collins *et al.*[9] relate the mass of PCB present to the sum of

$$\frac{\text{Peak height} \times \text{retention time}}{\text{Peak height of DDE} \times \text{retention time of DDE}}$$

for all peaks in the standard and sample. They also analyze using silver-impregnated silica gel layers with reflectance densitometric measurement of the silver spot. In this laboratory densitometry is employed to give quantitation of 0.1 $\mu g \pm 10\%$ Rel.S.D. with silica gel thin layers or 0.01 $\mu g \pm 10\%$ Rel.S.D. using alumina layers[7].

Other methods of quantitation commonly employed are: pyrolysis followed by coulometry or conductance after gas chromatography (GC–Coulson)[10] and conversion to perchlorobiphenyl with antimony pentachloride followed by GC–ECD[11]. Neither method is particularly useful in toxicological studies. The former should, if pyrolysis conditions are severe enough, give a response related to the chlorine content of the residue, not to the mass of PCB present. Since the amount of chlorine present is less important in determining toxicity than is the arrangement of the chlorine substituents (which has a profound effect on the metabolism of PCB isomers[5,8]), the result of GC–Coulson will not be directly related to the toxic potential of the residue. The perchlorination method, while it simplifies clean-up problems and thus probably improves analytical precision, only gives an estimate of the total mass of biphenyl skeletal material present including unchlorinated biphenyl. Hence its usefulness for evaluating the toxicological importance of a residue is small, and of course toxicology is fundamentally the reason for doing the PCB analysis.

The object of the work presented here was to determine which of the three methods available to us (TLC, GC–ECD, and GC–Coulson) gives results which are most closely related to the mass of PCB present. Unfortunately, pure synthetic samples of the major components of Aroclor 1254, the mixture we have studied, are not available, and so Aroclor 1254 itself was fractionated by preparative partition and adsorption chromatography.

EXPERIMENTAL

Materials

Aroclor 1254 was kindly provided by Monsanto (St. Louis, Mo., U.S.A.). Samples of nine pure PCB homologues and isomers were purchased from Analabs

* Notional values give a notion or general indication.

(North Haven, Conn., U.S.A.). All solvents were Nanograde quality. Mineral oil was U.S.P. quality. Thin-layer plates were manufactured by Schleicher and Schuell (Keene, N.H., U.S.A.).

Fractionation of Aroclor 1254

Mineral oil (8%), was loaded on to Celite (Gas-Chrom Q, 80–100 mesh; Applied Science Labs., State College, Pa., U.S.A.) using hexane. The dried packing was tamped into a glass column (2.5 × 100 cm), and a solution of acetonitrile–methanol–acetone–water (2:2:0.9:0.1), saturated with mineral oil, was pumped upward through the column at a rate of 2 ml/min for 48 h using an AutoAnalyzer proportioning pump with Solvaflex tubing (Technicon, Tarrytown, N.Y., U.S.A.). Fractions (10 ml) were collected, and the hydrocarbon layer was monitored by TLC after the addition of 2% sodium sulfate solution (25 ml) to each tube. Fractions which appeared to be similar were bulked, and four fractions (A, B, C and D) resulted (Fig. 1).

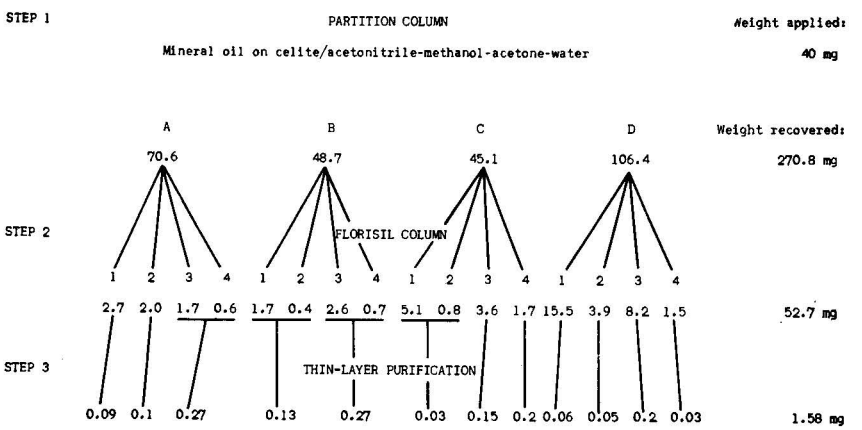

Fig. 1. Fractionation scheme for Aroclor 1254: partition fractionation followed by column adsorption and thin-layer purification.

Florisil columns were used to remove mineral oil from each fraction. The Florisil (Fisher Scientific, Fair Lawn, N.Y., U.S.A.) was activated at 450° for 4 h and packed when cool into 1 × 15-cm glass columns. For calibration, a solution of Aroclor 1254 (25 mg) plus mineral oil (25 mg) dissolved in hexane (10 ml) was applied to the top of a column previously wetted with hexane. Fractions (5 ml) were collected in weighed tubes during elution with hexane. The hexane was evaporated in a stream of filtered dry air, and the content of each tube was weighed using a Cahn electrobalance (Ventron, Paramount, Calif., U.S.A.) and assayed by TLC for PCB.

Fractions A, B, C, and D were purified by an identical Florisil column procedure. Several distinct fractions resulted as indicated by their TLC chromatograms (Fig. 1). The total mass recovered indicated that some mineral oil was still present, particularly in the D fractions, and so the last traces of oil were removed from all fractions by TLC on silica gel G 1500 layers, eluting with hexane, scraping zones

from the plates, and eluting with hexane into weighed tubes. Four fractions large enough to be weighed resulted (Fig. 1).

Because of the disappointing yield (1.58 mg from 40 mg) from this experiment, another fractionation was carried out solely by adsorption chromatography on silica gel thin-layer plates. Aroclor 1254 dissolved in hexane (1 g/l) was applied to ten G 1500 plates as streaks. After development in hexane for 20 cm, the six separated zones were located by chromogenesis of markers, scraped from the plate, and extracted from the adsorbent with hexane.

Weighing

The ten resultant fractions (4 partition, 6 adsorption) were weighed accurately with the Cahn balance by taring a small aluminum foil cup (5-mm diameter, 2.5 mm deep), adding the material in hexane, evaporating in a stream of filtered dry air, and re-weighing, taking care not to jar the balance pan downward. The precision achieved was $\pm$ 0.02 mg. The masses of the fractions obtained by this process and by preparative TLC are given in Table I.

TABLE I

MASS OF AROCLOR 1254 FRACTIONS

Fraction (partition)	*Mass (mg)*	*Fraction (adsorption, TLC)*	*Mass (mg)*
A	0.46	1	0.28
B	0.40	2	0.22
C	0.38	3	0.45
D	0.34	4	0.10
Total	1.58	5	0.23
		6	0.18
		Total	1.46

Gas chromatography–electron capture detection

A Hewlett-Packard 7600A system with electron capture detector was used. Glass columns (1.5 m $\times$ 0.2 mm I.D.) were packed with 2% Apiezon L on Gas-Chrom Q (80–100mesh). As carrier gas argon–methane (95:5) was employed. The column temperature was 205°. A gas chromatogram of Aroclor 1254 on Apiezon L is shown in Fig. 2.

The integrator read-out was processed in one of two ways: (1) the data were printed and converted into bar charts using a programmable calculator (Wang, Tewksbury, Mass.,) U.S.A. or (2) the data were recorded on paper tape using the Hewlett-Packard data acquisition package and the minicomputer incorporated into the 7600A system, the tape being used with a Fortran program to calculate the notional μg/g values and their mean.

Gas chromatography–Coulson detection

A Fisher-Victoreen Series 4400 gas chromatograph (Fisher Scientific, Pittsburgh, Pa., U.S.A.) was used with a Coulson detector (Tracor, Houston, Texas, U.S.A.) for the conductivity detection. The glass column (2 m $\times$ 1 mm I.D.) was

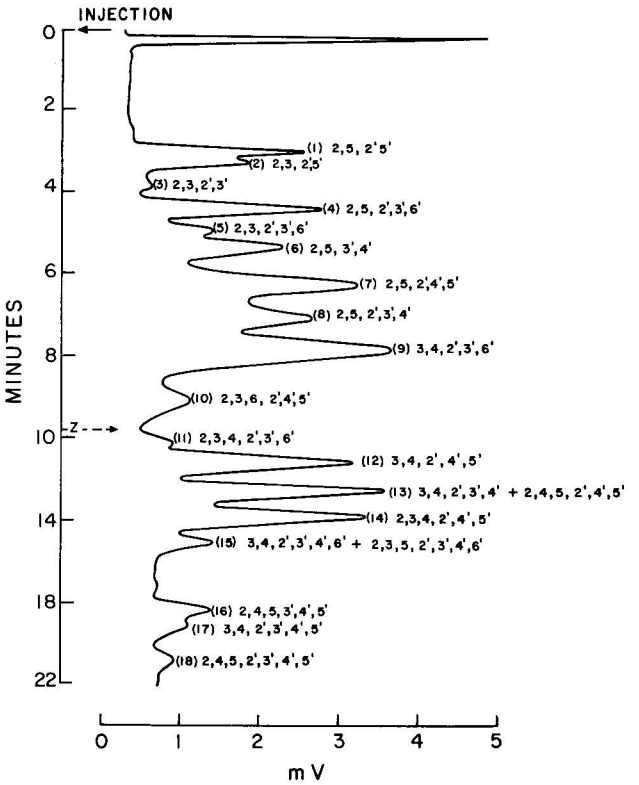

Fig. 2. Gas chromatogram of Aroclor 1254 on Apiezon L. At point Z the chart speed was changed from 0.5 to 0.25 in./min.

packed with 2% Apiezon L on Gas-Chrom Q (80–100 mesh). Detector conditions were as follows. Mode: reducing, no catalyst. Temperatures: column 200°, inlet 210°, transfer 220°, furnace 900°. Gases: carrier, nitrogen at 40 p.s.i., 60 ml/min; reactant, hydrogen at 20 p.s.i., 40 ml/min. Conductivity bridge: 30 V; attenuation, 2. Recorder: 1 mV at 30 in./h.

RESULTS

The relative responses of the three systems for the chromatographic and pure synthetic fractions are shown in Table II and summarized in Table III. The relative performance of the methods was also tested with real samples. When wildlife samples were analyzed, DDE was removed by oxidation with a mixture of chromium trioxide and acetic acid[7]. Chlordane and dieldrin are not removed by this clean-up procedure, and residues of the former are common in grain-eating birds. The nine peaks of commercial chlordane could, however, be separated from the eighteen peaks of Aroclor 1254 mixture by using a temperature program: 4 min at 160°, then 10°/min to 200° and hold for 30 min. Only a slight shift in baseline was observed, and the integrator is designed to compensate for such changes. Residues were confirmed by TLC. Fig. 4

TABLE II

RESPONSE RATIO SAMPLE: AROCLOR 1254

TCBP = tetrachlorobiphenyl; PCBP = pentachlorobiphenyl.

Sample fraction	*TLC*	*GC–ECD*	*GC–Coulson*
Partition			
A	0.28	0.44	0.37
B	0.61	0.82	0.60
C	1.07	0.63	0.44
D	1.62	1.48	0.45
Adsorption			
1	0.60	0.38	0.42
2	0.70	0.32	0.49
3	0.90	0.57	0.75
4	1.10	0.80	0.81
5	0.75	0.61	0.60
6	0.81	0.56	0.39
Pure synthetic PCB			
2,3-2′,3′-TCBP	0.64	0.60	1.01
3,2-2′,5′-TCBP	0.66	0.50	1.01
3,4-2′,4′-TCBP	1.10	0.50	1.09
2,3-5′,6′-TCBP	0.60	0.95	1.04
2,5-2′,5′-TCBP	0.58	0.40	0.86
3,4-3′,4′-TCBP	0.67	0.50	0.69
2,5-3′,4′-TCBP	0.84	0.80	1.38
2,3,4,5,6-PCBP	1.01	0.90	0.81
2,3,4-2′,5′-PCBP	0.80	0.95	1.52

TABLE III

MEAN RESPONSE RATIO SAMPLE: AROCLOR 1254

Fractions	*TLC*	*GC–ECD*	*GC–Coulson*
Partition fractions	0.90	0.84	0.47
Adsorption fractions	0.81	0.54	0.58
Synthetic PCB	0.65	0.80	1.00

shows bar charts of typical samples (wildlife and experimental toxicology) handled by this laboratory.

The ratio of response of each modified fraction to the response of a known mass of Aroclor 1254 was determined by TLC. The spot response to a known mass of each fraction was compared with the spot response to an identical mass of Aroclor 1254 on the same plate, taking the mean of the ratios of five pairs of spots to determine the ratio for each fraction.

For the Coulson detector, the total area under the response curve for each modified fraction was compared with the total area given by an identical mass of Aroclor 1254. The detector has too high a dead volume to allow for resolution of the individual peaks of the chromatogram.

DISCUSSION

It is clear from Table II that for pure synthetic PCB the Coulson detector gives the best estimate of the mass of PCB present, using Aroclor 1254 as the standard. With the chromatographic fractions, however, GC–Coulson yields variable results. TLC is least accurate with pure samples, perhaps because pure compounds give compact circular zones which would be expected to cause a TLC scanner response different from that caused by the diffuse elongated zone given by Aroclor 1254 and the chromatographic fractions.

ECD appears to give a good estimate of the mass present for three of the partition fractions but not for the adsorption fractions. This may be due to the different characteristics of the two fractionating systems. The partition system has been shown to separate according to degree of chlorination[5] (Fig. 2), whereas adsorption systems differentiate more according to steric arrangement around the biphenyl skeleton. Table II (synthetic PCB) illustrates how dependent is the electron affinity on substitution pattern.

Fraction D most resembles Aroclor 1254 after modification by living organisms (Figs. 3 and 4). It consists mostly of hexa- and heptachlorinated biphenyls, and the high response of both TLC and GC–ECD to this fraction (Table II) indicates that our analysis of such samples, while consistent by either method, will be approximately 50% too high.

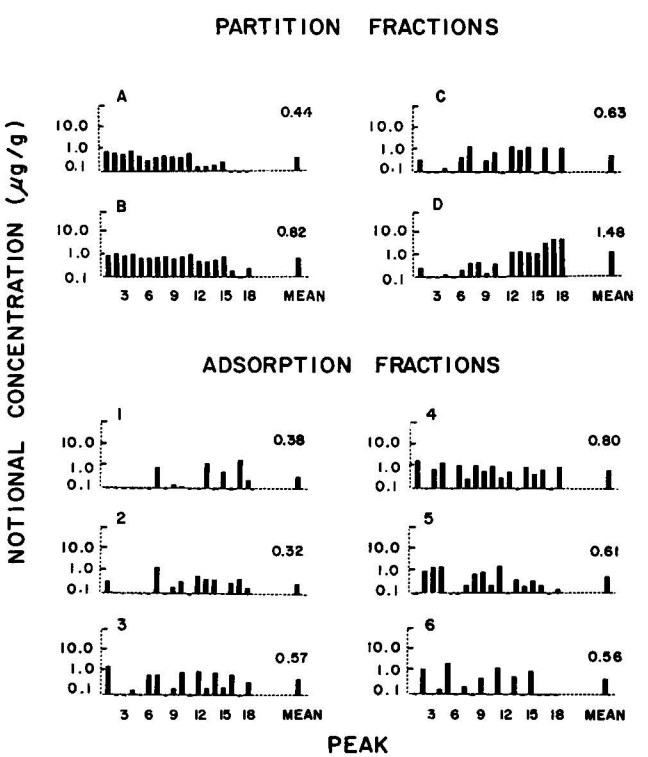

Fig. 3. Bar charts of the composition of fractions determined by GC–ECD.

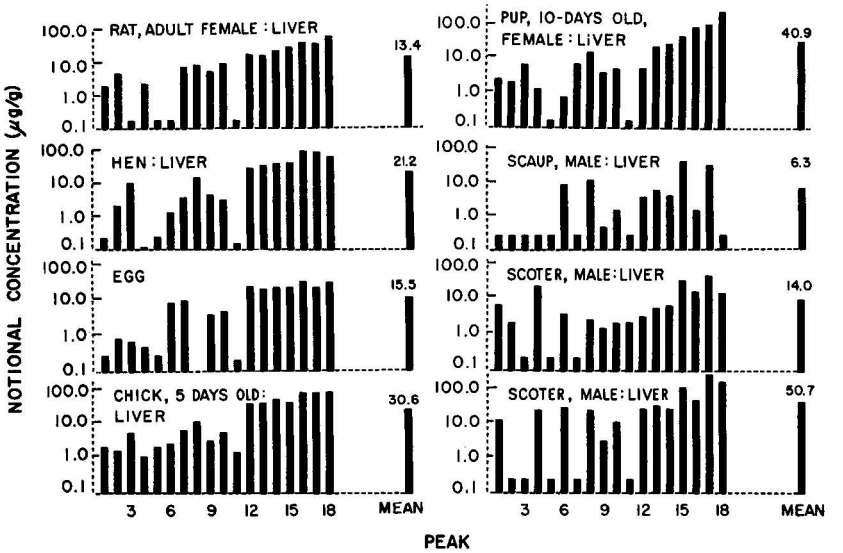

Fig. 4. Typical bar charts of biologically modified PCB determined by GC–ECD.

Correlation tests[12] using the Wang Statistical Package on the Wang Programmable Calculator (Table IV) show that the TLC and GC–ECD methods generally agree but the Coulson detector does not respond to PCB residues in the same way at all. Although the intercept figures (a_0) are small, except for the rat tissues the slopes of the regressions (a_1) are significantly different from unity, which would be obtained from two populations yielding exactly similar results. In all cases except for the rat tissues, TLC gives a lower estimate of PCB concentration than GC–ECD. If the synthetic samples are eliminated from the comparison, the value of a_1 rises to 0.88 ($r = 0.80$) for the ten chromatographic fractions.

TABLE IV

STATISTICAL COMPARISON OF TLC, GC–ECD AND GC–COULSON RESULTS

Symbols: v, degrees of freedom: $N-2$, where N is the number of pairs of results; r, coefficient of correlation between the two populations; a_1, regression slope: first-named population *vs.* second; a_0, regression intercept on axis of first-named population; P, probability that the observed correlation does not occur by chance.

Population	*Assays compared*	*Statistical values*				
		v	r	a_1	a_0	P
Fractions plus synthetic samples	TLC *vs.* GC–ECD	17	0.65	0.67	0.35	>0.99
	TLC *vs.* GC–Coulson	17	0.33	—*	—	<0.90
	GC–ECD *vs.* GC–Coulson	17	0.15	—	—	<0.90
Wildlife samples	TLC *vs.* GC–ECD	14	0.77	0.37	2.3	>0.99
Rat tissues from a PCB toxicology experiment	TLC *vs.* GC–ECD	13	0.94	1.0	0.5	>0.99
	TLC *vs.* GC–Coulson	13	0.24	—	—	<0.90
Hen eggs from a PCB toxicology experiment	TLC *vs.* GC–ECD	18	0.93	0.79	10	>0.99

* Blanks indicate no correlation.

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SÉPARATION D'HERBICIDES PAR CHROMATOGRAPHIE EN PHASE LIQUIDE À HAUTE PERFORMANCE

INFLUENCE DE L'EAU

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SUMMARY

Separation of herbicides by high-performance liquid chromatography. Influence of water

The influence of the amount of water dissolved in dichloromethane on the chromatographic separation of herbicides was studied. The selectivity of the mobile phase was demonstrated and compared to another system, dichloromethane modified with 2-propanol. The high efficiency of microporous packing was also demonstrated.

INTRODUCTION

En chromatographie sur couches minces, il a été montré[1] pour certains systèmes particuliers, que la modification de l'activité de l'adsorbant pouvait influer de façon importante sur les séparations et améliorer la sélectivité des systèmes chromatographiques utilisés. De même, en chromatographie liquide à grande vitesse, Engelhardt et Wiedemann[2] ont montré l'utilisation de systèmes de teneur en eau variable pour la séparation de pesticides chlorés et de stéroïdes.

Les solutés étudiés dans ce travail sont des herbicides de la famille des urées substituées. Des composés de la même série ont déjà été séparés selon une technique de chromatographie liquide–liquide[3], la phase stationnaire étant le β,β'-oxydipropionitrile déposé à 1 % sur de la silice (37–44 μm). La sélectivité de systèmes de chromatographie liquide–solide est montrée dans ce travail. Cette technique, de mise en oeuvre simple, permet, grâce à l'utilisation de supports de très fine granulométrie (5 μm), l'obtention de très grandes efficacités. Elle permet, d'autre part, d'éliminer le problème de la durée de vie des colonnes.

PARTIE EXPÉRIMENTALE

Chromatographie sur couches minces

Les couches utilisées sont des plaques Merck prêtes à l'emploi, Silica gel 60 F_{254}. Les solutés testés se dégradant à température élevée, il n'a pas été possible d'activer

les couches à l'étuve avant leur conditionnement aux différents taux d'humidité. D'autre part, il est désormais admis que l'activation des couches suivie du dépôt des solutés était inutile, la couche se désactivant pendant les dépôts.

L'activation des couches avant le conditionnement a été réalisée en laissant les plaques 3 h (avec les solutés déposés) dans un dessiccateur contenant de l'anhydride phosphorique, sous vide. Des solutions de H_2SO_4 de concentrations variables sont ensuite utilisées pour conditionner les couches à différentes humidités.

Dans le cas des mélanges isopropanol–dichlorométhane une présaturation de 30 min par les vapeurs de solvant est effectuée dans tous les cas de façon à éliminer les phénomènes de démixion.

Le volume des dépôts est de 2 μl pour les solutés en solution à 2% dans le dichlorométhane. Les conditionnements, présaturations et développements sont réalisés dans une cuve Vario KS. Les solutés sont révélés en UV à 254 nm.

Chromatographie en colonne

L'appareillage utilisé comprend: une pompe Orlita DMP 15; un injecteur Siemens à commande pneumatique; le volume d'injection est de 10 μl; un détecteur UV LDC 1205, 254 nm; une colonne de Lichrosorb Si 60 (5 μm), longueur 15 cm, diamètre interne 4.6 mm. Les colonnes sont remplies par voie humide suivant une technique décrite précédemment[4].

Réactifs. Les solvants utilisés sont des produits Merck (pour analyse). Le dichlorométhane saturé en eau est obtenu par agitation soutenue avec un excès d'eau pendant 1 h. Il a été montré que 15 min étaient en général suffisantes pour obtenir la saturation[5]. La teneur en eau du dichlorométhane est déterminée par la méthode de Karl Fischer à l'aide d'un appareil Metrohm AG, Herisau E. 547.

Les solutés étudiés sont des herbicides de la famille des urées substituées (Tableau I, No. 1–6). Ils sont commercialisés soit par la société Du Pont de Nemours, soit par la société Pepro. Deux produits inactifs provenant de la dégradation de l'isoproturon sont également analysés (Tableau I, No. 7 et 8).

RÉSULTATS

Chromatographie sur couches minces

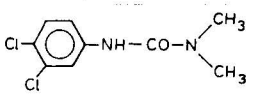
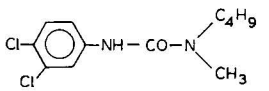
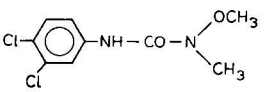
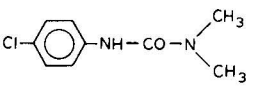
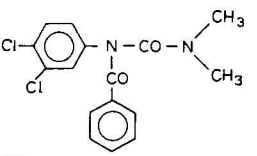
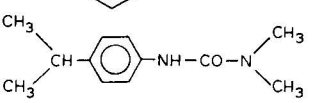
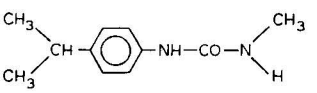
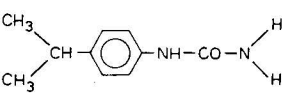
La Fig. 1 montre le chromatogramme obtenu avec les cinq pesticides (Tableau I, No. 1–3, 5 et 6) après développement avec le dichlorométhane pour différents taux d'humidité de l'adsorbant. Sur les couches conditionnées à de faibles humidités relatives, les produits sont trop retenus sur la couche pour avoir une bonne séparation. Celle-ci devient satisfaisante aux taux d'humidité relative plus élevés, *i.e.* 56%.

Dans le cas de l'isoproturon et de ses deux produits de dégradation, l'adsorption est beaucoup trop forte en raison de la présence des groupements $-N(H)CH_3$ et $-NH_2$ pour avoir une séparation satisfaisante même aux taux d'humidité élevés. Dans ce dernier cas, le système dichlorométhane–isopropanol (85:15) + 1‰ acide acétique s'est révélé efficace. Les valeurs des R_F observés sont données dans le Tableau II.

Dans les deux systèmes de phase mobile choisis, on peut considérer que l'eau et l'isopropanol jouent le même rôle. Pour les faibles taux d'humidité relative, les

TABLEAU I

HERBICIDES ÉTUDIÉS

No.	*Substance*	*Structure*
1	Diuron	
2	Néburon	
3	Linuron	
4	Monuron	
5	Phénobenzuron	
6	Isoproturon	
7		
8		

solutés sont fortement adsorbés sur la silice et le dichlorométhane n'est pas suffisamment polaire pour les en déloger.

Aux teneurs en eau supérieures, l'eau neutralise les sites les plus actifs de la silice[6]. On arrive alors à avoir une surface d'adsorbant moins active mais beaucoup plus homogène. Les adsorptions irréversibles pouvant se produire sur ces sites très réactifs n'ont plus lieu. Les solutés ne sont plus totalement retenus sur la couche. On considère généralement que cette homogénéité de la couche est atteinte lorsqu'on a fixé de 1/2 à 1 monocouche d'eau sur l'adsorbant ce qui correspond dans le cas présent à un conditionnement de 35 à 45 % d'humidité relative ou 10 % (g/g) de teneur en eau de l'adsorbant.

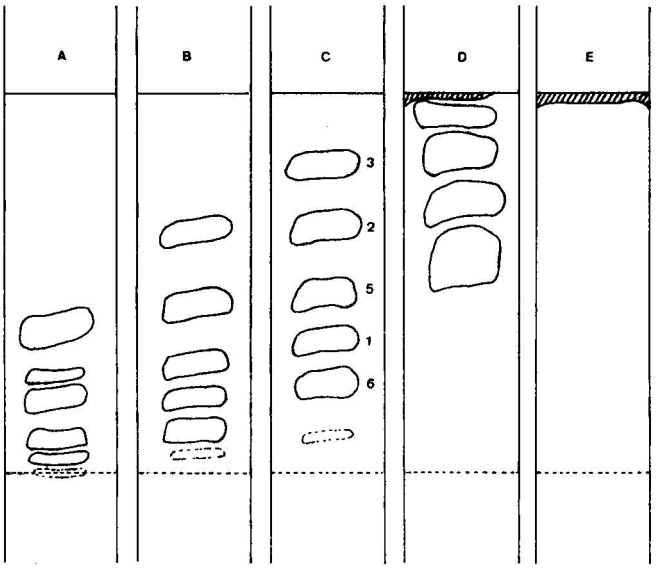


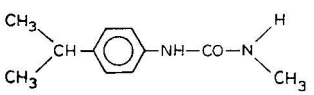
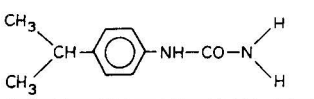
Fig. 1. Chromatographie sur couches minces des herbicides 1–3, 5 et 6 (*cf.* le Tableau I) pour différents taux d'humidité relative. A = 21%; B = 35%; C = 56%; D = 74.5%; E = 88%. Phase mobile: dichlorométhane pur.

Sur la Fig. 1, on voit bien que les séparations des solutés sont les plus intéressantes à partir de 35% d'humidité relative. Pour les taux d'humidité les plus élevés, l'eau arrive à recouvrir totalement la surface de l'adsorbant et on passe progressivement à un processus de partage. Les solutés, insolubles dans l'eau (qui devient alors la phase stationnaire), sont totalement élués avec le front de solvant. L'isopropanol, dans le système dichlorométhane–isopropanol, peut agir de la même façon en se fixant sur les groupements actifs du support. Dans ce cas, ces molécules d'isopropanol se fixent tout d'abord sous forme vapeur pendant le conditionnement de la couche.

Ces résultats confirment ceux obtenus par Viricel[7] dans une étude systématique de l'influence de l'eau sur les différents paramètres chromatographiques en CCM.

TABLEAU II

R_F DE L'ISOPROTURON ET SES DEUX PRODUITS DE DÉGRADATION

Substance	*Dichlorométhane–isopropanol (85:15) + 1‰ acide acétique*	*Dichlorométhane–isopropanol (90:10) + 1‰ acide acétique*
Isoproturon	0.74	0.61
	0.60	0.47
	0.34	0.26

Chromatographie en colonne

Système dichlorométhane modifié par l'eau. Les colonnes étant remplies par voie humide, on ne pourra fixer la teneur en eau de la silice que par une méthode *in situ*. Si on fait passer dans la colonne du dichlorométhane de teneur en eau constante, un état d'équilibre est atteint au bout d'un certain temps, état d'équilibre entre la teneur en eau de la phase mobile et teneur en eau de l'adsorbant. Cet équilibre peut être atteint de deux façons: soit partir d'un adsorbant anhydre (de forte activité) et faire passer dans la colonne une phase mobile de teneur en eau donnée, soit partir d'un adsorbant désactivé et utiliser une phase mobile anhydre. Nous avons utilisé la première méthode.

La Fig. 2 montre que lorsque la teneur en eau de l'adsorbant augmente, les valeurs des k' (facteur de capacité) des différents solutés diminuent, les variations les plus importantes se produisant pour les k' les plus grands. Les k' se stabilisent lorsqu'on arrive à un état d'équilibre entre la teneur en eau de l'adsorbant et la teneur en eau du solvant: équilibre isotonique[8]. Partant d'un adsorbant de forte activité et de dichlorométhane saturé en eau (teneur en eau: 2100 ppm à 21°), l'équilibre est atteint pour le système utilisé au bout de 10 h.

La Fig. 3 montre la séparation de la série des herbicides étudiés dans les conditions indiquées, lorsque l'équilibre est obtenu.

En accord avec des résultats déjà publiés[5,6,9], il semblerait que la forte diminution des k' pour les produits les plus retenus corresponde au recouvrement pro-

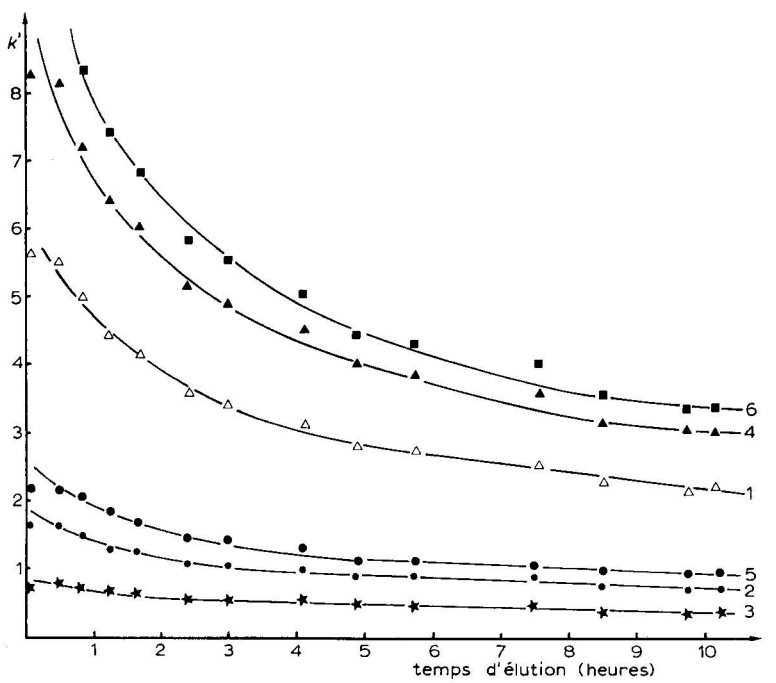


Fig. 2. Variation des facteurs de capacité k' des solutés 1–6 (*cf.* le Tableau I) en fonction du temps d'élution. Phase mobile, dichlorométhane saturé en eau; colonne, 25 cm × 4.6 mm de diamètre; support, Lichrosorb Si 60, 5 μm; débit, 1.5 ml/min.

gressif de la surface de la silice par les molécules d'eau. Les sites silanols les plus actifs sont les premiers "neutralisés" et les adsorptions des solutés possédant des groupements –NH très actifs ne sont plus aussi importantes. La surface de l'adsorbant est beaucoup plus homogène, le recouvrement en eau est alors de 0.1 g/g de silice. Il apparait qu'une teneur en eau de 10% en poids doit être fixée sur l'adsorbant pour obtenir une bonne résolution. Cette quantité d'eau correspond à la monocouche[5] et elle est obtenue en utilisant le dichlorométhane saturé en eau comme phase mobile jusqu'à obtention de l'équilibre.

Du point de vue pratique, il suffira donc de faire passer dans la colonne le dichlorométhane saturé en eau, jusqu'à obtention de l'équilibre. La colonne est alors prête à l'emploi pour des injections successives des herbicides. Un autre système a été testé et dans ce cas, il est modifié non plus par l'eau mais par l'isopropanol.

Système dichlorométhane modifié par l'isopropanol. Le chromatogramme obtenu

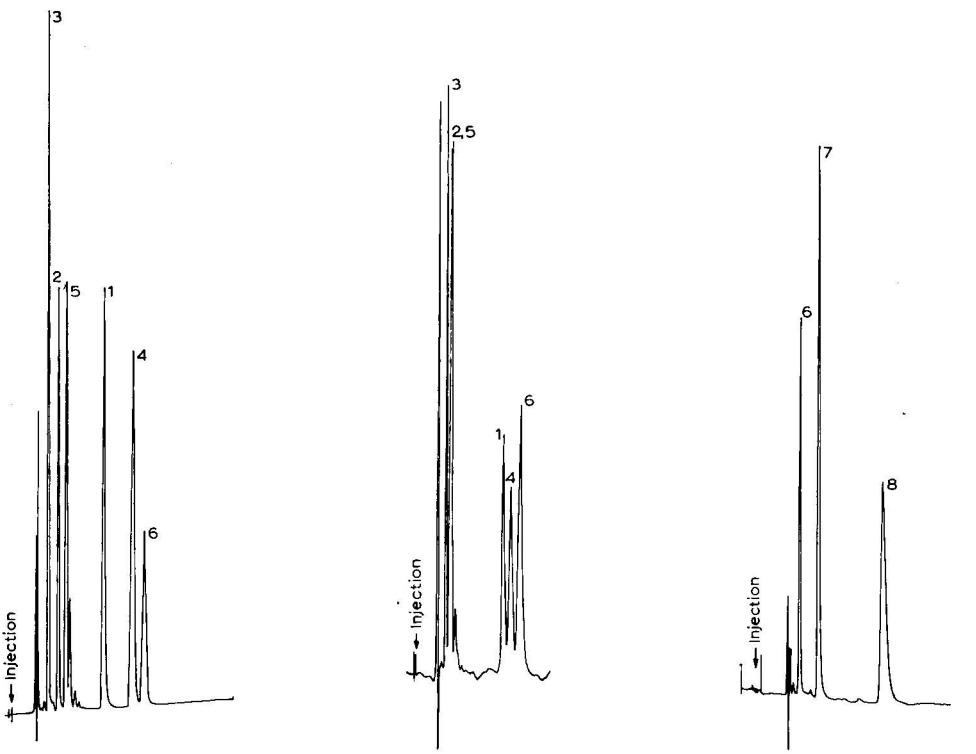


Fig. 3. Séparation des herbicides 1–6. Colonne, 14.5 cm × 4.6 mm de diamètre. Support, Lichrosorb Si 60, 5 μm; injection, 10 μl; défilement, 6 mm/min; P = 65 bars; détection, UV LDC, 254 nm; sensibilité = 0.32; phase mobile, dichlorométhane saturé en eau.

Fig. 4. Séparation des herbicides 1–6. Colonne, 25 cm × 4.6 mm de diamètre; support, Lichrosorb Si 60, 5 μm; injection, 10 μl; défilement 6 mm/min; P = 100 bars; détection, UV Zeiss, 254 nm; phase mobile, dichlorométhane–2-propanol (98.5:1.5).

Fig. 5. Séparation de l'isoproturon et de ses deux produits de dégradation (Tableau I, No. 7 et 8). Mêmes conditions que pour la Fig. 4. Phase mobile, dichlorométhane–2-propanol (85:15) + 1‰ acide acétique.

pour le même mélange de solutés avec une phase mobile dichlorométhane–isopropanol (98.5:1.5) est représenté sur la Fig. 4. La sélectivité de ce système est moins bonne que dans le cas précédent. Aucune forme anormale de pic n'a été observée sur les différents chromatogrammes. Ces observations ont été faites pour certains solutés très adsorbés lorsque les phases mobiles utilisées sont le dichlorométhane modifié par le métanol ou le dichlorométhane modifié par le 2-propanol[9].

L'isoproturon et ses deux produits de dégradation sont beaucoup trop retenus si l'on utilise les deux systèmes précédents. Dans ce cas, la phase mobile utilisée a la composition suivante: dichlorométhane–2-propanol (85:15). L'acide acétique est introduit à 1‰ dans le mélange pour éliminer les traînées (Fig. 5).

CONCLUSION

Les systèmes testés, en particulier le système dichlorométhane modifié par l'eau, se montrent très sélectifs pour la séparation des herbicides étudiés. La teneur en eau n'intervient pas de la même façon pour tous les systèmes. Il a été montré[3,10] que la dépendance des valeurs du facteur de capacité k' (ou des valeurs de R_F en chromatographie sur couches minces) était d'autant plus faible que le solvant était plus polaire.

La mise au point de systèmes d'adsorption pouvant remplacer des systèmes de partage est avantageuse dans la mesure où leurs facilités d'utilisation sont plus grandes. Les efficacités obtenues (HETP = 17–20 μm, Fig. 3) avec les silices de fine granulométrie sont très satisfaisantes.

Dans tous les cas, une première approche rapide pour la recherche du système approprié est faite sur couches minces. Le passage en colonnes est ensuite réalisé en optimisant la force éluante du solvant ou d'autres paramètres si cela s'avère nécessaire.

RÉSUMÉ

L'influence de l'eau présente dans la phase mobile, le dichlorométhane, sur la séparation d'herbicides est étudiée dans ce travail. Les solutés, de la famille des urées substituées, sont thermodégradables. La sélectivité du premier système est comparée à un deuxième dont la phase mobile est le dichlorométhane modifié par le 2-propanol. De grandes efficacités sont obtenues avec l'utilisation de supports microporeux (5 μm).

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CHROM. 8276

AUTOMATED CHROMATOGRAPHIC DETERMINATION OF CHLORHEXIDINE IN PHARMACEUTICAL PREPARATIONS

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SUMMARY

An automated method for the determination of chlorhexidine (Hibitane) and its salts in formulated pharmaceutical products is described. The equipment consists of a high efficiency liquid chromatograph, a variable wavelength high sensitivity ultraviolet spectrophotometric detector, the output of which is monitored simultaneously by a suitable recorder, and a digital computer. The sample is automatically introduced on to a 10-cm silica gel column by use of a slide valve. Results are calculated and printed out by the computer.

INTRODUCTION

The assay of chlorhexidine (Hibitane*) formulations in current use in this laboratory requires extraction of the active agent from the formulation followed by a colourimetric procedure with alkaline hypobromite[1]. The procedure is accurate and selective but has the disadvantage in that it is time consuming, and as the complexity of the formulation increases so the sophistication of the required extraction procedure increases. The application of high-efficiency liquid chromatography in pharmaceutical analysis has already been described[2]. Recent advances in the technology of packing materials (particularly alumina and silica) have made possible the separation of chlorhexidine from its synthesis and degradation products. This separation is utilised to provide a specific analysis of the concentration of chlorhexidine and its salts in formulated products.

EXPERIMENTAL

The basic instrumentation of the automated liquid chromatograph comprises the following parts: coil pump; 10-cm glass column (4 mm internal diameter, 6.5 mm external diameter) of 11-μ silica gel (Partisil) packed by a modified tap procedure; "Servomex SV220" slide valve; "Cecil" sample changer; "Cecil 212 UV monitor";

* The word "Hibitane" is a trade mark, the property of Imperial Chemical Industries Limited.

suitable recorder; "Datachrom" computer system; the Interface is described in Fig. 1.

The coil pump supplies eluent phase at column pressure to the injection valve by way of a cut off valve activated by either a power failure or by a digital output from the computer, the digital output operating a relay switching the electrical supply to the valve. A manual override is fitted into this circuitry to enable the chromatograph to be operated manually if required. The slide valve has two solvent (5 μl) passages and is arranged such that the eluent phase can flow through one whilst the sample passes through the other, injection being achieved by switching the slide valve by pneumatic controller operated by means of a signal switched from the timer unit of the Cecil sample changer. The Cecil sample changer is switched by means of an optical coupler operating on a digital output from the computer, capability for manual override being retained. The computer is then notified of injection by means of a signal from the timer unit of the Cecil.

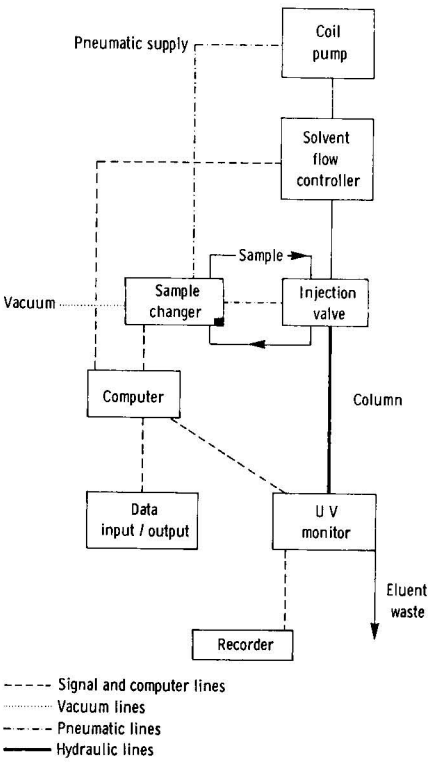


Fig. 1. Schematic diagram of automated high-pressure liquid chromatograph.

The chromatogram resulting from the separation on the column and the subsequent detection of the chlorhexidine by its ultraviolet absorption is monitored by both the recorder and the Datachrom computer system. The chromatograms (Figs. 2 and 3) show the separation of chlorhexidine from excipients in lotion and patch testing kits, demonstrating the degree of selectivity obtainable and enabling the chlorhexidine concentration in the samples to be calculated by comparison of the

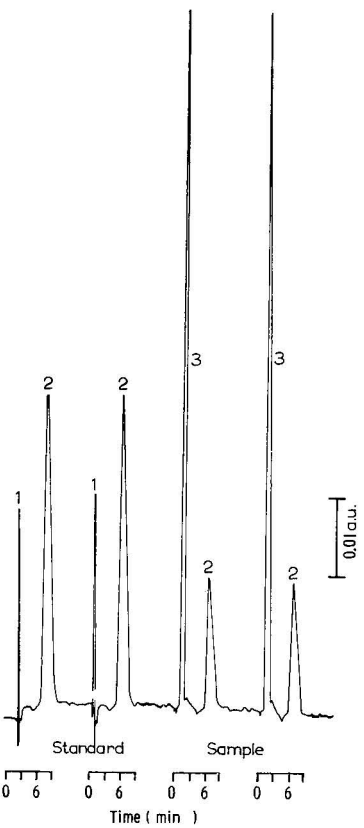
height of the chlorhexidine peak of the sample with that of a standard of known concentration.

The computer analyses the data acquired from the monitoring of the sample chromatogram according to the computer method. The acquired data are then referenced to the data acquired during the chromatographic run of a standard and the composition of the sample calculated in accordance with the computer method. The results are then presented to the operator as a printout.

Linearity of response with changes of concentration was checked over the range 0–600 μg/ml using solutions of chlorhexidine in methanol (Fig. 4).

Standard preparation

Transfer an accurately weighed quantity of standard chlorhexidine diacetate (50 mg) to a 200-ml flask, dissolve in and make to volume with methanol.



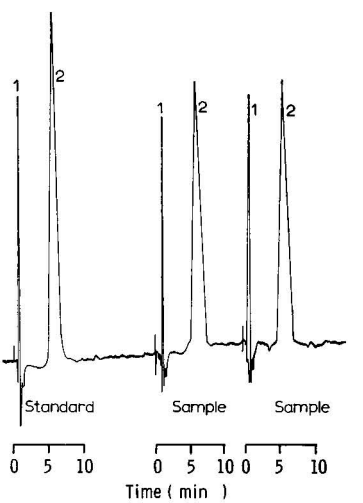


Fig. 2. Chromatogram of chlorhexidine standard and an extract of Savlon baby lotion. 1 = Solvent; 2 = chlorhexidine; 3 = excipient and solvent.

Fig. 3. Chromatogram of chlorhexidine standard and a sample from a patch testing kit. 1 = Solvent; 2 = chlorhexidine.

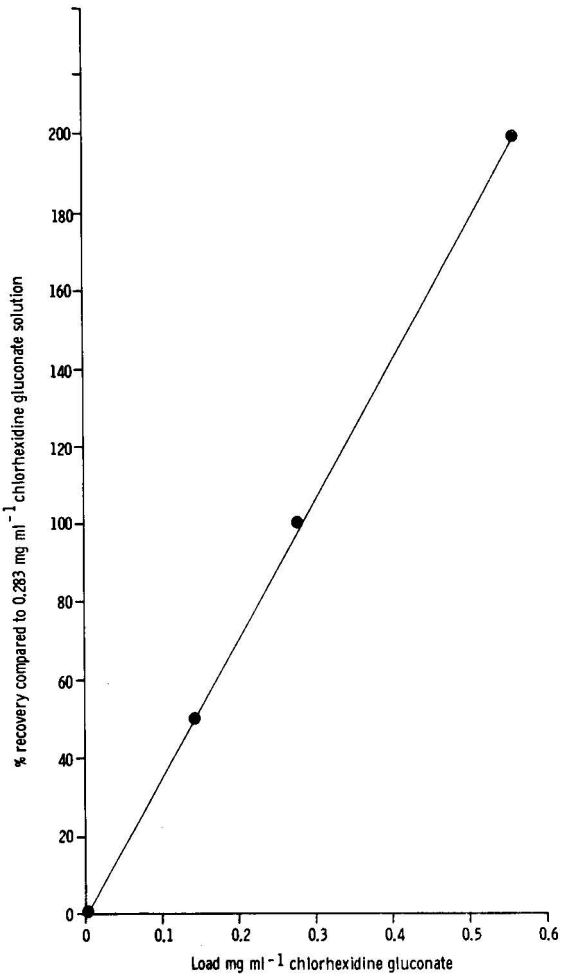


Fig. 4. Graph demonstrating linearity of chromatograph response to chlorhexidine.

Preparation of typical sample solutions

Savlon liquid antiseptic.* Transfer 10.0 ml of the sample to a 100-ml flask, dilute and make to volume with methanol.

Savlon hospital concentrate. Transfer 2.0 ml of sample to a 100-ml flask, dilute and make to volume with methanol.

Hibitane dental gel. Transfer an accurately weighed quantity of sample (1 g) to a 100-ml flask, disperse and make to volume with methanol. Use the supernatant solution.

Hibitane medical concentrate. Transfer 1.0 ml of the sample to a 200-ml flask, dilute and make to volume with methanol.

* The word "Savlon" is a trade mark, the property of Imperial Chemical Industries Limited.

*Hibiscrub**. Transfer an accurately weighed quantity (1 g) of sample to a 100-ml flask, dilute and make to volume with methanol.

Savlon antiseptic lozenges. Determine the average weight of a lozenge. Grind the lozenges to a fine powder, transfer an accurately weighed quantity (2 g) of the powder to a 100-ml separating funnel. Add 0.1 *M* sodium hydroxide (50 ml) and shake the mixture until dispersed. Extract the sodium hydroxide with three successive 25-ml volumes of diethyl ether. Combine the diethyl ether washings and evaporate to dryness. Dissolve the residue in a 1% solution of 1,5-gluconolactone in methanol, transfer this solution to a 25-ml flask and make to volume.

Savlon baby talc. Transfer an accurately weighed quantity (5 g) of sample to a 50-ml flask, disperse and make to volume with methanol. Shake the flask vigorously for 5 min. Allow the dispersion to settle and use the supernatant solution.

Savlon babycare lotion. Transfer an accurately weighed quantity (5 g) of sample to a separating funnel, disperse the sample in 25 ml of methanol, add 25 ml of isooctane and shake vigorously. Run the lower methanolic layer into a second separating funnel, and re-extract with a further 25 ml of isooctane. Transfer the methanolic layer to a 50-ml flask and make to volume with methanol.

Hibitane obstetric cream. Use the procedure as for Savlon babycare lotion with a sample weight of about 1 g and adjust the final volume to 100 ml.

Chromatography

The conditions for chromatography were as follows: column, 10 cm, glass, packed with 11-μ silica gel "Partisil" by a modified tap procedure; eluent, acetonitrile (general-purpose reagent)–0.02 *N* sulphuric acid in water (Analar) (91.5:8.5); pressure, 300 p.s.i.; flow-rate, 1 ml/min; temperature, 25°; UV detector wavelength, 254 nm; UV detector attenuation, 0.1 a.u.f.s.

The sample solution, prepared as previously described, was transferred to the sample vials of the automatic sample changer in duplicate. A standard, prepared as previously described, was placed at the beginning of the run of samples, in the middle, and at the end of the run. Then the automatic chromatograph was initiated.

RESULTS AND DISCUSSION

The automated high-efficiency liquid chromatographic procedure has been applied to a range of production and development samples and gives results indistinguishable from the colourimetric procedure. Degradation products and other impurities in chlorhexidine are separated by the chromatographic column with the following retention times: chlorhexidine, 320 sec; methanol, 50 sec; 4-chloroaniline, 80 sec.

The precision of the proposed analytical procedure was checked on a sample of Savlon liquid antiseptic and the results are tabulated in Table I. Recovery experiments of the three most widely used formulations were carried out. The results are shown in Table II.

* The word "Hibiscrub" is a trade mark, the property of Imperial Chemical Industries Limited.

TABLE I

RESULTS OF A SERIES OF REPLICATE ANALYSES ON A SAMPLE OF LIQUID ANTISEPTIC

Chlorhexidine gluconate (%, w/v)	*Mean (%)*	*Variance*
0.327	101.7	2.89
0.320	99.7	0.09
0.320	99.7	0.09
0.317	98.8	1.44
0.327	101.7	2.89
0.321	100.0	0.00
0.314	97.8	4.84
0.328	102.2	4.84
0.323	100.6	0.36
0.317	98.8	1.44

Standard deviation, 1.45%
Mean, 0.321% (w/v) chlorhexidine gluconate

TABLE II

RECOVERY OF CHLORHEXIDINE FROM PLACEBO FORMULATIONS

Sample	*Chlorhexidine gluconate (%, w/v)*		*Recovery (%)*
	Added	*Found*	
Savlon liquid antiseptic	0.282	0.283	100.4
Savlon hospital concentrate	1.50	1.48	98.9
Hibiscrub	3.95	3.94	99.8

TABLE III

COMPARATIVE ANALYSES OF SAVLON LIQUID ANTISEPTIC

Sample No.	*Chlorhexidine gluconate (%, w/v)*	
	Colourimetric result	*Chromatographic result*
1	0.31	0.32
2	0.32	0.33
3	0.32	0.32
4	0.32	0.31
5	0.32	0.32
6	0.32	0.33
7	0.33	0.31
8	0.31	0.30
9	0.32	0.30
10	0.31	0.29
11	0.32	0.31
12	0.32	0.32

TABLE IV

COMPARATIVE ANALYSES OF SAVLON HOSPITAL CONCENTRATE

Sample No.	*Chlorhexidine gluconate (%, w/v)*	
	Colourimetric result	*Chromatographic result*
1	1.52	1.52
2	1.56	1.54
3	1.51	1.50
4	1.50	1.49
5	1.59	1.57
6	1.36	1.36
7	1.50	1.53

TABLE V

COMPARATIVE ANALYSES OF SAVLON BABYCARE TALC

Sample No.	*Chlorhexidine hydrochloride (%, w/w)*	
	Colourimetric result	*Chromatographic result*
1	0.19	0.19
2	0.19	0.19
3	0.18	0.18

TABLE VI

COMPARATIVE ANALYSES OF HIBITANE MEDICAL CONCENTRATE

Sample No.	*Chlorhexidine gluconate (%, w/v)*	
	Colourimetric result	*Chromatographic result*
1	5.2	5.10
2	5.4	5.42
3	5.4	5.08

TABLE VII

COMPARATIVE ANALYSES OF HIBISCRUB

Sample No.	*Chlorhexidine gluconate (%, w/v)*	
	Colourimetric result	*Chromatographic result*
1	3.96	4.10
2	4.02	4.08

TABLE VIII

COMPARATIVE ANALYSES OF SAVLON BABYCARE LOTION

Sample No.	*Chlorhexidine gluconate (%, w/w)*	
	Colourimetric result	*Chromatographic result*
1	0.10	0.10
2	0.10	0.10
3	0.10	0.10
4	0.10	0.10

TABLE IX

COMPARATIVE ANALYSES OF MISCELLANEOUS CHLORHEXIDINE CONTAINING FORMULATIONS

Nature of sample	*Colourimetric result*	*Chromatographic result*
Hibitane obstetric cream	1.00% (w/w) Chlorhexidine gluconate	1.03% (w/w) Chlorhexidine gluconate
Hibitane acetate solution	0.10% (w/v) Chlorhexidine acetate	0.10% (w/v) Chlorhexidine acetate
Hibitane antiseptic lozenges	5.16 mg Chlorhexidine acetate/lozenge	5.30 mg Chlorhexidine acetate/lozenge
Hibitane dental gel	0.97% (w/w) Chlorhexidine gluconate	0.97% (w/w) Chlorhexidine gluconate

The results of comparative assays between the colourimetric and chromatographic procedures on a variety of formulations are given in Tables III–IX. The results demonstrate the wide applicability of the automated chromatographic procedure to the analysis of chlorhexidine containing formulations.

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CHROM. 8220

QUANTITATIVE DETERMINATION OF INDOLIC COMPOUNDS IN THE RAT BRAIN USING *p*-DIMETHYLAMINOCINNAMALDEHYDE AS REAGENT

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SUMMARY

A method is proposed for determining the metabolites of radioactive 5-hydroxytryptophan and of endogenous tryptophan in the rat brain. The compounds are separated by thin-layer chromatography and made visible with *p*-dimethylaminocinnamaldehyde (DACA) reagent. The conditions studied are those which allow the use of DACA reagent for the quantitative determination of tryptophan metabolites in brain tissue. Amounts of 5 ng of serotonin and 20 ng of tryptophan can be determined by densitometry.

INTRODUCTION

The indoleamine hypothesis of depression states that the level of serotonin (5-hydroxytryptamine, 5-HT) may be decreased in the brain of depressives. Therapy with the precursor tryptophan (Trp) has been attempted[1], as the concentration of Trp in the brain[2] is one of the factors that determines the synthesis of 5-HT. On the other hand, only a small part of Trp is converted into this amine. Thus, 5-hydroxytryptophan (5-HTP), an intermediate in the formation of serotonin, but not detectable in the brain, has been tried as a therapeutic agent for depression[3] and schizophrenia[4]. If radioactive serotonin is applied centrally (it does not cross the blood–brain barrier), it disappears from the brain within a few hours[5]. As the decarboxylation of 5-HTP is not the rate-limiting step of serotonin synthesis, it may be of importance to determine the metabolism of 5-HTP in view of its therapeutic value. The aim of this work was to develop a simple method for determining the metabolism of applied 5-HTP in the rat brain and its influence on endogenous serotonin metabolism.

Developing a thin-layer chromatographic (TLC) method for this purpose, the *p*-dimethylaminocinnamaldehyde (DACA) reagent of Harley-Mason and Archer[6] was used to stain the indolic compounds. In an earlier paper, the separation of some of the metabolites of Trp was reported[7]. In a semi-quantitative approach, Baumann

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and Narasimhachari[8] described the high sensitivity of the DACA reagent on cellulose but not on silica gel.

The use of the DACA reagent for the quantitative assay of Trp metabolites has not yet been proposed. The main purpose of this study was to test the highly sensitive DACA reagent as a tool for the quantitative measurement of tryptophan metabolites by TLC.

EXPERIMENTAL

Materials and reagents

The following equipment was used: scintillation counter, Packard Tri-Carb 3214; centrifuge, Sorvall RC-2; sample applicator, Waessle and Sandhoff prototype of the Linomat (Camag, Muttenz, Switzerland); electrically driven thin-layer plate coater, Camag; and densitometer, Zeiss (Oberkochen), complementary to the PMQ II. The compounds used were *p*-dimethylaminocinnamaldehyde (Schuchardt, Munich, G.F.R.), indolic compounds (Fluka, Buchs, Switzerland), microcrystalline cellulose (Avicel) (Merck, Darmstadt, G.F.R.) and cellulose MN 300 (Macherey, Nagel & Co., Düren, G.F.R.). Other reagents were Merck p.a. grade chemicals.

Radioactive compounds used were [3-^{14}C]D,L-5-hydroxytryptophan ([^{14}C]-5-HTP), 3.8 mCi/mmole (NEN, Boston, Mass., U.S.A.), [3-^{14}C]-5-hydroxytryptamine creatinine sulphate monohydrate ([^{14}C]-5-HT), 56 mCi/mmole (Radiochemical Centre, Amersham, Great Britain) and [1-^{14}C]-5-hydroxyindoleacetic acid ([^{14}C]-5-HIAA), 64 mCi/mmole (NEN, Boston, Mass., U.S.A.).

Method

In a typical experiment, 10 μl of a solution containing 58 μg (1 μCi) of [^{14}C]-5-HTP is injected intracisternally[5] in rats, with the aid of a 50-μl Hamilton syringe. Analyses of the endogenous compounds tryptophan and serotonin, and of the radioactive metabolites 5-HTP, serotonin and 5-HIAA, are performed on the whole brain or on parts of the brain after decapitation. The fresh brain samples are collected in ice-cold 0.4 *N* perchloric acid, then rinsed with 0.9% sodium chloride solution. One part of brain tissue is homogenised in 1.5 parts (w/v) of methanol. Alternatively, for small brain samples, the mixture is homogenised by sonication, by pressing the sound source on the outer wall of an Eppendorf reagent tube (1.8 ml) containing the mixture. As this procedure takes about 3 min, the sonication is performed in an ice-bath. The homogenates are centrifuged for half an hour at 31,000 *g* in the Sorvall centrifuge, then 100 μl extract are counted directly in a PPO–POPOP solution containing methanol. Extracts without radioactivity may occur if the intracisternal injection was unsuccessful; these extracts are not chromatographed.

Chromatography

An 18-g amount of Avicel cellulose is thoroughly mixed for 1 min with 100 ml of water with a ESGE mixer. This suspension is sufficient to coat seven thin-layer plates with the Camag coating apparatus. First, the 20 × 20 cm × 4 mm glass plates are cleaned with acetone so as to ensure the fixation of the 0.3 mm thick paste. The plates are dried in air.

In order to eliminate effects due to a possible uneven coating, the applications

of the mixture of standard substances and of the tissue samples are made alternately, with the Waessle and Sandhoff sample applicator. In order to obtain spots with the same width after the development, tissue samples are applied to a width of 26 mm and the standards to a width of 14 mm. On one plate, two 50-μl sample extracts together with three standard mixtures (15, 25 and 35 μl) of solutions containing, as a rule, 4 ng/μl of Trp, 4 ng/μl of 5-HTP and 1 ng/μl of 5-HT may be applied. The standards are dissolved in dilute methanol. The plates are developed in Desaga chromatography tanks with the following solvent mixture: butanone-2–acetone–2.5 *N* acetic acid (40:20:20). The running time for a distance of 12.5 cm is about 1.5 h. After the development, the plates are dried in air. The hR_F values already reported[7] are restated here: L-Trp, 50; 5-HT, 65; 5-HIAA, 98; and L-5-HTP, 35.

Densitometric measurement

A 2-g amount of DACA is dissolved in a mixture containing 100 ml of 6 *N* hydrochloric acid and 100 ml of ethanol. The reagent can be stored in a refrigerator for several weeks. The dry chromatograms are sprayed evenly with the reagent, but not until transparency occurs in order to avoid too high a background. Then, the plates are dried for 2 min at 60° in a drying cabinet. After this, the chromatograms are measured with the Zeiss densitometer, using the following settings: slit width of the monochromator, 0.06; slit length, 10 mm; wavelengths, 610 nm for blue spots (*e.g.*, serotonin) and 595 nm for violet spots (*e.g.*, Trp); scanning speed, 3; and recorder speed, 10.

The scanning proceeds in the same direction as the development of the chromatogram, after adjusting the baseline to $A = 0$. Each spot is scanned separately after first adjusting manually the point of maximal absorption of the spot. For quantitative evaluations, the peaks recorded were cut out and weighed. With the aid of the standard curve obtained, the values of the tissue samples could be determined, either mathematically or graphically.

Measurement of the radioactivity

For measurement of the radioactivity, Packard scintillation counting vials are filled with 15 ml of thixotropic gel, of which the background is determined. The thixotropic gel is prepared by dissolving 25 g of PPO and 1.5 g of dimethyl-POPOP in 5 l of pure toluene. To 1 l of this mixture, 40 g of thixotropic gel powder are added. After the densitometric measurements, the stained spots are scraped out and sucked into the vials under a light vacuum[9]. As the coloured spots may produce a high quenching, a quench curve must first be determined. For the measurement by the channel ratio method, the settings used are: windows, 50–1000 and 90–1000; gain, 15%. For carbon-14, recoveries of 40–80% are obtained under these conditions, depending on the quenching samples.

The results are given as mean values $\pm$ standard deviation.

RESULTS

Development of the method

Methanol was chosen as the solvent for homogenisation because it has a satisfactory volatility; acidic solvents would corrode the brass parts of the sample appli-

cator (Waessle and Sandhoff) and acidic solvents produce smearing of the spots on the chromatograms. As already reported[7], the proposed solvent system for TLC was the best for separating the most common indolic compounds in the rat brain. Urea (hR_F 57), giving a bright red spot with the DACA reagent, does not interfere under these conditions. Avicel cellulose was superior to MN 300 cellulose, as chromatography on the latter produces double spots for serotonin. In fact, the organic phase separates from the aqueous phase on the thin layer. The 5-HT spot is situated at the lower "front" (on top of the water layer) on Avicel cellulose. On MN 300, serotonin forms one spot on the organic and one on the water layer. Avicel cellulose also has the advantage of separating optical isomers. Fig. 1 shows the absorption spectra of 5-HT and Trp after staining the cellulose plates with DACA reagent. The spectra were obtained by measuring the absorption at discrete wavelengths at the point with the highest absorbance of the spot against the background. The uncorrected maxima, at a band width of 5 nm, are at 610 nm for serotonin and at 595 nm for Trp.

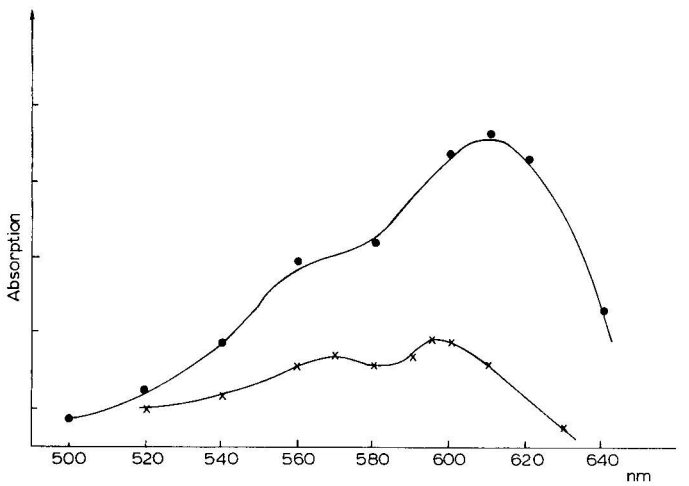

Fig. 1. Spectra of 5-HT (●) and Trp (×) after staining with DACA reagent on cellulose.

Harley-Mason and Archer[6] mentioned that the background may disturb the measurement. In Fig. 2, the results of four chromatograms, each with spots of 35 ng of serotonin, are shown. At the time 0 min, the plates are sprayed with DACA, then dried for 2 min with a hair-dryer. The first measurement occurred 3–4 min after the spraying procedure. This measured value was taken as 100% and other values were expressed as a percentage of this first value. During this experiment, the plates were kept in the dark, as light enhances the formation of a background. Fig. 2 shows that with increasing background the values decrease comparatively, the results differ from one plate to another, and the best time to measure the plates is between 10 and 30 min after staining.

The Zeiss densitometer allows measurements of both transmission and remission. In Fig. 3, these techniques are compared, showing standard curves for Trp and serotonin. It can be seen that 5 ng of 5-HT and 10 ng of Trp are still measurable. The sensitivity is higher in transmission than in remission, but the background is more stable in the latter, as in this case it is less dependent on the unevenness of the plates.

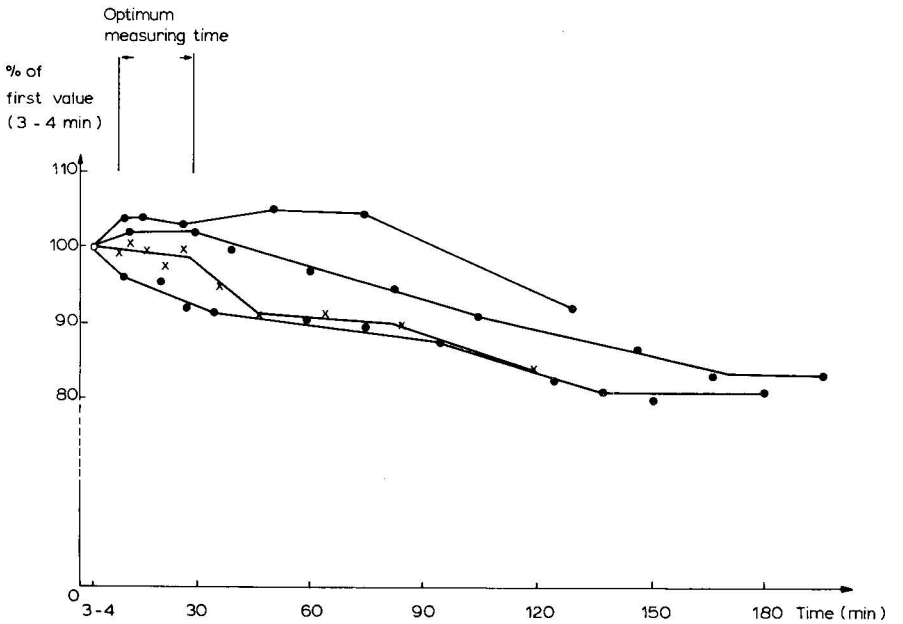

Fig. 2. Stability of the 5-HT–DACA reaction product as a function of time. Identification as in Fig. 1.

Of course, the sensitivity may still be augmented by applying smaller spots and closing the diaphragm. In this way, less uniform spots are obtained, giving less reproducible results and the Lambert–Beer law is no longer adhered to. This is clearly shown in an experiment where the same amount of 5-HT was applied as a band of 7 or 15 mm, and measured densitometrically after chromatography and detection with the DACA reagent with slit widths of 6, 10 and 14 mm (Fig. 4). As expected, the sensitivity can be increased by concentrating the substance on to a band of 7 mm and measuring with a slit width of 6 mm. However, at the same time, the reproducibility decreases, as shown by the widespread values of the three measured spots in this case. Therefore, final spots of 14 mm width, measured at a slit width of 10 mm, were preferred.

The blue spots and especially the reddish background formed by DACA produce a high quench. Thus, a cellulose plate was sprayed with DACA. To vials containing thixotropic gel and [^{14}C]-toluene increasing amounts of sprayed cellulose were added. By the ratio method, the quenching was established. The quench curve was used for the experiments, as the recovery may vary from 40 to 80%.

The recoveries of 5-HTP, 5-HT and 5-HIAA were estimated by adding the radioactively labelled compounds to four rat brains separately. After homogenisation and chromatography, the corresponding spots were scraped out and counted. The following recoveries were obtained: [^{14}C]-5-HTP, 91.4 $\pm$ 8.4%; [^{14}C]-5-HT, 68.9 $\pm$ 4.0%; [^{14}C]-5-HIAA, 79.1 $\pm$ 2.8%. For calculation, it was assumed that the brain has a density of unity and that the radioactive compound is equally distributed between the liquid and solid phases.

By this method, and by varying the extraction procedure and the solvent system for TLC, many indolic compounds could be determined quantitatively. Two examples are given here, *viz.*, the determination of 5-HT and Trp in the rat brain.

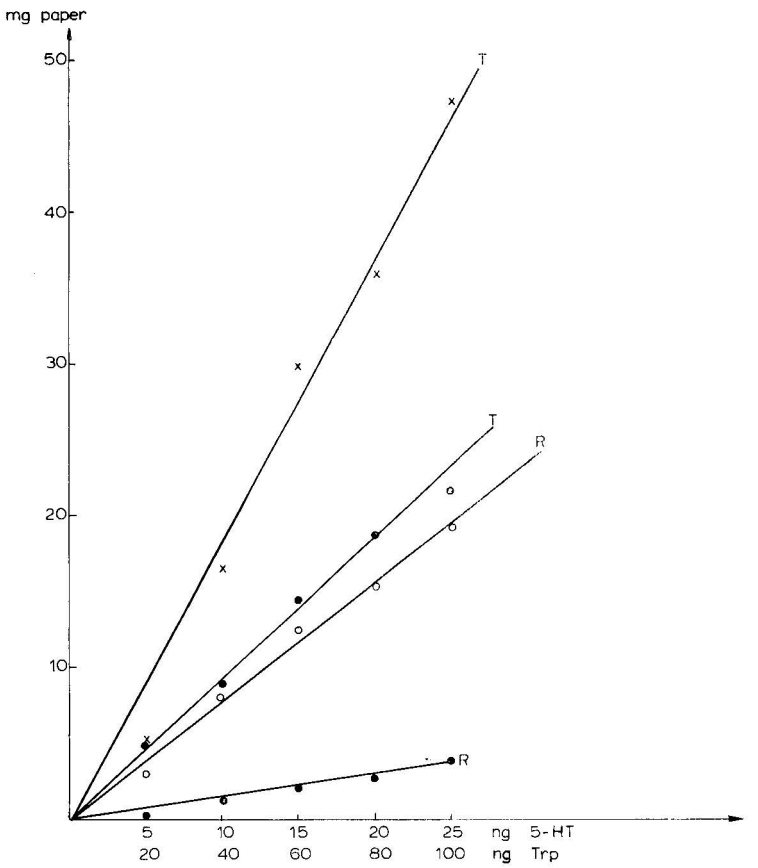

Fig. 3. Standard curves for 5-HT (at 610 nm) and Trp (at 595 nm) measured in transmission (T) and remission (R) applied as 14-mm spots on Avicel cellulose. (●), 5-HT; (○, ×), Trp.

For the determination of serotonin in the brainstem of Sprague-Dewley rats, three brainstems were pooled (mean weight 0.45 g) and homogenised in methanol. Eight double determinations on eight plates were carried out on the extract. On each plate, spots containing 15, 20 and 25 ng of serotonin as standards and two spots of 75 μl of the extract were applied. Fig. 5 shows the mean standard curve for the eight plates and the mean value of the eight double determinations of the tissue extract. The calculated value is 28.4 $\pm$ 3.8 ng (13%) per 75-μl extract. Considering the recovery, the 5-HT content of the brainstem is 1340 $\pm$ 175 ng per gram of tissue. For the determination of Trp in the brainstem, six double determinations on six plates were carried out, by applying 60, 100 and 140 ng of Trp and twice 50 μl of the supernatant. Table I gives the measured values. The recovery is 5.35 $\pm$ 0.33 μg of Trp per gram of tissue.

Note: the considerable variations in the absolute values from one plate to another indicate the necessity for comparing standards on the same plate.

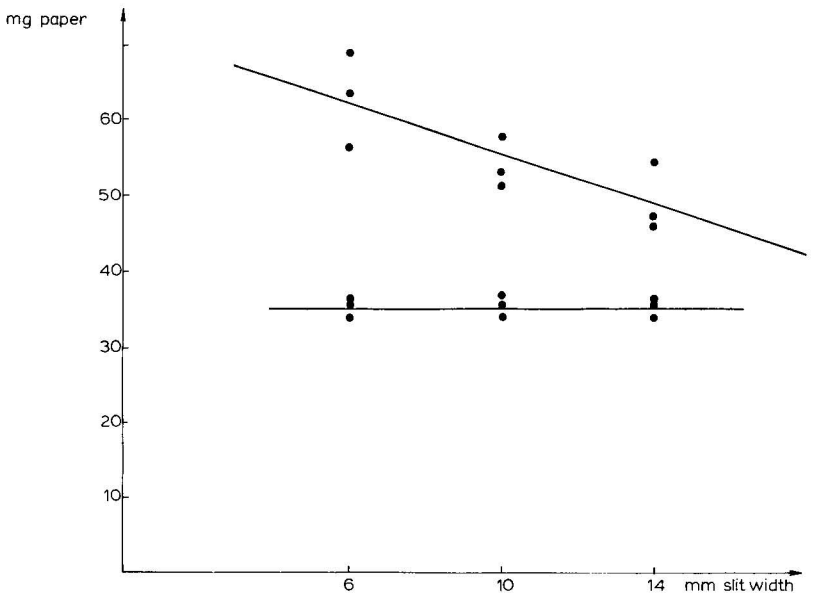

Fig. 4. Dependence of the measured value (expressed as milligrams of paper) on the slit width and the width of the applied spot. The same amount of serotonin was applied as a band of 7 mm (upper line) and of 15 mm (lower line).

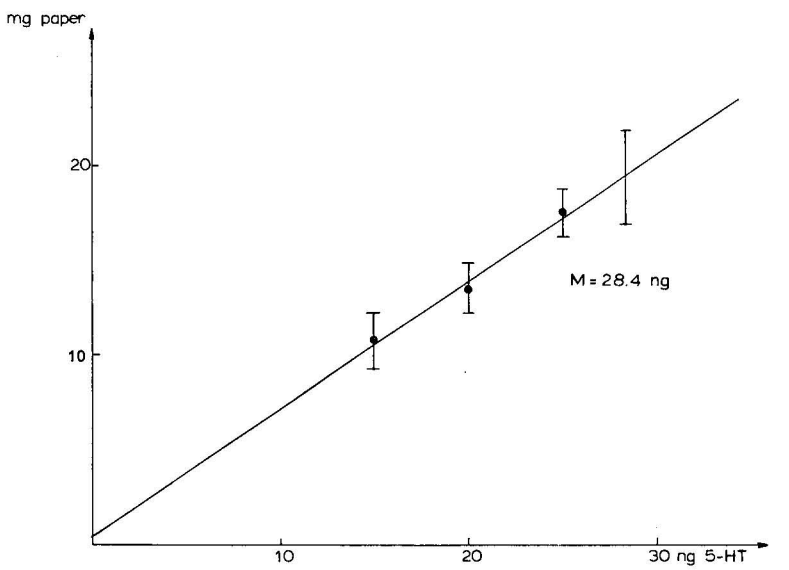

Fig. 5. Determination of 5-HT in three pooled brainstems of rats. The standard curve and the tissue value are means of eight double determinations. The standard curve is derived from 5-HT spots of 15, 20 and 25 ng.

TABLE I

DETERMINATION OF TRYPTOPHAN IN FOUR POOLED BRAINSTEMS OF RATS

The measured values are expressed as mg "peak weight".

Chromatogram	*Measured values of the standard curve*			*Mean value of 2 × 50-μl brain extract*	*Mean values (ng)*
	60 ng	*100 ng*	*140 ng*		
1	8.7	26.2	59.4	25	90
2	11.5	13.4*	27.8	16.2	86
3	17.4	25.2	33.0	21.8	82
4	24.1	49.0	68.4	37.2	80
5	18.2	41.3	72.9	28.8	77
6	16.9	—	62.6	26.4	78
					Mean: 82 ± 5 ng per 50 μl extract

* 80 ng.

Application

This method permits a pharmacokinetic study to be made of the metabolism of 5-HTP, serotonin and 5-HIAA and the influence of drugs upon it.

In an experiment, one group of five Sprague-Dawley rats was not pre-treated, while another group of six animals received an intracisternal injection of 58 μg of [^{14}C]-5-HTP (1 μCi). One hour later, the eleven rats were decapitated and assayed for [^{14}C]-5-HTP, [^{14}C]-5-HT, [^{14}C]-5-HIAA, 5-HTP, Trp and 5-HT. Fig. 6 shows a typical chromatogram from this experiment and its densitometric measurement. The left-hand curve replicates the measurement of the first line, with the following standards:

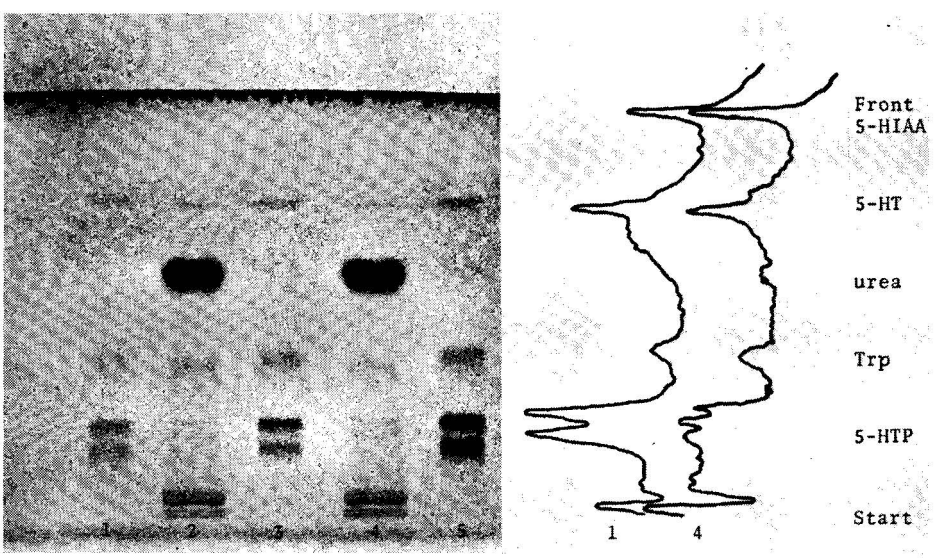

Fig. 6. Chromatogram and graphs of its densitometric measurement in transmission of a brain tissue extract and standards.

5-HTP (60 ng), 5-HT (15 ng) and Trp (60 ng). The good separation of the optical isomers of 5-HTP can be seen; in isolated experiments with the D- and L-forms of 5-HTP, it appeared that the D-form moves faster. This clear separation could not have been achieved when tissue extracts were chromatographed: the right-hand curve shows the measurement of line 4. As the recording was carried out at 610 nm, the bright red spot of urea was filtered out almost quantitatively. In the two curves, the high background at lower R_F values than that of 5-HT is striking, and may be due to water-soluble impurities in the cellulose. Results are given in Table II.

In untreated animals, 5-HTP is not detectable, which is in agreement with previous work[10]. The radioactive 5-HTP is still measurable densitometrically 1 h after its injection. In the last columns, the proportion of each radioactive compound is expressed as a percentage of the total remaining radioactivity. 5-HTP remains predominant. The newly formed 5-HT is rapidly metabolised, which is shown by its relatively small proportion. By application of [^{14}C]-5-HTP, the total 5-HT does not increase in comparison with the value in untreated animals.

DISCUSSION

Several papers have described the separation of indolic compounds on silica gel[11–23], but only Contractor and Wragg[24] studied the resolution of Trp metabolites on cellulose. Applications of the quantitative assay of indolic compounds by densitometry are rare[12,18]. Although the DACA reagent has been widely used as a spray reagent for qualitative measurements[6,8,20,22], no attempt has been made to prove its use in the quantitative determination of indolic derivatives by densitometry.

By densitometry (Fig. 3), the visual impression of the high sensitivity of the reagent has been confirmed. Standard curves on a single chromatogram show that linearity exists for the ranges 5–25 ng of 5-HT and 20–100 ng of Trp. On the other hand, all experiments failed to obtain comparable standard curves from one plate to another. Fig. 2 shows that the stability of the product may vary from one plate to another. In this regard, the DACA reagent is less advantageous than 4-dimethylbenzaldehyde reagent, where the reaction products are relatively stable[12]. As is clearly shown by Table I, it is strongly recommended that standards are carried on the same plate as the extract. However, as the DACA reagent is about ten times more sensitive than Van Urk's reagent, it may be useful for problems where a high sensitivity is especially needed, such as for measurements of indoles in brain samples. In this respect, it may be compared with the OPT reagent[8]. Nevertheless, after TLC, the OPT reaction products have only been determined after extraction of the silica gel[16,17] and never by densitometry. Paraformaldehyde could be an equally sensitive reagent as DACA, as it gives a fluorescent product with serotonin at the nanogram level[11]. Until now, no application with paraformaldehyde reagent has been reported.

It is noteworthy that, as the reaction with DACA takes place at the C-2 position of the indole ring, the composition of the C-3 side-chain does not determine the colour formation among the tested compounds. More important is the C-5 position, because if this position is not occupied a violet colour is produced (DMT, tryptamine, Trp, indoleacetic acid). A hydroxy or methoxy group at the C-5 position is responsible for a higher reaction rate, which may be explained by the positive mesomeric effect caused by the oxygen. This appears as a more intense blue colour after

TABLE II

FATE OF 58 μg (1 Ci) OF [^{14}C]-5-HTP IN THE RAT BRAIN AFTER INTRACISTERNAL INJECTION AND ITS INFLUENCE ON THE ENDOGENOUS TRYPTOPHAN METABOLISM

Group	*5-HTP*			*5-HT*			*Trp (ng/g tissue)*	*5-HIAA (dpm/g tissue)*	*Percentage of total radioactivity*		
	ng/g tissue	*dpm/g tissue*	*dpm/ng*	*ng/g tissue*	*dpm/g tissue*	*dpm/ng*			*5-HTP*	*5-HT*	*5-HIAA*
Controls (5 animals)	–	–	–	1171±188	–	–	3880±860	–	–	–	–
[^{14}C]-5-HTP-treated (6 animals)	1840±350	135500±38500	73±13	1180±300	6125±540	5.4±1.7	4500±1100	22500±2000	82.47±1.63	3.74±0.21	13.79±1.53

spraying serotonin, 5-hydroxytryptophan, 5-methoxy-N,N-dimethyltryptamine, etc., with the DACA reagent. The reaction may be similar to that described by Dibbern and Rochelmeyer[25] for the Van Urk reagent.

The extraction procedure with methanol–water is not very specific but permits the extraction in a single step of all of the substances tested. Other compounds that may interfere on the chromatogram occur in the brain in very low concentrations. Melatonin, highly concentrated in the pineal gland[26], is not synthesized in the remainder of the brain. Chase *et al.*[27] injected serotonin intracisternally. The neutral metabolites (5-hydroxytryptophol?) formed were only about 7% of the mean metabolite 5-HIAA. Information about 5-hydroxytryptophol in the rat brain is scarce. Klein and Notides[28] showed *in vitro* that after 24 h the principal pineal gland derivative formed from serotonin is 5-HIAA. The 5-hydroxytryptophol formed was only about 1.5% of the incubated serotonin.

By the present method, 1340 and 5350 ng/g of 5-HT and Trp, respectively, were measured in the brainstem. Maickel *et al.*[29], Curzon and Green[30] and Shellenberger and Gordon[31] found 1230, 1030 and 941 ng/g of serotonin, respectively, in the midbrain. Tagliamonte *et al.*[32] showed the Trp level in the rat brain to be 4.56 μg per gram of tissue.

The application with [^{14}C]-5-HTP presented here is one of a series in which the disappearance of radioactive 5-HTP and its metabolites as a function of time will be studied. However, it already appears that serotonin is not accumulated after intracisternal injection of [^{14}C]-5-HTP, but is rapidly metabolised to 5-HIAA.

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POLYAMIDE COLUMN CHROMATOGRAPHY FOR RESOLUTION OF COMPLEX MIXTURES OF ANTHOCYANINS

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SUMMARY

A method for the separation of anthocyanins on polyamide columns is described. This procedure makes possible the detection and enrichment of minor components and the separation of derivatives which are difficult to separate by paper or thin-layer chromatography. Separation of acylated anthocyanins was effected with minimal degradation. Microcolumns gave excellent resolution of extracts from as little as 15 mg tissue.

INTRODUCTION

Since 1955 it has been well established that polyamide of the polycaprolactam type is suitable for chromatography of phenolic compounds because of its ability to adsorb polar substances through hydrogen bonding[1–4]. Complex mixtures of glycosides and aglycones of isoflavones, flavones, flavonols, dihydroflavonols, and flavanones have been successfully separated with a water–methanol system[5]. From a crude leaf extract of *Cucurbita*, Strack and Reznik[6] purified 10 different flavonolglycosides using water–methanol elution and re-chromatography with a mixture of methanol and chloroform.

Until now, little is known about the application of polyamide column chromatography to anthocyanins. Previous investigations have indicated that polyamide was useful only for preliminary purification of crude anthocyanin extracts[7–9] and offered no advantage over proven classical anthocyanin chromatography methods[10]. Fuleki and Francis[11] attempted to develop a quantitative purification method on polyamide columns, but they observed extensive diffusion on the column and some pigment was lost through degradation.

Insoluble polyvinylpyrrolidone (PVP), which has been used mostly for binding phenolic compounds during purification of protein extracts[12], is the first adsorbent which showed both purification and resolution of anthocyanin extracts on columns[13]. Previously PVP was used for preliminary purifications of extracts of anthocyanins

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(*e.g.*, separation from impurities such as sugars, cinnamic acid derivatives or other flavonoid compounds). Other workers have also reported PVP to be preferable for anthocyanin purification (*c.f.*, Fuleki and Francis[11]). Separation of five different anthocyanin-3,5-diglucosides on a PVP column has been reported by Hrazdina[14]. Van Teeling *et al.*[13] partially purified and resolved simple mixtures of anthocyanin derivatives from black raspberry (*Ribes occidentalis*), poinsettia (*Euphorbia pulcherrima*), and cranberry (*Vaccinium macrocarpon*).

Our purpose in this work was to develop a method for anthocyanin chromatography which fulfils the following requests: (1) separation from impurities; (2) resolution of complex natural mixtures; (3) enrichment of minor compounds; (4) purification of fractions containing acylated anthocyanins. Therefore we tested red radish (*Raphanus sativus*) and red cabbage (*Brassica oleracea*), known for containing extremely complex mixtures of different anthocyanins[15–17].

We also tested this procedure on the red genotype (11HHP^{r}P^r) of *Impatiens balsamina*. All the major anthocyanins in the petals have been identified and two acylated derivatives occur[18]. There are also several minor compounds whose identity is putative but through the enrichment procedure described herein, we were able to isolate enough of these compounds to confirm their identity.

EXPERIMENTAL

Pigment extraction

With the exception of *Impatiens* (genotype 11HHP^{r}P^r), which was grown from seed stocks derived from Hagen[18,19], all other plant material was purchased from local merchants. Tissue was extracted overnight at room temperature with 95% ethanol containing 1% (v/v) HCl. The extracts were concentrated in a rotary evaporator at 40° to near dryness and redissolved in a small volume of 0.01 *N* HCl.

Polyamide column chromatography

Polyamide. Polyamide-CC 6 for column chromatography (grain size 0.07 mm) was purchased from Macherey, Nagel & Co., Düren, G.F.R.

Columns. Dimensions of 2 × 25 cm and 2 × 50 cm were used with polyamide bed volumes of 100 and 200 ml, respectively, containing 25 and 50 g polyamide. Pasteur pipets were used for the microcolumns.

Elution. Water and various mixtures of water–methanol with descending polarity were used, each mixture containing 0.01 *N* HCl. Flow-rates were in the range 1–2 ml/min.

Control of elution. The absorption of the anthocyanins was monitored at 505 or 520 nm in a Beckman spectrophotometer DB-G equipped with a flow cell. The column was monitored continuously and the eluant was collected in 10-ml fractions.

Regeneration of the polyamide. 5 ml of 4 *N* NaOH were washed through the polyamide bed and then the column was washed with 0.01 *N* HCl until the eluent was acidic. In some columns a yellow-brown band near the top remained after recharging. This section was discarded before another pigment extract was applied.

Identification of pigments

Tentative characterization of the isolated pigments was based on comparison

of paper chromatography R_F values in at least four different solvent systems[10,20], absorption maxima, calculations of the extinction ratios from spectroscopic data[21], and color and fluorescence under ultraviolet light (UV). Identification of the acyl substituents of the acylated anthocyanins was done after mild basic hydrolysis (0.01 *N* NaOH, 5 min at 50°). The hydrolysate was re-acidified with HCl and shaken against several aliquots of diethyl ether. The ether fraction was dried, redissolved in methanol and spotted on Sigmacell type 20 thin-layer plates. The plates were developed in toluene–acetic acid (2:1, saturated with water) and 2% formic acid. After drying the spots were located under UV light. Color changes in the presence of ammonia vapor and after spraying with diazotized *p*-nitroaniline oversprayed with 2 *N* NaOH were recorded and compared with known cinnamic acids.

RESULTS AND DISCUSSION

Using polyamide column chromatography we have been able to resolve complex natural mixtures of anthocyanins extracted from red radish roots (*Raphanus sativus*) (Fig. 1), leaves of red cabbage (*Brassica oleracea*) (Fig. 2), and lateral petals of *Impatiens balsamina*, genotype 11HHP^{r}P^r (Fig. 3).

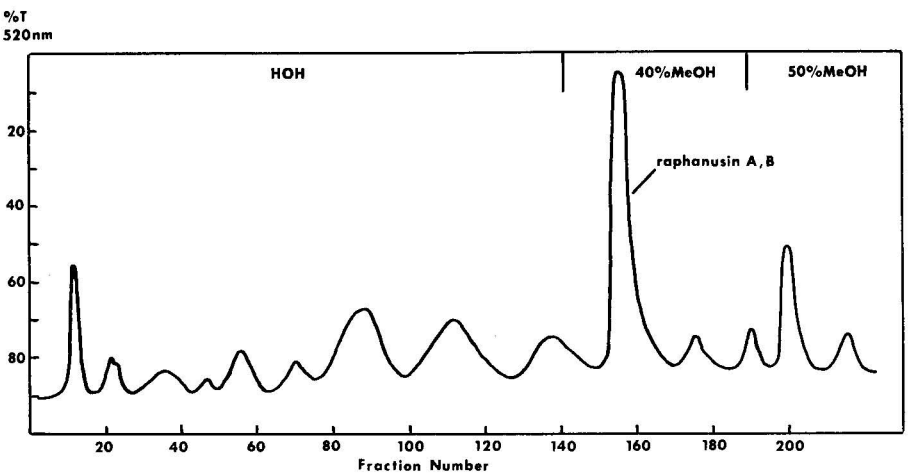

Fig. 1. Elution profile of an alcoholic extract of red radish (*Raphanus sativus*). Column: 25 g polyamide-CC 6 (0.07 mm), 25 × 2 cm. Solvent: water–methanol mixtures with descending polarity; flow-rate: 1–2 ml/min; 10-ml fractions collected.

The behaviour of anthocyanins on a polyamide column is similar to other types of flavonoids, *e.g.*, flavonolglycosides[6]. Less polar or highly soluble anthocyanin derivatives (3-sophoro-5-monoglucoside or 3,5-diglucoside) migrate most rapidly and are eluted with water (Figs. 3 and 4). Anthocyanins possessing only one sugar are more strongly retained and can be eluted only with methanol–water mixtures. Most strongly bound are the anthocyanidins, and these can be removed only with methanol concentrations approaching 100% (Fig. 4).

If there are different anthocyanidins with the same type of glycosylation (*e.g.*, pelargonidin, cyanidin and malvidin 3-monoglucoside) in the extract, malvidin-3-

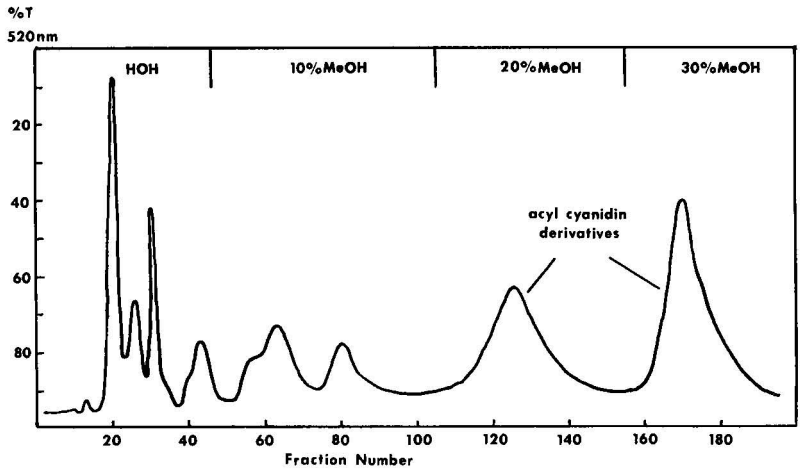

Fig. 2. Elution profile of an alcoholic extract from leaves of red cabbage (*Brassica oleracea*). Column: 50 g polyamide-CC 6 (0.07 mm), 50 × 2 cm. (Other conditions as in Fig. 1).

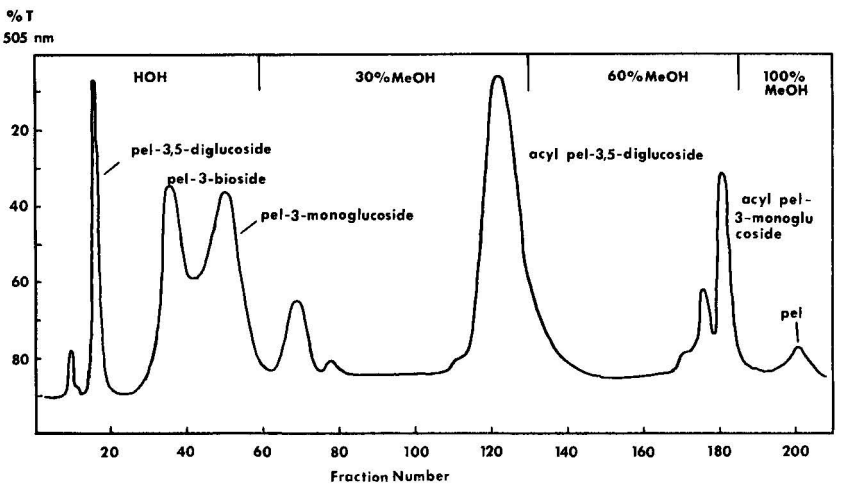

Fig. 3. Elution profile of an alcoholic extract from the lateral petals of *Impatiens balsamina* (genotype 11HHP^{r}P^{r}). For conditions see Fig. 1.

monoglucoside is eluted first, pelargonidin somewhat later followed closely by cyanidin glucoside.

Difficulties are often observed when there are different derivatives of anthocyanidins differing in polarity; this might be the limiting feature of both polyamide and PVP column chromatography.

Van Teeling *et al.*[13], using PVP columns, obtained incomplete separation and overlapping bands with an extract from poinsettia bracts which contain the 3-monoglucoside and 3-rutinoside of both pelargonidin and cyanidin. We also attempted to resolve the poinsettia pigments and obtained results similar to those of Van Teeling *et al.* We were able to separate partially cyanidin-3-rutinoside and pelargonidin-3-

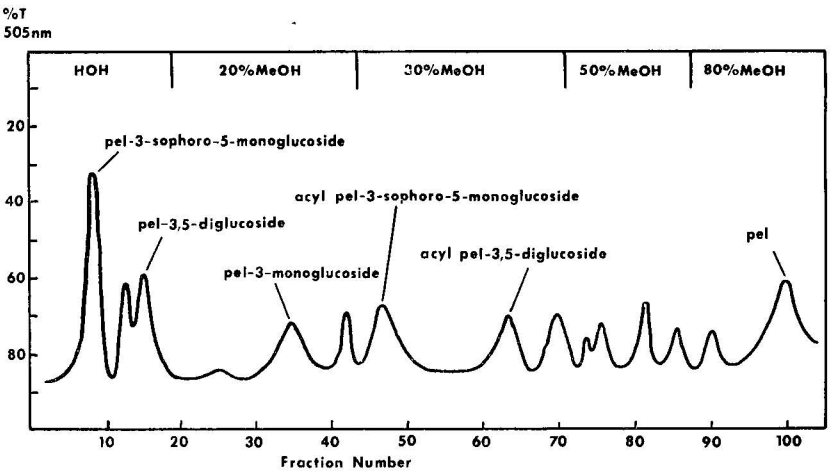

Fig. 4. Elution profile of products from a mild acid hydrolysis of the main fraction of red radish (raphanusin A and B). For conditions see Fig. 1.

monoglucoside but with a large overlapping volume. Pelargonidin-3-monoglucoside and cyanidin-3-monoglucoside from strawberry extracts were eluted in bands which overlapped in the middle 8 of 40 fractions. Thus the intersection of two characteristics of this type of chromatography can be seen: the solubility of the anthocyanin in the solvent, and the adsorption strength of polyamide.

As with PVP chromatography, polyamide columns demonstrate that the nature of the sugar moiety has a greater effect on the rate of movement of the pigment than the hydroxylation pattern of the B ring (*c.f.*, Van Teeling *et al.*[13]). The best resolution is obtained from mixtures of anthocyanins containing different derivatives of the same anthocyanidin (Figs. 3 and 4).

The retention time for a complex mixture of anthocyanins increases in the following sequence: high glycosylation, low glycosylation, acyl-high glycosylation, acyl-low glycosylation, aglycone. The acyl anthocyanins also show a very similar chromatographic mobility to the corresponding flavonol glycosides. Table I shows that the acyl pelargonidin-3-monoglucoside moves together with kaempferol-3-monoglucoside.

The present work opened the possibility of purifying and enriching derivatives of anthocyanins which occur in extremely low amounts. From red radish, red cabbage, and *Impatiens* we were able to purify and enrich the very minor anthocyanins. Fuleki found thirteen different pelargonidin derivatives in red radish roots[17]. In leaves of red cabbage 5 different anthocyanins have been described[15,16]. From a red radish extract we obtained 14 separate bands showing absorption at 520 nm (Fig. 1). Using a longer polyamide column we found eight 520-nm absorbing fractions from red cabbage (Fig. 2). In the lateral petals of *Impatiens balsamina* (red genotype 11HHP^{r}P^r) Hagen[18,19] has described ten different anthocyanins, derivatives of pelargonidin. Our investigations of this tissue has confirmed these previous findings. They were resolved clearly and we obtained those anthocyanins which had been given a putative identification.

TABLE I

RESOLUTION OF ANTHOCYANIN AND FLAVONOL DERIVATIVES OF A STAGE 3* FLOWER PETAL OF THE RED GENOTYPE 11HHP^{r}P^r OF *IMPATIENS BALSAMINA* ON A MICRO-SCALE

Conditions: Polyamide-CC 6 (0.07 mm), 6.5 × 0.5 cm; flow-rate, 0.3 ml/min; alcoholic extract from 15 mg of tissue. Compounds: pelargonidin: 4 = 3,5-diglucoside; 5 = 3-monoglucoside; 6 = acyl 3,5-diglucoside; 37 = 3-bioside; 41 = aglycone; 52 = acyl 3-monoglucoside. Kaempferol: 2 = aglycone; 7 = 3-monoglucoside; 8 = 3-bioside.

Solvent	*Fraction (ml)*	*Compound*								
		4	*37*	*5*	*6*	*8*	*7*	*52*	*41*	*2*
water	5	+								
	25		+	+						
40% methanol	15				+	+				
50% methanol	12						+	+		
80% methanol	10								+	
100% methanol	10									+

* Flower stage and compound number after Hagen[18,19].

This technique makes it thus possible to determine more accurately the total number of anthocyanins present in a plant organ. It is also likely that the qualitative anthocyanin content of many well known plants will be found to be greater than previously thought, since this procedure permits the isolation of most minor compounds in amounts sufficient for further investigations.

Mabry *et al.* observed polyamide columns separating flavone and flavonol derivatives which could not be separated by paper chromatography[5]. Our results confirm the same to be true for anthocyanins. In petals of *Impatiens* there are two pelargonidin derivatives (putative 3-diglycoside and acyl-3,5-diglucoside) which have nearly identical R_F values in four different solvent systems. These compounds can only be distinguished by their fluorescent properties under UV light. The polyamide elution profile (Fig. 3) shows that these derivatives have very different retention times and are easily separated.

During the identification of the major compounds from radish we carried out a mild acid hydrolysis (15 min at 100° in 1 *N* HCl) of the raphanusin A and B fraction (Fig. 1). This hydrolysate was re-chromatographed on polyamide and numerous anthocyanin derivatives were found (Fig. 4). Each of the pigments labelled in Fig. 4 were identified by paper chromatography.

In recent investigations of the kinetics and turnover of the anthocyanins during development, we obtained a rapid and quantitative resolution of the alcoholic extract of *Impatiens balsamina*, genotype 11HHP^{r}P^r (Table I). All compounds listed are clearly resolved and excellent reproducibility can be obtained if definite volumes of solvents are used. Even kaempferol-3-monoside and 3-bioside, which show very similar rates of migration on polyamide columns[2,6], are quantitatively separated.

An important parameter for obtaining good resolution is the grain size of the polyamide powder. In earlier experiments with the regular grain size of 0.16-mm broadening of the bands was observed which resulted in great overlapping and contamination of the individual fractions.

Since anthocyanins are weakly retained on polyamide compared with other

types of flavonoids[7] and this behaviour might be strengthened by using HCl in the solvent (*c.f.*, Mabry *et al.*[5]) for stabilization of the pigments, the water–methanol gradient must be as low as possible.

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CHROM. 8225

GEL CHROMATOGRAPHY OF ACETYLACETONE AND ITS METAL(II, III) COMPLEXES IN THE MERCKOGEL OR-2000–TETRAHYDROFURAN SYSTEM

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SUMMARY

The gel chromatographic behaviour of acetylacetone and its metal(II, III) complexes was studied, using the polyvinyl acetate gel (Merckogel OR-2000)–tetrahydrofuran system. Seven metal complexes, *viz.*, cobalt(III), iron(III), chromium(III), aluminium(III), copper(II), nickel(II) and beryllium(II), of acetylacetone were investigated. Experiments with some *n*-alkanes were also carried out for comparison.

The elution characteristics, *viz.*, K_{av}, HETP and skew ratio, were obtained with high precision with the aid of an on-line data processing system. The correlation between K_{av} values and molar volumes of the solutes is discussed.

INTRODUCTION

Gel chromatography has a unique separation mechanism based on differences in the molecular sizes of the sample components. It has become a powerful method especially for the separation and characterization of high-molecular-weight substances.

In comparison with other liquid chromatographic methods based mainly on chemical or physical interactions in the column system, gel chromatographic separations, without any of those interactions (so-called secondary effects), can be carried out under such mild conditions that any labile compounds do not suffer from undesirable influences. These attractive features will expand the applications of this method not only to high- but also to low-molecular-weight substances, especially metal-containing compounds.

The application of this method to metal-containing substances has been reported. Most of the studies described[1–6] were carried out using Sephadex in aqueous media.

Metal complexes that are insoluble in aqueous media are important in analytical chemistry, where the formation of such complexes is frequently utilized for the separation and determination of some metal ions. It is expected that the combination of the advantages of gel chromatography with the usefulness of the metal complexes will expand their analytical utility further.

However, gel chromatographic studies of such complexes have rarely been made. Nevertheless, a few workers have reported on some neutral metal chelates in organic media. Yamamoto *et al.*[7] investigated tris(acetylacetonato)chromium(III) and -cobalt(III) using some organic solvent–polystyrene gel systems. We have previously studied[8] tris(acetylacetonato)chromium(III) in the polyvinyl acetate gel (Merckogel OR-2000)–chloroform system. In order to expand the utility of gel chromatography to such metal compounds, more systematic fundamental studies must be carried out.

Separations in gel chromatography result from the preferential diffusion of small solute molecules into the porous gel structure with the exclusion of large molecules. These solutes are characterized by the equation[9,10]

$$V_e = V_0 + K_d V_i \tag{1}$$

The elution volume, V_e, is equal to the sum of the void (interstitial) volume, V_0, and a fractional volume, V_i, which is related to the solvent volume taken up by the gel beads. K_d is normally derived from the above equation and is similar to a distribution coefficient.

The void volume, V_0, is assumed to be the peak elution volume of a sufficiently high-molecular-weight compound. However, the exact determination of the volume V_i is very difficult.

Eqn. 1 shows that for the chromatographic determination of V_i it is necessary to use a reference material for which K_d is unity. In general, *n*-pentane or acetone is selected as such a reference material for gel chromatography using organic solvents. However, in the treatment of low-molecular-weight molecules, it is not safe to assume that the K_d value of these reference materials is unity because their molecules are too large.

The following equation was derived by Laurent and Killander[11]:

$$V_e = V_0 + K_{av} V_x \tag{2}$$

where V_x is the volume of the gel phase and K_{av} corresponds to a distribution coefficient of solute between the interstitial solution phase and the swollen gel phase. The volume V_x is equal to the sum of the volume of the gel matrix, V_g, and the volume V_i, and can easily be determined by the equation

$$V_x = V_t - V_0 \tag{3}$$

where V_t is the total volume of the column system (bed volume) and is a measurable parameter. Consequently, K_{av} is a more appropriate parameter than K_d for the expression of the elution characteristics of small molecules such as metal complexes.

In this work, K_{av} values of acetylacetone (AA) and its metal complexes, $M(AA)_n$, were measured in the Merckogel OR-2000–tetrahydrofuran (THF) system. Acetylacetone is a typical β-diketone that is well known as an important chelating agent in analytical chemistry. The metal complexes studied were $Co(AA)_3$, $Fe(AA)_3$, $Cr(AA)_3$, $Al(AA)_3$, $Cu(AA)_2$, $Ni(AA)_2$ and $Be(AA)_2$. In addition, the K_{av} values of some *n*-alkanes were also obtained for comparison.

EXPERIMENTAL

Materials

Merckogel OR-2000 (E. Merck, Darmstadt, G.F.R.) was washed with re-distilled water, acetone and methanol (with *ca.* 50 ml per gram of gel in each case), in that order. The gel was dried under reduced pressure and further over silica gel for 3 days. The resulting solid mass was then ground to a fine material using an agate mortar and then finally sieved to obtain 200–300 mesh fractions.

The metal(II, III) acetylacetonates, except for $Be(AA)_2$, were prepared and purified as reported elsewhere[12,13]. $Be(AA)_2$ was reagent-grade material (Dojindo Co., Kumamoto, Japan).

According to the C, H elemental analyses for the materials finally applied in this work, all of the complexes, except that of Ni(II), were anhydrous; the Ni(II) complex was a dihydrate, *i.e.*, $Ni(AA)_2 \cdot 2H_2O$.

Acetylacetone was purified by washing the reagent-grade material with 1 *M* ammonia solution and distillation after drying over anhydrous sodium sulphate.

Polystyrene, used as a reference material to measure the column void volume, V_0, was monodisperse polystyrene standard (Pressure Chemical Co., Pittsburgh, Pa., U.S.A.), with mol. wt. 200,000 and $M_w/M_n < 1.06$ (Batch No. 1c).

THF was purified by re-distillation of the reagent-grade material (Wako, Osaka, Japan) over iron(II) sulphate at 66.0°.

Apparatus

The apparatus used is shown schematically in Fig. 1. As acetylacetone has a high reactivity with metals, all parts that come into contact with liquid are made not of metal but of PTFE or Pyrex in order to avoid undesirable influences, except for the pump, which is made of stainless steel.

The column consists of 100-cm sections of 5-mm I.D. Pyrex tubing with a water-jacket. The column is kept at 25.00 ± 0.02° by a Haake Model FK-2 constant-temperature circulator. The Jasco Model FLC-350 pumping system (Japan Spectroscopic Co., Tokyo, Japan) is a syringe-type high-pressure pump. The sample injection

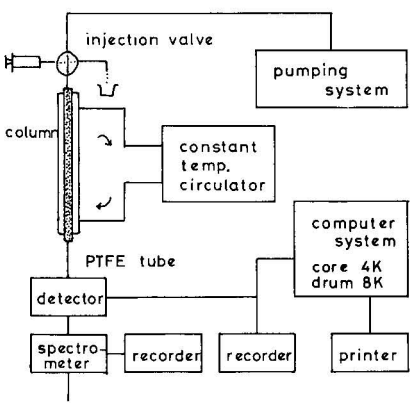

Fig. 1. Schematic diagram of the chromatographic system.

valve (Jeol, Tokyo, Japan) is calibrated to 40-μl. A refractometric detector (Laboratory Data Control, Riviera Beach, Fla., U.S.A.) is used, and the detection signal is fed to a JEC-5 computer system (Jeol) for on-line data processing. The effluent flowing out the detector is fed to a Jasco Model UVIDEC-I spectrophotometer with micro-flow cells made of PTFE (8 μl).

The details of the present system and the data processing software will be published elsewhere.

Procedure

Merckogel OR-2000, after being swollen in THF for at least 24 h, was packed into the column using a packing reservoir supplying the solvent at a flow-rate of 0.20 ml/min. After the gel had settled, the solvent was further pumped through the column at a flow-rate of 0.20 ml/min for 24 h so as to ensure complete setting of the gel bed under normal operating conditions, and this flow-rate was maintained throughout the experiments.

Sample solutions were prepared by dissolving the mixture of a metal complex or *n*-alkane and polystyrene in 5 ml of THF, as shown in Table I. Polystyrene acts as an internal reference. A 40-μl portion of a sample solution was fed into the column by the injection valve.

TABLE I

COMPOSITION OF SAMPLE SOLUTIONS

No.	*Sample*	*Amount taken (mg*)*	*Polystyrene added (mg*)*
1	$Co(AA)_3$	15.35	8.70
2	$Fe(AA)_3$	15.91	6.82
3	$Cr(AA)_3$	15.04	6.11
4	$Al(AA)_3$	15.40	7.19
5	$Cu(AA)_2$	10.75	7.13
6	$Ni(AA)_2 \cdot 2H_2O$	13.90	6.30
7	$Be(AA)_2$	14.64	6.87
8	Acetylacetone	29.80	7.00
9	*n*-Hexadecane	58.50	6.30
10	*n*-Heptane	100 μl	6.36
11	*n*-Pentane	50 μl	6.80

* In 5 ml of solution.

UV absorption profiles of the effluent were recorded on the spectrophotometer in order to identify the species being eluted.

The computer printed out the data on the chromatographic behaviour of the sample components each time the elution of all of the components of an injected sample was completed. Each experiment on a sample was carried out in triplicate.

RESULTS AND DISCUSSION

The elution volumes of the sample components increased in the following order: polystyrene, *n*-hexadecane, *n*-heptane, $Al(AA)_3$, $Cr(AA)_3$, $Co(AA)$ $Fe(AA)_3$,

n-pentane, $Ni(AA)_2$, $Cu(AA)_2$, $Be(AA)_2$, acetylacetone. Some examples of the chromatograms obtained are shown in Fig. 2.

According to the measurements of the gel bed volume, V_t, and the elution volume of polystyrene, the void volume, V_0, and the gel phase volume, V_x, were calculated by eqn. 3 as $V_t = 19.63$, $V_0 = 6.82$ and $V_x = 12.81$ ml.

These values are based on the assumption that the K_d and K_{av} values for polystyrene are zero, and these are the results corrected for the dead volume (0.24 ml), which is increased by the internal volume of the tubing and the detector.

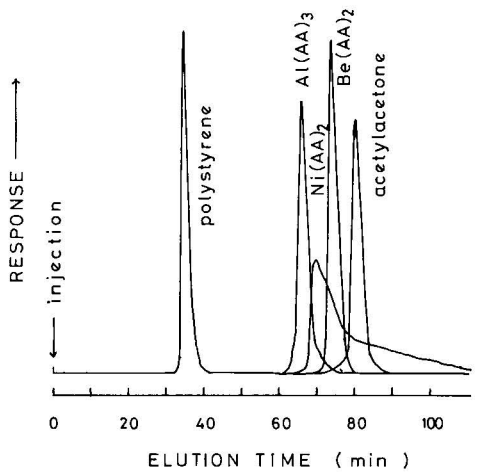

Fig. 2. Chromatograms of acetylacetone and some of its metal complexes. Polystyrene is added as the reference material in each sample solution. Merckogel OR-2000 column, 100 cm × 5 mm I.D., THF solvent at 0.20 ml/min, 25.0°.

The distribution coefficients, K_{av}, of the solutes were obtained by means of eqn. 2 and are listed together with the skew ratio, R, and HETP in Table II. The reproducibility of the K_{av} values is satisfactory. The relative standard deviation in each case is not more than 0.7%. The values shown in Table II are the average values of triplicate measurements.

The skew ratio is a convenient parameter for expressing the shapes of elution peaks, and is defined as the ratio of the magnitude of the slopes of the trailing and leading edges at their points of inflection[14]; thus, when the peak is symmetrical, the skew ratio is unity; however, when the leading slope is steeper than the trailing slope, the skew ratio is less than unity. According to the results for the skew ratios, the complexes of the trivalent metals, except iron, show symmetrical peaks. The value for $Fe(AA)_3$ is 0.58, which indicates a skewed peak. However, the HETP of $Fe(AA)_3$ is less than those for the other complexes and therefore it can be said that $Fe(AA)_3$ gives a sharp peak with a somewhat trailing shape.

Of the complexes of divalent metals, $Be(AA)_2$ gives a sharp and symmetrical peak, but the complexes of copper and nickel had small skew ratios. The complex of nickel gave a particularly distorted elution peak, as shown in Fig. 2. The UV absorption profile of the trailing fraction of the effluent was different from the profile of the

TABLE II

OBSERVED VALUES OF DISTRIBUTION COEFFICIENT, K_{av}, HEIGHT EQUIVALENT TO A THEORETICAL PLATE, HETP, AND SKEW RATIO, R

Column: Merckogel OR-2000, 100 cm × 5 mm I.D., 25.0°. Solvent: THF, 0.20 ml/min.

No.	*Substance*	K_{av}	*HETP (mm)*	R
1	$Co(AA)_3$	0.523	0.36	0.97
2	$Fe(AA)_3$	0.536	0.19	0.58
3	$Cr(AA)_3$	0.517	0.32	0.97
4	$Al(AA)_3$	0.497	0.32	0.89
5	$Cu(AA)_2$	0.571	0.22	0.58
6	$Ni(AA)_2$	0.557	—	0.28
7	$Be(AA)_2$	0.620	0.28	0.99
8	Acetylacetone	0.714	0.17	0.77
9	*n*-Hexadecane	0.252	0.22	0.97
10	*n*-Heptane	0.464	0.17	0.94
11	*n*-Pentane	0.539	0.16	0.95

original solution of nickel acetylacetonate. Nickel acetylacetonate is well known as a labile complex, and decomposition may occur in the column.

$Be(AA)_2$ is tetrahedral and the central metal atom is surrounded by the ligands. Therefore, the complex can be assumed to be a spherical molecule. The bis(acetylacetonato) complexes of copper and nickel, however, are square-planar. The differences in the elution behaviour of these complexes must be also related to stereochemical effects. In addition, the nickel complex used in this work is a dihydrated crystal, whereas the other complexes are anhydrous.

According to the principles of gel chromatography, a larger solute molecule is expected to have a smaller K_{av} value. It is not easy to measure the exact sizes of the solute molecules. Therefore, the molar volume of each solute, which is known, is adopted in the following discussion.

Irving[15] showed that the molar volumes of $M(AA)_3$ in organic solvents ($Al(AA)_3$, 271; $Cr(AA)_3$, 267; $Fe(AA)_3$, 269; $Co(AA)_3$, 261) are not very different from those for the solid state. The molar volume of $Be(AA)_2$ is calculated to be 184 when the empirical relationship between the molar volume of acetylacetone, V_{AA}, and that of its metal complex, $V_{M(AA)_n}$, expressed by the equation[15,16] $V_{M(AA)_n}/V_{AA} = 0.9\,n$, is used. Wakahayashi *et al.*[16] showed that the partition behaviour of acetylacetone can be explained satisfactorily by assuming that the molar volume of acetylacetone is 102*.

The molar volumes of *n*-pentane, *n*-heptane and *n*-hexadecane are 116, 147 and 295, respectively[17]. The correlations between the K_{av} values and the molar volumes, V, of the solutes are clearly shown in Fig. 3.

Comparing the V values for the *n*-alkanes (group A) with the K_{av} values, the order of decreasing molar volume is the same as that for increasing K_{av} values. A similar correlation is also obtained for acetylacetone and its metal complexes (group B).

However, it seems that the elution behaviour of the two groups is different.

* From density measurements in our laboratory, the molar volume of acetylacetone is calculated to be 103.4 at 25.0 °.

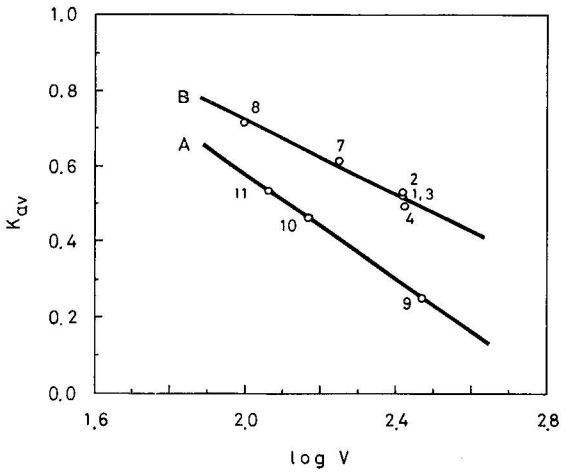

Fig. 3. Correlation between K_{av} and molar volume, V. A, n-Alkanes; B, acetylacetone and its metal complexes. The numbers correspond to those in Table II.

For example, the molar volume of n-heptane is apparently less than that of $M(AA)_3$, but the K_{av} value of the former is not greater than that of the latter.

Considering that n-alkanes are inert compounds, the elution behaviour of the group B compounds can be explained not only by the sieving effect but also by some interactions between the solute and gel phase and also by the difference in the geometrical structures of the molecules. For the elucidation of the chromatographic behaviour of these compounds, it will be necessary to investigate the effects of solvents and gels more extensively, and such studies will be published elsewhere.

From a practical point of view, the K_{av} and HETP results indicate that the separation of some of these metal complexes will be successful under more appropriate conditions, *e.g.*, lower flow-rate of the solvent and longer column length.

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CHARACTERIZATION OF SYNTHETIC CARRIER AMPHOLYTES FOR ISOELECTRIC FOCUSING

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SUMMARY

The synthesis of carrier ampholytes suitable for isoelectric focusing is described. The mixture of hexamethylenetetramine (HMTA), triethylenetetramine (TETA), tetraethylenepentamine (TEPA) and pentaethylenehexamine (PEHA) ampholytes closely resembles commercial Ampholine, and covers the pH range 3–9.5. We have been able to detect focused ampholytes in a gel slab, taking advantage of their different refractive indices, and to assess their relative amounts along the pH gradient.

PEHA ampholytes contain up to 20% of chromophoric structures, with two UV peaks at 368 and 315 nm, in a pH-dependent equilibrium, associated with a very weak nitrogen function having a pK of 1.1. This could be the pK_6 of the last amino group in PEHA. However, NMR spectra failed to reveal any nitrogen heterocyclic structure formed during the synthesis.

This mixture of ampholytes exhibits good conductivity, produces smooth pH gradients and allows sharp protein separations in the pH range 3–9.5. Their synthesis is very easy and their cost is extremely low. Their availability should make feasible large-scale preparative isoelectric focusing, and attract more interest to continuous-flow techniques, where large amounts of ampholytes are required.

INTRODUCTION

The development of the technique of isoelectric focusing (IEF) represents a major breakthrough in the field of high-resolution separations of amphoteric molecules. IEF is an equilibrium method in which amphoteric substances are segregated according to their isoelectric points (pI) in a pH gradient. In a series of theoretical papers, Svensson[1–3] laid the foundations of IEF in its present form: he introduced the idea of developing a "natural" pH gradient from amphoteric molecules having closely spaced pIs and high conductivities. Under an electric field, these ampholytes would be distributed according to their pI values to form a pH gradient increasing monotonically from the anode to the cathode.

In the early work on IEF, an extensive search for commercially available ampholytes that might be useful as carrier ampholytes was not very successful.

Svensson[2] published a list of these substances, but was unable to find suitable ampholytes with good conductivity and buffering capacity in the pH range 3.9–7.3. In subsequent experiments, Vesterberg and Svensson[4] were able to obtain, by hydrolysis of proteins, oligopeptides with different p*I* values and rather good electrochemical properties. However, even these preparations lacked satisfactory carrier ampholytes in the pH range 4.5–6, and they also had the drawback of exhibiting properties that resembled those of proteins too closely.

Efforts were then directed toward synthetic processes. Vesterberg[5] synthesized a mixture of a large number of homologues and isomers of aliphatic polyamino polycarboxylic acids with different p*K*s and p*I*s closely spaced in the pH range 3–10. Their synthesis involves the coupling of propionic acid residues to polyethylene polyamines. Carrier ampholytes with suitable properties are obtained when appropriate amounts of acrylic acid are allowed to react with different polyethylene polyamines in water at 70° until all of the acrylic acid has been consumed. The synthesis proceeds via an anti-Markovnikov addition after the Michael reaction and therefore β-amino acids are obtained. These carrier ampholytes, encompassing the pH range 3–10, are commercially available from LKB (Stockholm, Sweden) under the trade-name Ampholine.

By utilizing polyamines with the amino groups more than three methylene groups apart, Lundblad *et al.*[6] were able to synthesize more alkaline ampholytes, with p*I* values up to 11.1, thus extending the range for protein separations. Some more acidic ampholytes have also been synthesized, and have proved useful[7] for protein separations down to pH 2.5. Recently, Vinogradov *et al.*[8] described a modification of the Vesterberg synthetic procedure, and obtained suitable carrier ampholytes in the pH range 4–8 by coupling pentaethylenehexamine with acrylic acid.

At present, most laboratories use isoelectric focusing routinely on an analytical scale, especially in gel media, because of the simplicity, reproducibility and speed of the method. We feel that progress in preparative isoelectric focusing, especially when using continuous-flow techniques[9], has been severely hampered by the high cost of Ampholine and by difficulties in recovering them after a run, especially when using sucrose density gradient stabilized columns. From this point of view, it is surprising that it took so long to "rediscover" Vesterberg's synthetic procedure, and that so far only few laboratories have adopted it.

In an attempt to attract more interest to "home-made" carrier ampholytes and to encourage further the preparative aspects of IEF, we describe here the synthesis of carrier ampholytes suitable for isoelectric focusing and report some of their physico-chemical properties. In the following paper, we describe a simple and reproducible method for fractionating the synthetic mixture in narrow pH ranges covering one or two pH units.

MATERIALS AND METHODS

Hexamethylenetetramine (HMTA), triethylenetetramine (TETA), tetraethylenepentamine (TEPA) and pentaethylenehexamine (PEHA) were obtained from Hoechst Italia (Milan, Italy) and acrylic acid, acrylamide and N,N′-methylenebisacrylamide (Bis) were obtained from Merck-Schuchardt (Munich, G.F.R.). Bis was recrystallized from acetone and the acrylamide from chloroform, as described by Loening[10].

UV–visible spectra were measured with a Cary 118 spectrophotometer (Varian, Palo Alto, Calif., U.S.A.). Fluorescence spectra were obtained with a Perkin-Elmer Model MPF-2A fluorescence spectrometer, fitted with a Hitachi recorder. Nuclear magnetic resonance (NMR) spectra were recorded with a Perkin-Elmer Model R 12 NMR spectrometer at 60 MHz. Optical rotatory dispersion (ORD) spectra were obtained with a Jasco ORD/UV 5 spectropolarimeter.

Isoelectric focusing was performed in a gel slab, using the LKB 2117 Multiphor and a pulsed power supply, as described by Righetti and Righetti[11]. Scans of pictures of focused ampholytes in a gel slab were obtained with a chromoscan from Joyce, Loebl & Co. (Burlington, Mass., U.S.A.) equipped with an automatic integrator. pH gradients were measured with a Radiometer pH meter fitted with a combination microelectrode, in 1 × 0.5 cm gel segments, eluted with 0.3 ml of 10 m*M* sodium chloride solution, at room temperature.

Conductimetric studies were carried out with a conductimeter from Instrumentation Laboratory, in a 2-ml cell thermostated at 25°.

RESULTS

Reagent distillation

Acrylic acid has to be distilled in order to remove the polymerization inhibitor (200 ppm of *p*-methoxyphenol). This is carried out under a stream of nitrogen under reduced pressure. Usually only the amount of acrylic acid that is needed for the coupling reaction with the polyamine is distilled. However, we have also tried batchwise distillations, immediately using the amount of acrylic acid needed and storing the remainder in a nitrogen atmosphere in a freezer. Under these conditions, we have not observed the formation of self-polymers (polymethacrylate).

Perhaps the most cumbersome operation during the synthesis of carrier ampholytes is the distillation of PEHA. This is a necessary step, in order to remove yellow compounds present in the commercial product. One could avoid distilling the various polyamines and then remove the chromophoric compounds present by repeated charcoal treatment, after the synthesis, as described by Vinogradov *et al.*[8]. However, we have observed that, in this case, the amount of "coloured" carrier ampholytes obtained is greatly enhanced, and that they tend to undergo a browning process upon storage. Fig. 1 depicts the distillation set up for purification of PEHA. This material has a very high boiling point and under a vacuum of 500 μmHg it distills in the temperature range 200–290°. The only efficient process was to use a sand-bath, with a multiple bunsen burner, and an extra bunsen burner for the Vigreux column. The vacuum pump is protected by a Fresenius tower containing concentrated sulphuric acid, followed by a calcium chloride trap. During the distillation process, the nitrogen used should be oxygen-free in order to prevent further oxidation of PEHA, which is not cooled in the condenser so as to avoid an increase in viscosity. To achieve this, the gas from the nitrogen tank flows through a catalytic burner (copper wire at 450°) and is subsequently cooled in a "serpentine" (see Fig. 1). This process effectively reduces the absorption spectrum of PEHA, although it does not completely eliminate the peak at 290 nm and a shoulder at 330 nm. As a comparison, free acrylic acid does not show any appreciable chromophore above 300 nm (see Fig. 2). The distillation of TETA, TEPA and acrylic acid is a much easier process, because of their lower boiling

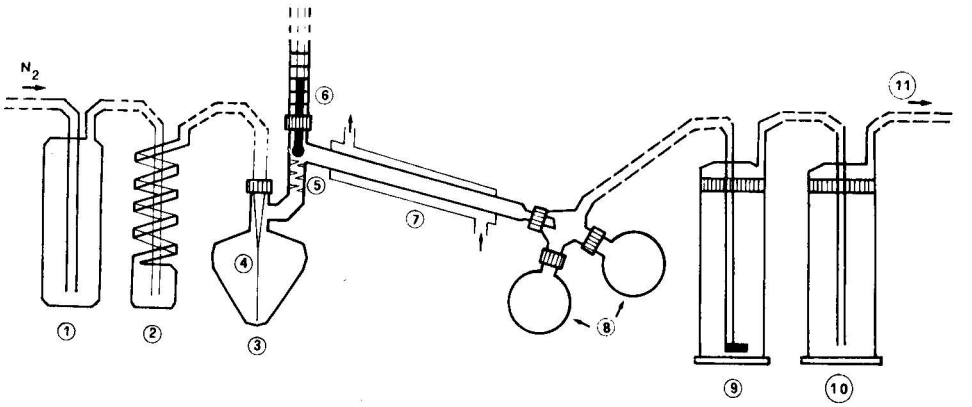

Fig. 1. Scheme of the distillation procedure. 1, Copper wire catalyst, kept in an oven at 450°; 2, cooling "serpentine"; 3, distillation flask; 4, capillary for nitrogen flushing; 5, Vigreux column; 6, thermometer; 7, condenser; 8, collection flasks; 9, Fresenius tower with conc. H_2SO_4; 10, $CaCl_2$ trap; 11, connection to the vacuum pump. The shaded regions represent ground-glass joints.

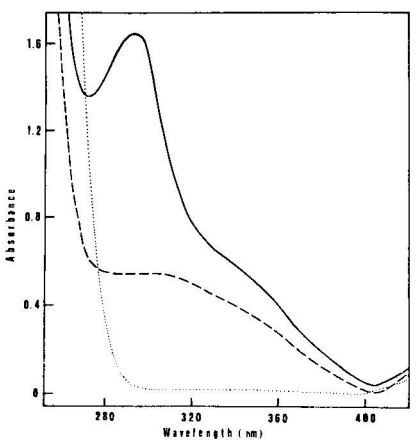

Fig. 2. UV spectra obtained with a Cary 118 spectrophotometer. Solid line: spectrum of commercial PEHA. Broken line: PEHA spectrum after distillation. Dotted line: acrylic acid spectrum. All samples were 1% (v/v) solutions.

points. HMTA was used as such, and not recrystallized, because its solutions did not show any appreciable chromophore in the 250–500 nm range.

Synthesis of carrier ampholytes

We have followed, as a general procedure, that outlined by Vesterberg[5]. The reaction was carried out in a two-necked flask, equipped with a capillary for nitrogen flushing and with a burette for the addition of acrylic acid, fitted with a side-arm as a gas outlet. The polyamine is diluted with water to 0.15 *M* in the flask, cooled and degassed. Then acrylic acid is added dropwise from a burette, with continuous stirring, over a period of 60 min so as to provide the desired nitrogen:carboxyl ratio. The reaction is exothermic, and during the addition it is critical to flush the system with

oxygen-free nitrogen in order to prevent yellowing and browning of the products. For this purpose, we use nitrogen purified through a catalytic burner, as shown in Fig. 1. After the addition of acrylic acid, the flask is stoppered, sealed with Parafilm and transferred to a Dubnoff shaker, thermostated at 70°, for 16–20 h. The reaction mixture is then cooled at room temperature and enough distilled water added to make a 40% (w/v) solution of the carrier ampholytes.

In agreement with the reports of Vesterberg[5] and Vinogradov *et al.*[8], we also found that the best carrier ampholytes in the pH range 3–10 were obtained with a nitrogen:carboxyl ratio of 2:1. Therefore, in coupling the various polyamines with acrylic acid, we always used this ratio. Under these conditions, when samples of the reaction mixture were tested for unreacted acrylic acid with potassium permanganate, as described by Vesterberg[5], virtually no free acrylic acid could be detected. In a few experiments with PEHA, we increased the amount of acrylic acid added, decreasing the nitrogen:carboxyl ratio to 1.5:1. Under these conditions, approximately 70% of the ampholyte population is shifted in the p*I* range 3–5.5, and more than 10% of the acrylic acid remains unreacted. This type of ampholyte can be useful to reinforce the acid side of the pH range, provided that the free acrylic acid is removed, for instance, by electrolysis (see the following paper). A further increase in the amount of acrylic acid added, to a nitrogen:carboxyl ratio of 1:1, leads to ampholytes of no practical value, and leaves more than 25% of unreacted acrylic acid (see Fig. 3).

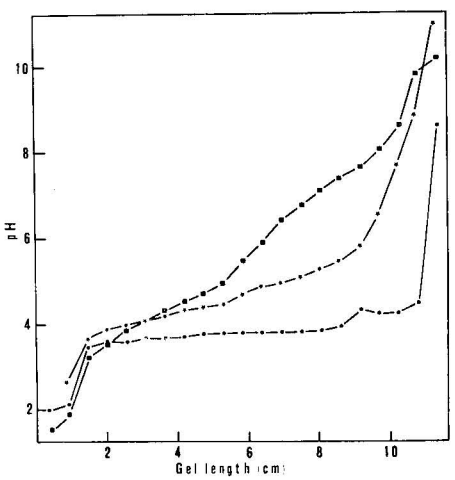

Fig. 3. pH gradients obtained with PEHA ampholytes in a polyacrylamide gel slab. PEHA ampholytes synthesized with a nitrogen:carboxyl ratio of: ■, 2:1; ★, 1.5:1, ●, 1:1. In the last case, no useful ampholytes are obtained.

The ampholytes thus synthesized are yellow-orange in colour. These coloured compounds are, in fact, amphoteric and they focus in the pH range 3–10. With PEHA ampholytes focused in a gel slab, we were able to detect 8–10 yellow focused bands, and we could collect and characterize six of them, having p*I*s of 2.8, 3.4, 4.5, 5.5, 6.2 and 8.5. They represent up to 20% of the entire ampholyte population. While they do not appear to interfere with protein separations and staining in polyacryl-

amide gels, they can in any event be substantially reduced by repeated (3–4 times) treatment with activated charcoal (2 g per 100 ml of 20% ampholyte solution). The mixture is de-gassed, kept under vacuum and heated at 80–90° for 10 min. After cooling and filtering, the ampholytes are stored frozen in brown bottles.

TETA ampholytes

TETA ampholytes, synthesized as described above, with a nitrogen:carboxyl ratio of 2:1, were used to polymerize a polyacrylamide gel slab to a final concentration of 5% of acrylamide and 2% of ampholyte. The gel was photopolymerized with riboflavin, as described by Vesterberg[12]. At equilibrium, taking advantage of the different refractive indices of ampholytes along the gel length, we were able to reveal them by photography, as shown in Fig. 4. The rope-like structures are clusters of focused ampholytes, and the valleys in between are regions with low concentrations of or

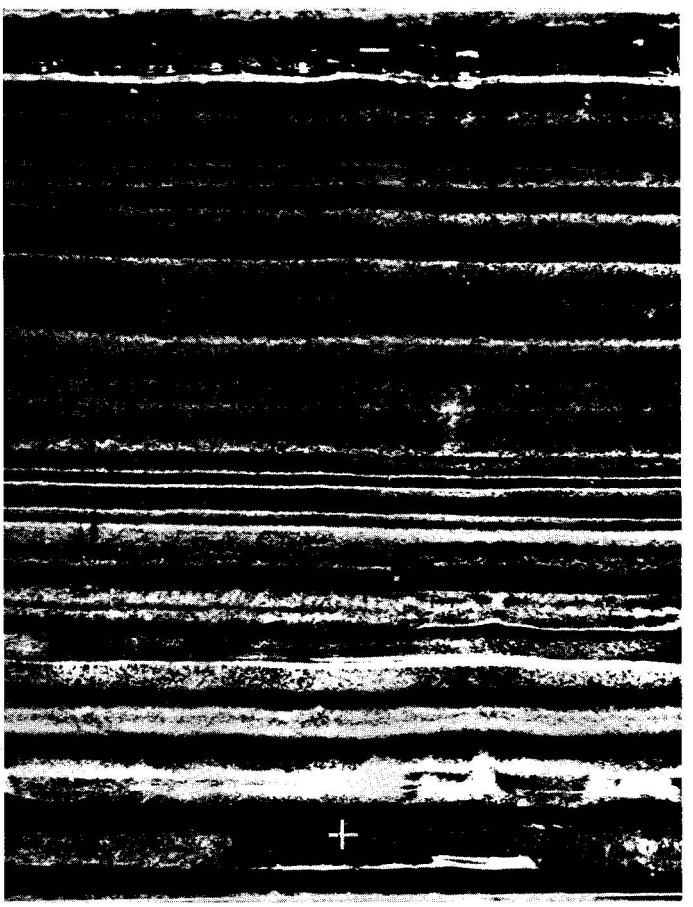

Fig. 4. TETA ampholytes focused in a gel slab. At equilibrium, the gel was photographed against a black background with side illumination. The rope-like structures are clusters of focused ampholytes. This is a picture of a transparent, unstained gel and the ampholytes are detected on the basis of different refractive indexes. The anode (+) and the cathode (−) are marked.

almost no ampholytes. The scan of it in Fig. 5 reveals the ampholyte clusters and the large gaps in between (indicated by arrows). From both figures it is evident that TETA ampholytes are rather poor along the whole of the pH gradient, as there are too few species and too many gaps between them.

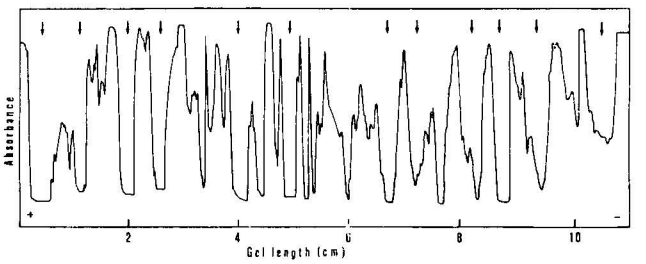

Fig. 5. Scan of focused TETA ampholytes. This scan was made on the print of Fig. 4, with a Joyce, Loebl & Co. chromoscan. The gel polarity is indicated by a + sign (anode, left-hand side) and a − sign (cathode, right-hand side). The arrows indicate gel regions of ampholyte gaps.

Similar conclusions can be drawn from Fig. 6, which gives the pH gradient, the conductance and the UV profiles at 280 and 360 nm obtained by focusing TETA ampholytes in a gel slab. It can be seen that the pH gradient is very uneven and that there are conductivity gaps in several regions of the gel. Therefore, TETA ampholytes do not appear to be suitable for isoelectric focusing, as reported by Vesterberg[5].

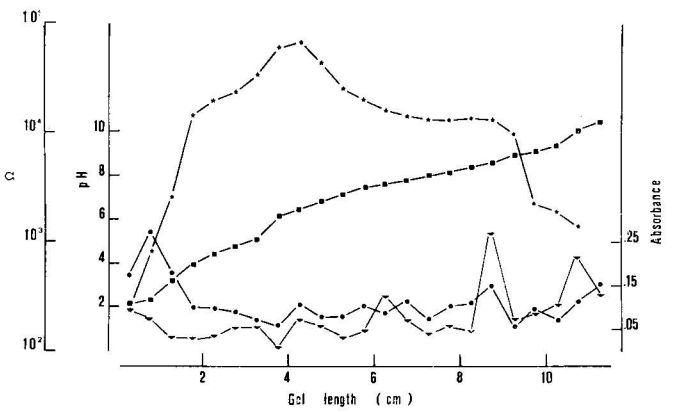

Fig. 6. Conductance (★—★) and pH (■—■) courses and UV profiles at 360 nm (▼—▼) and 280 nm (●—●) of focused TETA ampholytes in a gel slab. For pH determinations, segments of 1 × 0.5 cm were cut. For conductimetry and UV absorbances, gel segments of 4 × 0.5 cm were cut and eluted in 3 ml of distilled water.

TEPA ampholytes

A gel slab containing 2% of TEPA ampholytes and 5% of acrylamide was polymerized and subjected to electrofocusing. A picture of the focused ampholytes (Fig. 7) shows a large improvement over TETA ampholytes. This is also confirmed by the scan in Fig. 8. It can be seen that the ampholyte clusters, especially in the acidic region of the gel, are tighter and closer to one another, with narrower channels

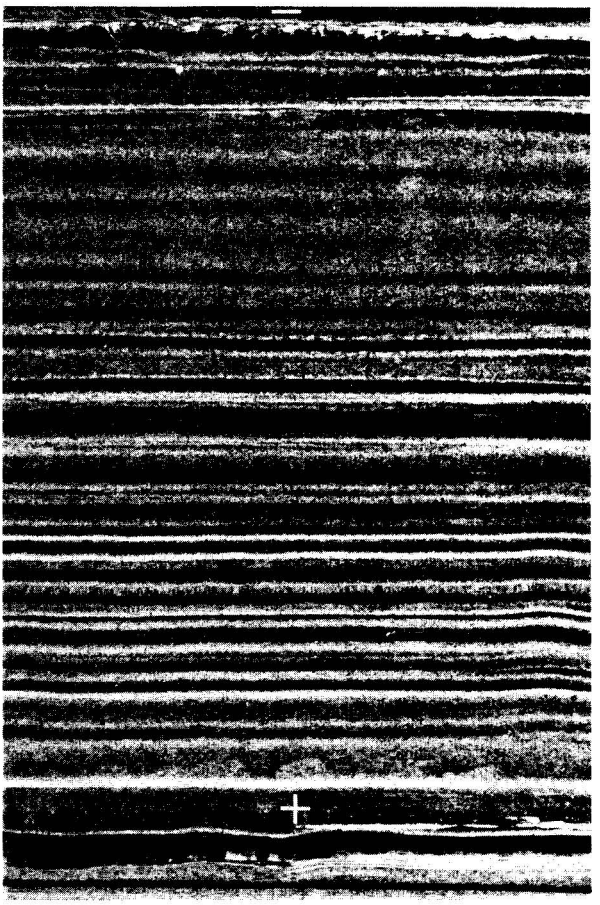

Fig. 7. TEPA ampholytes focused in a gel slab. The direction of the pH gradient is indicated by + (anode) and − (cathode). All other conditions as in Fig. 4.

in between, except for a few, wider gaps indicated by arrows in Fig. 8. The basic region of the gel, while definitely improved in comparison with TETA ampholytes, still shows the presence of less ampholyte species than the acidic region, with wider gaps and lower concentrations of individual clusters of ampholytes. This is also apparent

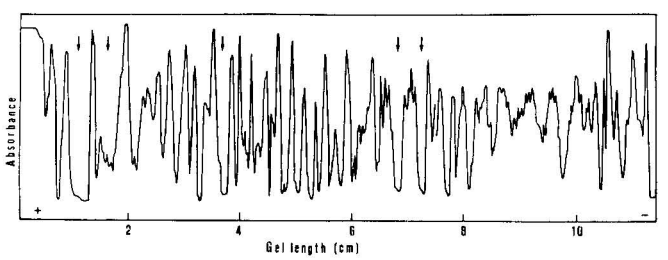

Fig. 8. Scan of focused TEPA ampholytes, from Fig. 7. The arrows indicate regions of low ampholyte concentration and the + and − signs the gel polarity. All other conditions as in Fig. 5.

in Fig. 8, which shows a progressive decrease in peak height in the region of basic ampholytes.

Fig. 9 gives the pH gradient, the conductance and the UV profiles at 280 and 360 nm obtained by focusing TEPA ampholytes in a gel slab. In comparison with Fig. 6, it can be seen that the pH gradient is smoother and that there are fewer conductivity gaps.

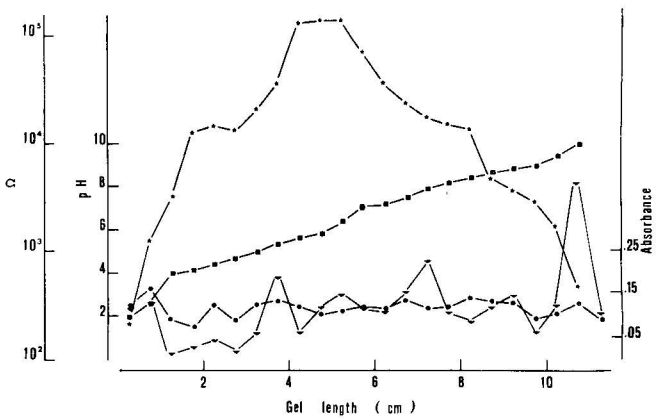

Fig. 9. Conductance (★—★) and pH (■—■) courses and UV profiles at 360 nm (▼—▼) and 280 nm (●—●) of focused TEPA ampholytes in a gel slab. All conditions as in Fig. 6.

PEHA ampholytes

A gel slab containing 2% of PEHA ampholytes and 5% of acrylamide was polymerized and electrofocused as described above. A picture of the focused ampholytes in the gel slab is shown in Fig. 10. This represents a still greater improvement over TEPA ampholytes. The acidic regions of the two types of ampholytes appear to be rather similar, with sharp, closely spaced peaks indicating a smooth pH gradient and good conductivity in this gel region. However, the basic region of PEHA ampholytes appears to be improved in comparison with TEPA ampholytes, with high-relief, tightly packed clusters, which only towards the very basic end of the gel become progressively shallower and more spaced. There are only three major valleys of low ampholyte concentration, two towards the neutral region of the gel, and one towards the alkaline end. They are indicated by arrows in Fig. 10.

Fig. 11 gives the conductance, the pH gradient and the UV profiles at 280 and 368 nm of PEHA ampholytes focused in a gel slab. It is immediately apparent that the conductivity is much more uniform and higher than with TETA and TEPA ampholytes. There is only a small conductivity dip in the pH region 5.5. Also, the pH gradient is smoother and encompasses the pH range 3–9.5. The UV profile at 368 nm shows 6–7 peaks, representing chromophoric ampholytes whose p*I*s were given in the section *Synthesis of carrier ampholytes* and whose properties are described later.

Fig. 12 shows the separation of sickle-cell and normal haemoglobins, together with free α- and β-chains, in a polyacrylamide gel slab in the presence of 2% of PEHA ampholytes. The bands are sharply focused and their p*I*s are the same as those obtained by Perrella *et al.*[13] when using commercial Ampholine.

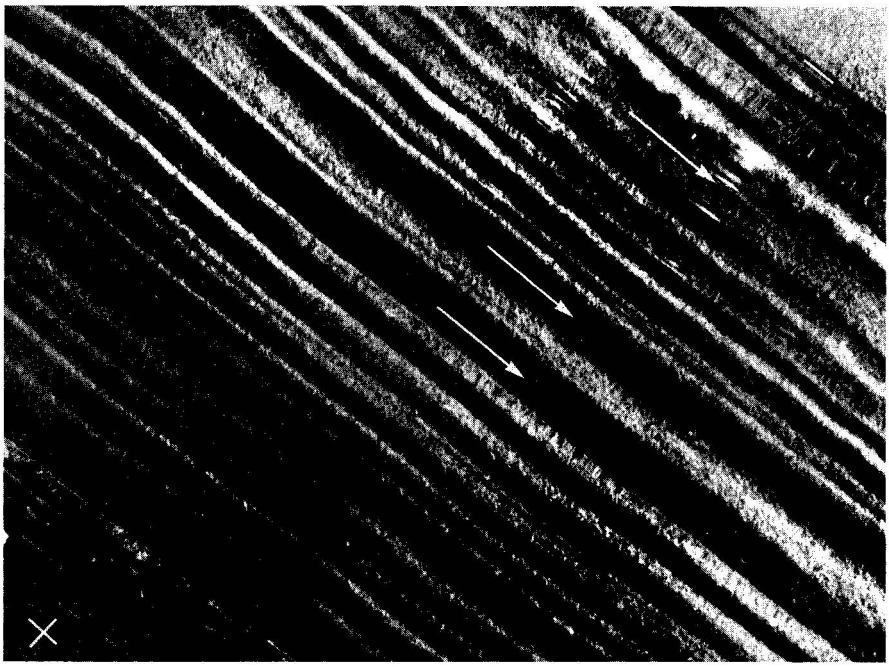

Fig. 10. Scan of focused PEHA ampholytes. The direction of the pH gradient is indicated by + (anode) and − (cathode). The arrows indicate ampholyte gaps. All other conditions as in Fig. 4.

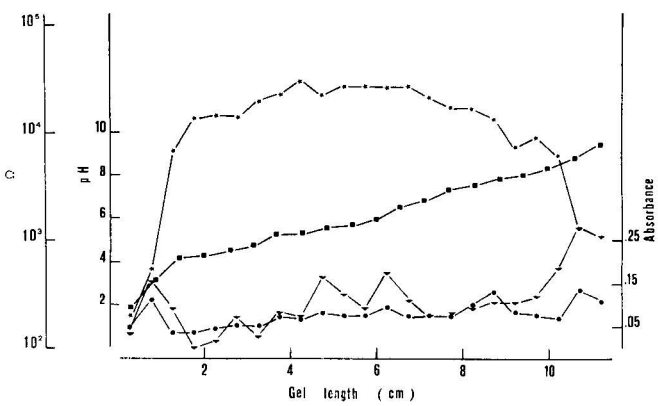

Fig. 11. Conductance (★—★) and pH (■—■) courses and UV profiles at 368 nm (▼—▼) and 280 nm (●—●) of focused PEHA ampholytes in a gel slab. All conditions as in Fig. 6.

Some physico-chemical properties of ampholytes

We have seen that in PEHA ampholytes, there are chromophoric structures, which, upon focusing, give 8–10 yellow bands, encompassing the pH range 3–9.5. These structures are formed during the synthesis, as PEHA and acrylic acid do not contain them (see Fig. 2). As these chromophoric compounds can sometimes represent up to 20% of the total ampholyte mixture, and as they cannot be completely removed by charcoal treatment, we thought it worthwhile to investigate their structure.

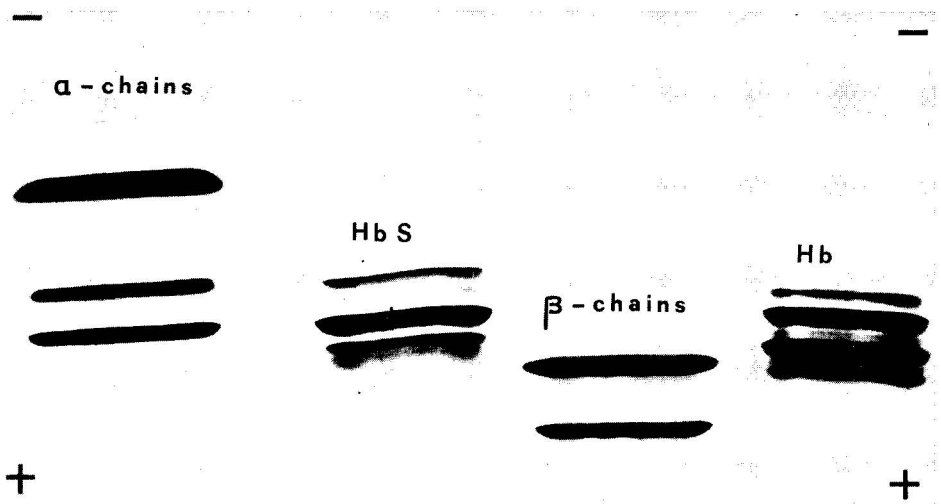

Fig. 12. Isoelectric focusing of normal human adult haemoglobin (Hb), sickle-cell haemoglobin (HbS) and free α- and β-chains from haemoglobin. The run was made in a 5% acrylamide gel slab and 2% PEHA ampholytes at 2°. All the samples were equilibrated in CO. The equilibrium was obtained in 80 min with a pulsed power supply at a final voltage of 1200 V.

Fig. 13 shows the UV spectra of these chromophoric ampholytes. These spectra are identical, irrespective of whether the entire mixture or the single, isoelectric ampholytes, isolated by gel slab isoelectric focusing, are analyzed. The spectrum shows a strong peak at 368 nm, which is progressively quenched from pH 9 to pH 1. Below pH 1.8, a new peak appears at 315 nm. The two structures are in a pH-dependent equilibrium, the 315-nm peak representing the protonated and the 368-nm peak the unprotonated species. Their isosbestic point is at 335 nm. When the solution is titrated to pH 0.5 and below, allowed to remain there for a few hours and then back-titrated,

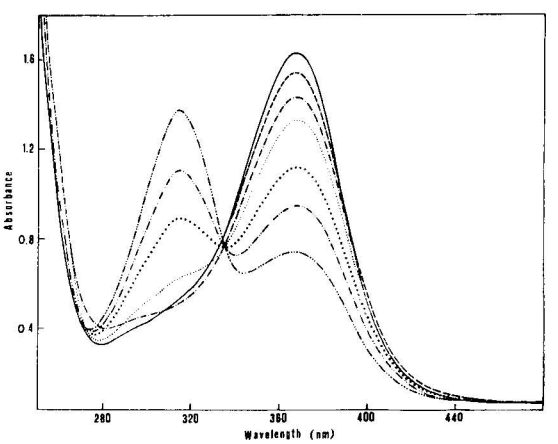

Fig. 13. UV titration spectra of "chromophoric" PEHA ampholytes. The two peaks are at 315 and 368 nm and the isosbestic point is at 335 nm. ———, pH 8.95; ---------, pH 6.8; –·–·–·–, pH 3.6; ·····, pH 1.75; ○○○○○, pH 1.28; –·–·–·–, pH 1.05; –···–···–, pH 0.83.

the spectrum is completely reversible. This rules out the possibility that the 315-nm peak is an irreversible degradation product, at least during the experimental period. If we now plot the peak maxima against their respective pHs, we obtain the two "pliers"-like curves in Fig. 14, whose intersection gives the pK (1.1) of the dissociating function connected with the two chromophores. This pK value has not been corrected as suggested by Adrien and Serjeant[14], because even at a pH of about zero it has not been possible to extinguish the chromophore attributed to the conjugated base.

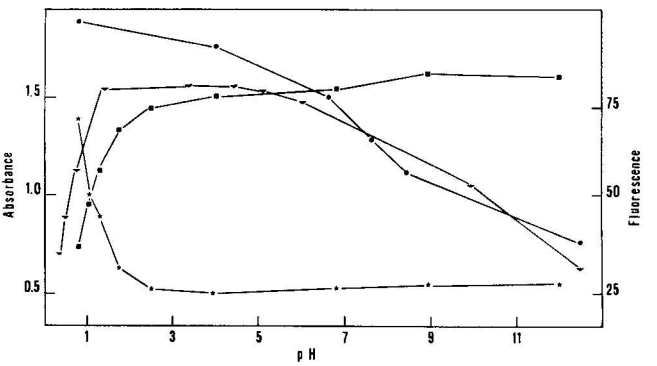

Fig. 14. pH dependence of UV and fluorescence spectra in "chromophoric" PEHA ampholytes. ★—★, UV peak at 315 nm; ■—■, UV peak at 368 nm. The intersection of these two curves gives the pK (1.1) of the dissociating group linked to the two chromophores. ●—●, pH dependence of the fluorescence peak obtained by excitation at 368 nm (emission peak at 455 nm); ▼—▼, pH dependence of the fluorescence peak obtained by excitation at 315 nm (emission peak at 420 nm).

In Fig. 14 we have also plotted the fluorescence titration curves obtained by excitation at the two peak maxima. Their behaviour is in agreement with the pH dependence of the respective UV chromophores. We also measured the ORD spectra of the ampholytes, but failed to observe any rotation of polarized light in the range 500–220 nm.

We were puzzled by the very low pK of the chromophore-associated function. From the known structure of ampholytes, it could be either a carboxyl group or a nitrogen function. We therefore lyophilized the chromophoric ampholytes and esterified the carboxyl group with methanol saturated with hydrogen chloride. The extent of methylation was followed with the hydroxamic acid test. However, the methylated ampholytes failed to show either a decrease of both peaks or the disappearance of one of them. We then blocked the nitrogen function with trifluoroacetic anhydride. The formation of the N-trifluoro derivative was followed by thin-layer chromatography. In this last case, both chromophores were greatly reduced, although not completely destroyed. Therefore, it appears that the two UV peaks are linked to a very weakly basic nitrogen function. Unfortunately, the pK values for the amino groups in PEHA are not known. However, Vesterberg[5] reported a pK_5 value of 2.7 for TEPA. Judging from the progressive decrease in pKs in TEPA, it is reasonable to expect a pK_6 value in PEHA of the order of 1.1, as found in the present work. We have been able to find these chromophores even in commercial preparations of Am-

pholine from LKB (see Fig. 2 in ref. 15), which suggests a similarity between our ampholytes and the commercial product. However, during that investigation[15], we were unable to link the two chromophores and to detect the nitrogen function, because the titration studies were discontinued at pH 1.75, just when the 315-nm peak begins to appear.

The behaviour of the two UV peaks cannot be readily explained on the basis of the known structure of ampholytes, which are supposed to be polyamino polycarboxylic acids. Previously, Righetti and Drysdale[16] and Vinogradov *et al.*[8], on the basis of UV and fluorescence spectra and of the behaviour of ampholytes on charcoal treatment, had suggested the presence of nitrogen heterocyclic structures, formed during the synthesis. This had also been hinted at by Haglund[17] in a review paper. To test this hypothesis, we performed NMR studies on our ampholytes and on the commercial product. We chose Ampholine in the pH ranges 3–5 and 4–6 because of their strong UV spectrum and their distinct yellow colour. As shown in Fig. 15, nothing can be detected in the region of aromatic structures (6–8.6 ppm), nor is there any hint of nitrogen heterocyclic structures anywhere along the spectrum. Therefore, the structure of the two UV chromophores linked to the nitrogen function remains to be explained.

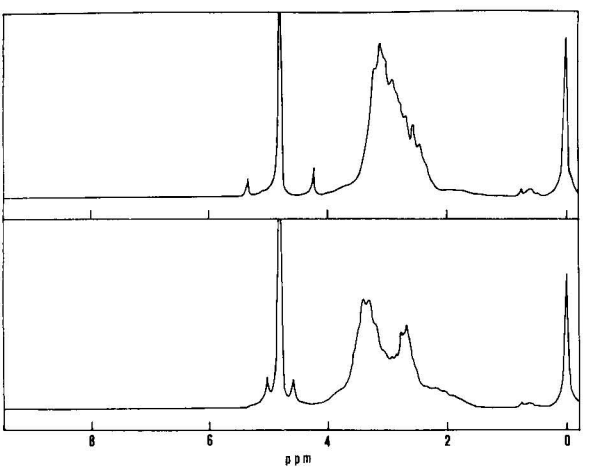

Fig. 15. NMR spectra of "chromophoric" PEHA ampholytes (above) and of LKB Ampholine, pH range 3–5 (below). The spectra were obtained with a Perkin-Elmer Model R 12 NMR spectrometer at 60 MHz in deuterium oxide.

DISCUSSION

The aim of the present investigation was to examine thoroughly the procedure for the synthesis of carrier ampholytes and to describe in more detail some of their physico-chemical properties. As already pointed out by Vesterberg[5] and Vinogradov *et al.*[8], it takes at least four amino groups in the polyamine to be able to synthesize ampholytes with acceptable properties. By a simple method of rendering focused ampholytes in a gel slab visible, we have been able to confirm this and to show the

actual distribution pattern of the ampholytes along the pH gradient. In going from Fig. 4 to Fig. 7 and to Fig. 10, one can see a progressive improvement in the ampholyte mixture, until in PEHA ampholytes almost all of the gaps in the gel disappear. These ampholyte patterns are well correlated with their respective conductimetric and pH profiles.

Other workers have described methods for the detection of focused ampholytes. Frater[18] reported a direct staining procedure, by precipitation in half-saturated picric acid and staining with Coomassie violet. However, by this method he was able to detect only acidic ampholytes, as the neutral and alkaline ampholytes are only poorly fixed. An interesting detection method, based on a glucose caramelization procedure on paper, was described by Felgenhauer and Pak[19]. By this technique, many more Ampholine peaks became apparent. Our detection method for ampholytes, based on differences in refractive indices, has been reported previously by Rilbe[20]. However, possibly because he used the liquid phase, the striations he was able to detect formed almost a continuous spectrum (see Fig. 3 in ref. 20). In our work, we have a pattern of sharp peaks that show up well in a densitometric scan. We realize that our method is not easily amenable to quantitation, as we can only scan a print, and therefore there is a transfer of errors from the actual gel to the negative and to the print finally used. Even so, we think that much can be inferred from a qualitative scan and that, in any event, although absolute quantitation cannot be made, the relative amounts in the various peaks can be easily appreciated. For instance, in Fig. 7 we could see a progressive flattening of the ampholyte peaks in the alkaline region. The scan in Fig. 8, in fact, demonstrates a progressive decrease in peak height, and therefore in relative amounts of basic ampholytes compared with acidic ampholytes. In any event, it is clear that what we see are not peaks of individual ampholytes, but probably, as already pointed out by Rilbe[20], clusters of carrier ampholytes. If we take that into account, and we examine the pattern complexity of Figs. 7, 8 and 10, it can be seen that Vesterberg's assumption[21] that more than 360 homologues and isomers could be generated during the synthesis, might not be too far from reality.

The reason why we investigated the properties not only of PEHA, but also of TETA and TEPA ampholytes, is that, in order to obtain a smoother pH gradient and an even conductivity, we synthesize the three types of ampholytes, and then mix them together and use this mixture as the wide ampholyte pH range, which can then be fractionated into narrow pH ranges (see the following paper). To this mixture we also add ampholytes obtained by coupling HMTA to acrylic acid. HMTA gives a very poor mixture of ampholytes, but they are clustered in the pH region 4–6, and therefore they are useful for reinforcing this pH zone.

The mixture of HMTA, TETA, TEPA and PEHA ampholytes that we have synthesized appears to be, in many respects, equivalent to the commercially available Ampholine. Perhaps a limitation is that our ampholyte mixture covers only the pH range 3–9.5, while LKB Ampholine covers the pH range 2.5–11. A great advantage, however, is that they are extremely easy to synthesize and extremely inexpensive, as the starting material is very cheap. The only difficult step in the synthetic procedure is the distillation of PEHA.

Recently, there have also been other approaches to the production of ampholytes suitable for isoelectric focusing. Thus, Pogacar and Jarecki[22] described the suitable coupling of TEPA and PEHA to either propane sulphone, vinyl sulphonate or

chloromethyl phosphonate. Their polyamino-polysulphonic ampholytes distribute into two groups, one covering the pH range 2–3.5 and the other the pH range 5.8–9.5 and there is therefore a gap in the pH region 3.5–5.8. While not useful *per se*, these "sulphonic ampholytes" could be a useful addition to the "carboxyl ampholytes" for extending their fractionation range down to pH 2.

An interesting approach has been described by Blanicky and Pihar[23], who prepared ampholytes from bactopeptone after removal of proteins by precipitation in 60% ethanol. After the removal of ethanol, a mixture of possibly several dozen different peptides remains, which appears to be rather good for isoelectric focusing. In the neutral region, the pH is improved by adding histidine. These types of ampholytes, however, are of the Rilbe type and therefore they suffer from the same inconvenience. A similar approach was used by Molnárová and Sova[25], who used casein hydrolyzates to focus different DNA species. While they were able to obtain good pH gradients down to pH 2.5, they did not present evidence to exclude binding of these peptide ampholytes to the DNA species isolated.

A completely new approach was described by Troitzki *et al.*[24]. Instead of using conventional polyamino-polycarboxylic acids or sulphonated derivatives, they used common buffers in gradients of organic solvents, such as ethanol, dioxane and glycerol, or in polyol gradients, such as mannitol, sucrose and sorbitol. Taking advantage of the pK variations of these buffers in different concentrations of these solvents, they are able to generate pH gradients of approximately 1.5 pH units in different regions of the pH scale. These pH gradients are stable for up to 12 days of isoelectric focusing. They achieved separations of rabbit haemoglobin in a pH gradient of 7–8.6 obtained with borate buffer in a mixed gradient of 0–5% glycerol and 0–30% sucrose, and of human serum albumin in a pH gradient of 4.5–5.8 obtained with acetate buffer in a 0–90% glycerol gradient. It is too early to assess whether or not this system might become of general use, but it certainly deserves further investigation.

We hope that the ease with which suitable ampholytes can be synthesized and characterized in a biochemical laboratory will attract more interest to the use of isoelectric focusing on a large preparative scale, such as in continuous-flow techniques.

ACKNOWLEDGEMENTS

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CHROM. 8224

FRACTIONATION OF CARRIER AMPHOLYTES FOR ISOELECTRIC FOCUSING

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SUMMARY

A simple method for fractionating synthetic carrier ampholytes is reported, based on the principle of continuous-flow isoelectric focusing in gel-stabilized layers. An 8% ampholyte solution, encompassing the pH range 3–9.5, is separated into 12 fractions in a chamber filled with Sephadex G-100 by a continuous-flow technique. We are thus able to obtain ampholytes of narrow pH range, encompassing approximately 2 pH units, whose resolving power is comparable with that obtained with commercial Ampholine covering similar pH ranges.

INTRODUCTION

In the preceding paper[1], we described the synthesis of carrier ampholytes suitable for isoelectric focusing. Acrylic acid is coupled to either hexamethylenetetramine (HMTA) or triethylenetetramine (TETA), or tetraethylenepentamine (TEPA) or pentaethylenehexamine (PEHA), using a nitrogen:carboxyl ratio of 2:1. The reaction has a yield greater than 98%. HMTA, TETA, TEPA and PEHA ampholytes are pooled to form a mixture of a great number of amphoteric compounds with closely spaced p*K*s and p*I*s in the pH range 3–9.5. This mixture produces smooth and stable pH gradients, and does not show any conductivity gap along the pH gradient formed. Detection of focused ampholytes along a gel slab, by a refractometric method, reveals sharp ampholyte gaussians closely spaced all along the separation support medium[1].

In this paper, we report the separation of these ampholytes, encompassing the pH range 3–9.5, into narrower pH ranges, covering 1–2 pH units. This fractionation technique is an electrophoretic method based on the continuous-flow system described by Fawcett[2].

MATERIALS AND METHODS

Sephadex G-100 was obtained from Pharmacia, Uppsala, Sweden. Samples of bovine pepsin, pig pepsin, chick pepsin and rennins from *Mucor mihei* and *Mucor pusillus* were kindly supplied by La Biotecnica, Trento, Italy.

The synthesis of carrier ampholytes was described in the preceding paper[1]. Isoelectric focusing in polyacrylamide gel slabs has been reported previously[3]. Continuous-flow isoelectric focusing was carried out essentially as described by Fawcett[2]. More details are given under Results.

RESULTS

Fig. 1 depicts the arrangement for continuous-flow isoelectric focusing of ampholytes. The separation chamber (D), 14 × 0.6 × 22.5 cm, is made of Plexiglass. The chamber has cooling jackets built in on both sides and is cooled by circulating water at 1° from a thermostat (A). To the bottom of the chamber are glued twelve outlet channels (3 mm I.D.) evenly spaced at 1-cm intervals. Against the bottom, from the inside of the chamber, is pressed a strip of porous polyethylene (average pore size 50 μm) to prevent drainage of the resin. On the two sides of the chamber are built the electrode compartments, 23 × 0.7 × 2.3 cm, which are filled with 1 *N* sodium hydroxide solution at the cathode and 1 *M* orthophosphoric acid at the anode. The electrical connections are made of platinum wire wound around two lucite strips, in widely spaced turns, going to the bottom of the electrode compartments. The anodic and cathodic vessels are sealed off from the separation chamber, except for the last

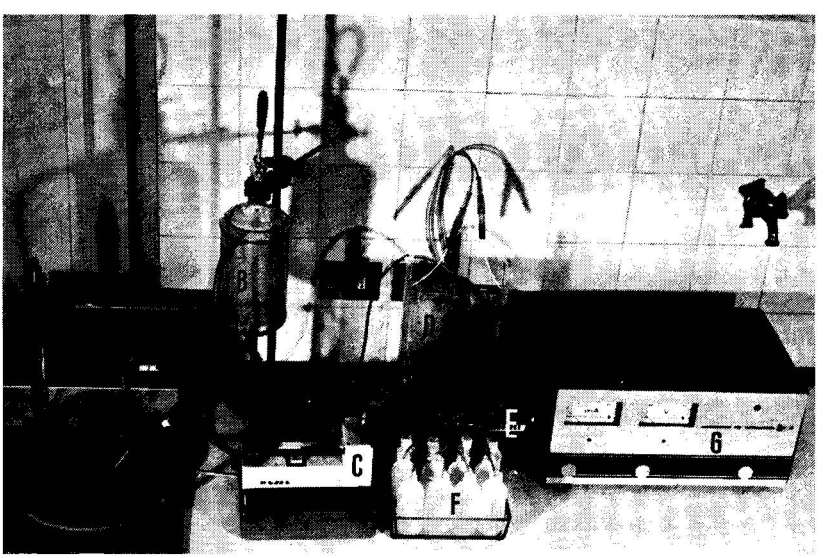

Fig. 1. Equipment for continuous-flow isoelectric focusing. A, Thermostat; B, Mariotte flask (from an LKB Uniphor column); C, stepping motor from a Razel syringe pump, Model A99; D, chamber for continuous-flow isoelectric focusing; E, modules of a Delta/6 pump (Watson-Marlow); F, collection bottles; G, power supply.

3 cm at the bottom, where the plastic wall is replaced with two rectangular pieces of porous polyethylene (2 × 3 cm), in order to allow electrical conductivity. The separation chamber is filled with 140 ml of a Sephadex G-100 slurry, swollen in a solution of 8% ampholyte mixture to be fractioned, and de-gassed. A 3-cm depth of free ampholyte solution is allowed above the gel surface.

Immediately after pouring the gel slurry, the electrode chambers are filled with anolyte and catholyte to the same liquid level as in the central chamber. The fractionation chamber is connected, via a plastic tube, to a Mariotte flask containing the same 8% ampholyte mixture to be fractionated. The Mariotte flask (taken from an LKB Uniphor column) is filled with 1.5 l of 8% ampholyte solution, and is fitted with a cooling jacket through which water at 1° from a thermostat is circulated. As the liquids in the reservoir and in the fractionation chamber are hydrostatically equilibrated, constant automatic sample feeding is provided, without any need for pumps. The twelve outlets of the chamber are connected with silicone rubber tubing to Delta/6 pump modules (from Watson-Marlow, Falmouth, Cornwall, Great Britain) that are driven by the stepping motor of a Razel Model A99 pump (Razel Scientific Instruments, Stamford, Conn., U.S.A.). The motor was removed from the Razel pump and connected mechanically to the axle of the Delta pump modules. This arrangement provided a stable, digitally pre-set flow-rate from 1 to 90 ml/h (total flow from twelve channels). The tightening of the tubing around the Delta pump is adjusted so that they deliver equal volumes. No recirculation of anolyte and catholyte, as suggested by Fawcett[2], is provided and therefore in the separation chamber the field strength rapidly decreases from the porous polyethylene membranes toward the top of the chamber.

At the start, a voltage of 200 V, with a corresponding current of 70 mA, is applied and the Delta pump is not activated. After 3 h, the voltage has increased to 300 V and the current has dropped to 50 mA. The zones of "chromophoric" ampholytes are now seen as vertical, focused bands. At this stage the Delta pump is started at a speed of 20 ml/h (total flow from twelve channels) and the fractions are collected in twelve plastic 100-ml bottles. The system then runs at a steady state, and can be left unattended.

Fig. 2 shows details of the central system, consisting of the electric motor (A), Delta units (C), separation chamber (B) and collection bottles (D), kept in a glass basin. This type of Delta pump can be run with many more channels, as blocks of ten channels can be linked together in a linear fashion to the electric motor. In Fig. 2 are shown three blocks of ten channels each (C), interposed between the fractionation chamber (B) and the collection bottles (D).

Fig. 3 shows the pH profiles in the twelve collection bottles when the system was run for 10 days without interruptions. Every 24 h the bottles were harvested and the pH values measured at room temperature. It can be seen that the pH is constant in each fraction, except for fraction 7, which shows an initial pH drift during the first 70 h. That the system was at equilibrium was also ensured by the constancy of the voltage and the current across the separation chamber. Also, the positions of the yellow lines of "chromophoric" ampholytes, marked on the wall of the chamber, did not shift during the experimental period (10 days). The separation shown in Fig. 3 was achieved from an 8% ampholyte solution, obtained by mixing HMTA, TETA, TEPA and PEHA ampholytes in a ratio of 1:2:2:3. All of these ampholytes were synthesized

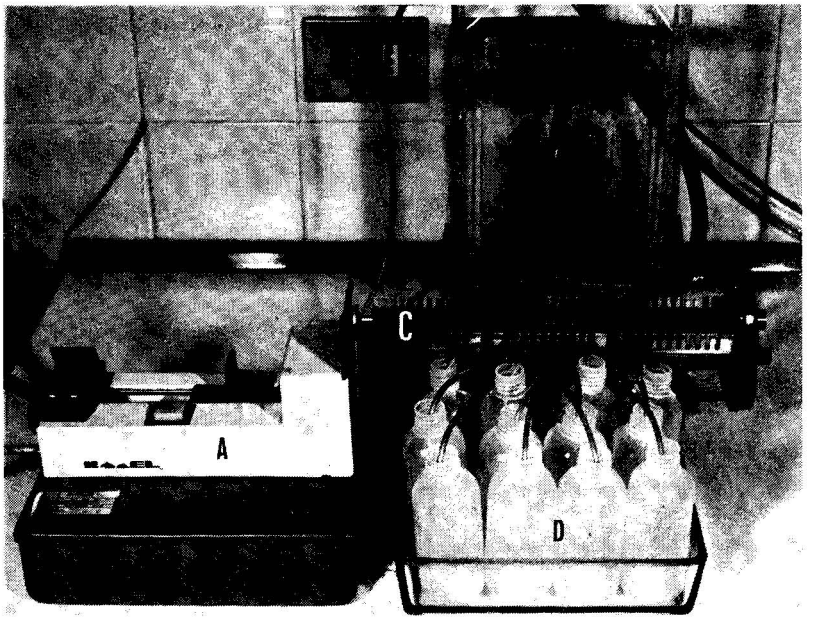

Fig. 2. Details of the central unit for continuous-flow isoelectric focusing. A, Stepping motor from a Razel syringe pump, with digital control unit for flow-rates; B, fractionation chamber for continuous-flow isoelectric focusing; C, blocks of three Delta units linked together, with a capacity for 30 channels (only 12 connected); D, collection bottles for the 12 fractions.

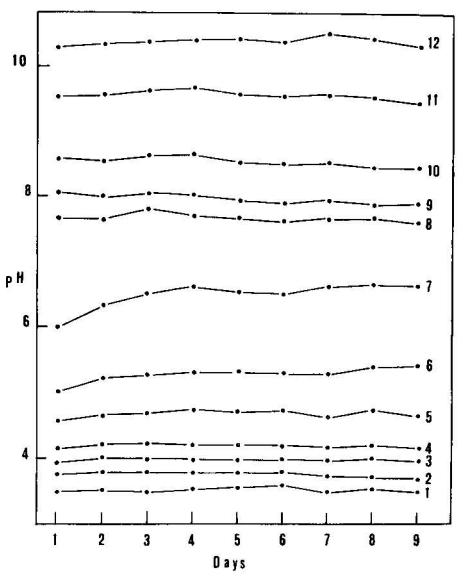

Fig. 3. Continuous-flow fractionation of a mixture of HMTA, TETA, TEPA and PEHA ampholytes in the ratio 1:2:2:3 for 9 days. The PEHA ampholytes were synthesized with a nitrogen:carboxyl ratio of 1.5:1 and with a nitrogen:carboxyl ratio of 1:1. The various curves give the pH in each fraction at 24-h intervals. The numbers on the right of each curve indicate the fraction number (from anode to cathode).

with a nitrogen:carboxyl ratio of 2:1, except for PEHA ampholytes, which were obtained from a batch with a nitrogen:carboxyl ratio of 1.5:1 and from a batch with nitrogen:carboxyl ratio of 1:1. This explains why so many fractions (bottles 1–6) contain acidic species in the pH range 3–5.5 (see also Fig. 3 in ref. 1).

We then tested the various fractions by polyacrylamide gel isoelectric focusing, in order to obtain the actual pH ranges encompassed by the separated ampholytes. Fractions 1 and 12 were discarded, as they were shown to be contaminated with orthophosphoric acid and sodium hydroxide, respectively. Fractions 2–6 were pooled in a single ampholyte batch, as they had p*I* values too close to one another and, separately, they would probably have formed pH gradients too shallow to be of practical use. The other fractions were used as such.

Fig. 4 gives the results of these experiments. The black triangles are the average pHs as measured in each fraction eluted by the continuous-flow technique. The vertical lines represent the actual pH gradient generated by the various fractions upon focusing in polyacrylamide gel. In this way, we were able to collect six different fractions, each covering a range of approximately 2 pH units, encompassing the pH range 3–9.5.

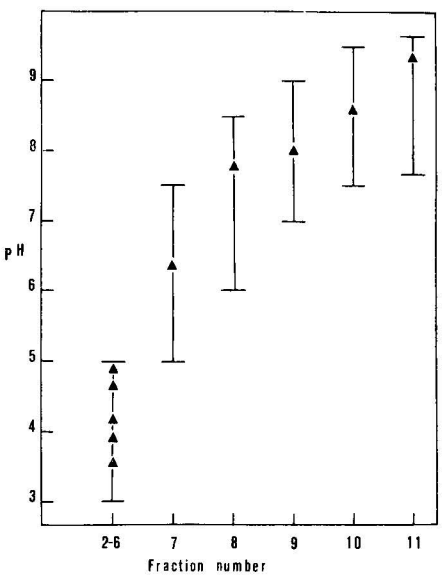

Fig. 4. Actual pH range of each fraction separated by continuous-flow isoelectric focusing. The black triangles indicate the average pH in each collection bottle, and the vertical lines the pH range generated by the various fractions upon isoelectric focusing in polyacrylamide gels. Fractions 1 and 12 were discarded, as they were contaminated by anolyte and catholyte, respectively. Fractions 2–6 were pooled.

Fig. 5 shows the separation of some commercial rennets in the acidic pH range, using the pooled fraction 2–6 from Fig. 4. In this particular case, as we wanted also to detect the four components in bovine pepsin, which are isoelectric around pH 2.6, one third of the ampholytes in the mixture was represented by LKB Ampholine "pH 2.5–4". Without it, except for the loss of bovine pepsin, the separation would

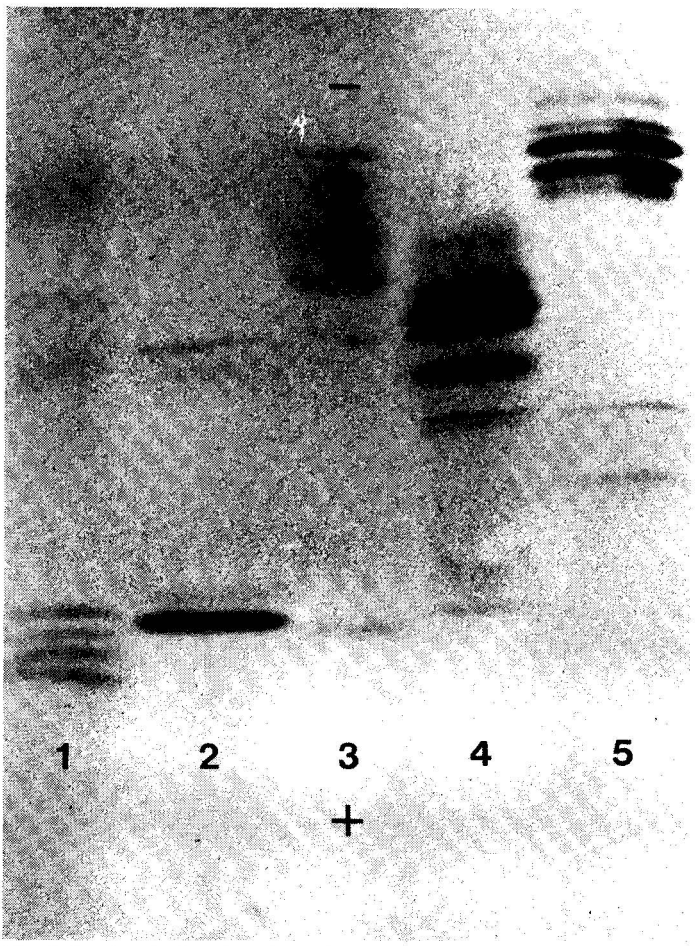

Fig. 5. Isoelectric fractionation of rennins in a polyacrylamide gel slab. As ampholytes we used a mixture of two thirds of the synthetic product (fractions 2–6 of Fig. 4) and one third of LKB Ampholine "pH 2.5–4". Samples: 1, bovine pepsin; 2, pig pepsin; 3, chick pepsin; 4, rennins from *Mucor pusillus*; 5, rennins from *Mucor mihei*.

have been identical. In fact, even pig pepsin would have been detected as a sharp band at the edge of the filter-paper strip of the anode. In any event, the number of components found and their respective p*I*s were the same as those obtained by performing the separation in a mixture of commercial Ampholine "pH 2.5–4" and "pH 4–6". The separation of rennins will be described elsewhere[4]. We have produced protein patterns with other ampholytes of narrow pH range fractionated by us (for instance, the separation of haemoglobins in fraction 8 and of human ferritins in fraction 7) and found an excellent correlation with the patterns obtained with commercial Ampholine encompassing similar pH ranges.

DISCUSSION

The present ampholyte fractionation system appears to offer several advan-

tages over multi-compartment electrolyzers, as described by Vesterberg[5] and Rilbe[6]. The latter apparatus seems to be difficult to build in an ordinary laboratory workshop. It is usually constructed with 20 cells and each cell has to have an independent stirring system. The fitting of the single cells with outer cooling jackets or inner cooling systems also appears to be difficult, as the entire apparatus should be built in such a way as to allow dismantling into single units. The membranes between pairs of cells have to be changed often and, during electrolysis, they can also give rise to undesirable polarization effects.

Another drawback of multi-compartment electrolyzers is the phenomenon of unbalanced osmotic pressure. If, during isoelectric focusing, one cell becomes particularly enriched in ampholytes, compared with neighbouring cells, this cell will show an increase in osmotic pressure. In order to counteract this effect, water will be drawn in until the osmotic pressure is balanced again. This phenomenon will adversely affect the pattern of focused ampholytes.

A further disadvantage of this system is that, at the end of the run, the electrolyzer has to be dismantled, cleaned, the membranes changed, and re-assembled for another run.

All of these problems are eliminated in our continuous-flow system. Once in operation, it can be run for several weeks and left unattended without risk, provided that adequate sample input is ensured. Assembly and dismantling of the equipment shown in Fig. 1 is easy. Gel stabilization of focused ampholytes during isoelectric focusing appears to be superior to membrane stabilization against convection and diffusion. Another advantage of our continuous-flow system is that the ampholytes, during the fractionation process, are kept in contact with the anolyte and catholyte only for a short time (*ca.* 1 h and 15 min respectively, at the flow-rate used). This prevents anodic oxidation and chemical modification of ampholytes. In multi-compartment electrolyzers, where the ampholytes are kept in the apparatus for at least 24 h, Vesterberg[5] reported the formation of yellow compounds, spreading from the anode. In order to prevent anodic oxidation, special precautions had to be taken[5].

We found it adequate, for our fractionation purposes, to have a twelve-channel outlet. However, if a finer resolution of ampholytes is needed, with collection of more fractions, our apparatus can easily be modified with interchangeable bottom outlets, containing as many channels as needed. Fawcett[2] (who built the continuous-flow chamber we have been using) reported fractionation of proteins in chambers containing as many as 54 channels.

We hope that the ease with which suitable carrier ampholytes can be synthesized and fractionated in narrow pH ranges, in a biochemical laboratory, will stimulate more research and applications in the field of large-scale preparative isoelectric focusing.

ACKNOWLEDGEMENTS

We thank Dr. J. Fawcett (Department of Experimental Biochemistry, Queen Mary College, London) for the extended loan of his continuous-flow chamber. We are indebted to Dr. L. Rossi-Bernardi for valuable suggestions and for his interest in this work.

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CHROM. 8222

CHROMATOGRAPHIC BEHAVIOUR OF PHENOLS ON THIN LAYERS OF CATION AND ANION EXCHANGERS

II. DOWEX 50-X4 AND REXYN 102

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(First received January 29th, 1975; revised manuscript received February 10th, 1975)

SUMMARY

The chromatographic characteristics of 58 phenols on Rexyn 102 and Dowex 50-X4 thin layers in both the acidic and sodium salt forms have been studied, using elution with water, water–alcohol mixtures and aqueous salt solutions at different pH values. The influence of the percentage of alcohol, the pH and the ionic strength on the chromatographic behaviour of these phenols was investigated.

The validity of the relationships among the R_F values, the pH of the eluent and the pK_a of the phenol has been verified on Dowex 50-X4 (Na^+) thin layers. It has been shown that from the $R_{F_{ac}}$, $R_{F_{alk}}$ and pK_a values of the different phenols, it is possible to predict their behaviour over the whole pH range and therefore to select the best conditions for their chromatographic separation.

INTRODUCTION

In Part I (ref. 1), 58 phenols were studied on Bio-Rad AG 3-X4A, PEI-cellulose and DEAE-cellulose thin layers. This second part concerns a study of the same phenols on Dowex 50-X4 and Rexyn 102 so that a complete picture of their chromatographic behaviour on cation exchangers with different supports can be seen.

EXPERIMENTAL

Preparation of the layers and of the solutions

The solutions of the phenols were prepared as described previously[1]. The layers of Dowex 50-X4 and Rexyn 102 (Fisher Scientific, Fair Lawn, N.J., U.S.A.) were prepared by mixing 3 g of the resin with 9 g of microcrystalline cellulose in 50 ml of water. Before use, the exchangers were rinsed with water and methanol and dried at room temperature. Water–alcohol mixtures in different ratios, neutral and alkaline salt solutions and equimolecular sodium acetate and acetic acid solutions were employed as eluents.

The chromatographic measurements were made at 25 ± 0.5°. The migration distance was 11 cm unless otherwise stated.

Detection

The detection of the phenols was effected as previously described[1]. The layers, however, must be sprayed with 1 *M* sodium hydroxide solution before their exposure to nitrogen dioxide vapour. Some phenols cannot be detected under such experimental conditions, as is shown in the results in the different tables.

When eluting with alkaline solutions, the detection of hydroquinone is not possible as it is probably oxidized. The same effect occurs, although to a lesser extent, by catechol, pyrogallol and gallic acid. These latter compounds, however, can be detected doubling their amounts on the layer.

RESULTS AND DISCUSSION

Dowex 50-X4 (H^+)

Table I shows the results for Dowex 50-X4 in the acid form when eluting with water and water–ethanol mixtures. On eluting with water, most phenols with a primary or a secondary amine group in the aromatic ring remain at the starting point, with the exception of 2-aminophenol-4-sulphonic acid and 2,4-dinitro-4′-hydroxy-diphenylamine-3′-sulphonic acid, which have high R_F values.

As regards the other phenols, the results in Table I show that they move from the starting point to different extents. The chromatographic behaviour of the phenols, when eluting with water, is determined by the presence of one or more substituent groups in the ring and overall by the nature of such substituents. With respect to phenol, the introduction into the ring of a chlorine atom or a methyl group causes a remarkable decrease in the R_F value and this decrease becomes larger as the number of substituents increases.

Also, mono- and dinitrophenols exhibit a higher affinity than phenol towards the exchanger. With dinitrophenols, however, it should be noted that their affinity towards the exchanger decreases as their acidity increases. Such characteristics are exemplified by picric acid, which, owing to its high acidity, almost runs with the solvent front.

The introduction of one or more hydroxyl groups into the ring, on the contrary, does not result in any essential change with respect to phenol.

With water–alcohol eluents, for most phenols an increase in R_F value is observed compared with water alone. An exception is shown by some phenols with an amine group, which remain at the starting point independently of the percentage of alcohol in the eluent. Anomalous behaviour is that shown by 2-aminophenol-4-sulphonic acid, whose R_F value decreases as the percentage of ethanol in the eluent is increased owing to its low solubility in this solvent.

With water–ethanol in a 1:2 ratio, a levelling of the R_F values of many phenols is observed. For this reason, we considered it useful to examine the chromatographic behaviour of these compounds with water–alcohol mixtures owing to the importance of such eluents in "solubilization chromatography".

Fig. 1 shows some peculiar trends of R_F values with increasing percentages of alcohol in the eluent. Such trends refer to phenols whose affinity towards the ex-

TABLE I

R_F VALUES OF PHENOLS ON DOWEX 50-X4 (H^+) AND REXYN 102 (H^+) THIN LAYERS

Phenol	*Dowex 50-X4 (H^+)*			*Rexyn 102 (H^+)*
	H_2O	*H_2O–C_2H_5OH (4:1)*	*H_2O–C_2H_5OH (1:2)*	*0.5 M HCl in H_2O–C_2H_5OH (4:1)*
Phenol	0.54	0.57	0.92	0.50
Guaiacol	0.41	0.45	0.92	0.45
Hydroquinone	0.56	0.61	0.94	0.68
Catechol	0.57	0.57	0.93	0.63
Resorcinol	0.54	0.60	0.94	0.65
Orcinol	0.40	0.48	0.94	0.56
Pyrogallol	0.61	0.65	0.89	0.71
Phloroglucinol	0.52	0.70	0.90	0.70
Pyrocathechic acid	0.37	0.49	0.90	0.50
Gallic acid	0.38	0.50	0.90	0.52
o-Cresol	0.35	0.43	0.92	0.41
m-Cresol	0.35	0.43	0.92	0.41
2,6-Dimethylphenol	0.01	0.02	n.d.	0.02
2,3-Dimethylphenol	0.25	0.32	0.90	0.27
3,4-Dimethylphenol	0.26	0.34	0.90	0.27
3,5-Dimethylphenol	0.26	0.34	0.90	0.27
m-Nitrophenol	0.28	0.30	0.89	0.31
o-Nitrophenol	n.d.*	n.d.	n.d.	n.d.
p-Nitrophenol	0.23	0.30	0.90	0.29
2,5-Dinitrophenol	0.22	0.28	0.88	0.21
2,4-Dinitrophenol	0.25	0.29	0.88	0.23
2,6-Dinitrophenol	0.35	0.38	0.90	0.23
Picric acid	0.90	0.90	0.96	0.28
m-Aminophenol	0.00	0.00	0.00	0.71
o-Aminophenol	0.00	0.00	0.00	0.68
p-Aminophenol	0.00	0.00	0.00	0.72
5-Aminosalicylic acid	0.00	0.00	0.00	0.55
4-Aminosalicylic acid	0.00	0.00	0.00	0.49
3-Hydroxyanthranilic acid	0.00	0.00	0.00	0.57
2-Aminophenol-4-sulphonic acid	0.91	0.85	0.76	0.87
4-Amino-2-nitrophenol	0.00	0.00	0.00	0.58
2-Amino-5-nitrophenol	0.00	0.02	0.13	0.53
2-Amino-4-nitrophenol	0.00	0.00	0.00	0.58
2-Amino-4,6-dinitrophenol	0.00	0.04	0.32	0.22
2-Amino-3,4,6-trichlorophenol	0.00	0.01	0.44	0.18
p-Chlorophenol	0.25	0.30	0.92	0.26
m-Chlorophenol	0.25	0.30	0.92	0.25
o-Chlorophenol	n.d.	n.d.	n.d.	n.d.
p-Bromophenol	0.20	0.23	0.91	0.19
o-Bromophenol	n.d.	n.d.	n.d.	n.d.
3,4-Dichlorophenol	0.11	0.14	0.91	0.10
3,5-Dichlorophenol	0.12	0.16	0.91	0.10
2,4-Dichlorophenol	n.d.	0.14	0.91	0.14
2,3-Dichlorophenol	n.d.	0.14	0.91	0.14
2,5-Dichlorophenol	n.d.	n.d.	n.d.	n.d.
2,6-Dichlorophenol	n.d.	n.d.	n.d.	0.18
β-Naphthol	0.08	0.14	0.86	0.10

(Continued on p. 368)

TABLE I *(continued)*

Phenol	*Dowex 50-X4 (H^+)*			*Rexyn 102 (H^+)*
	H_2O	*H_2O–C_2H_5OH (4:1)*	*H_2O–C_2H_5OH (1:2)*	*0.5 M HCl in H_2O–C_2H_5OH (4:1)*
α-Naphthol	0.07	0.13	0.86	0.10
1,5-Naphthalenediol	0.08	0.14	0.86	0.20
2-Hydroxy-1-naphthaldehyde	0.04	0.07	0.78	0.03
7-Amino-2-naphthol	0.00	0.00	0.00	0.34
1-Amino-7-naphthol	0.00	0.00	0.00	0.34
5-Amino-1-naphthol	0.00	0.00	0.00	0.36
4-Hydroxydiphenylamine	0.00	0.01	0.03	0.42
3-Hydroxydiphenylamine	0.00	0.01	0.43	0.21
2,4-Dinitro-4′-hydroxydiphenylamine	0.01	0.07	0.80	0.03
2,4-Dinitro-4′-hydroxydiphenylamine-3′-sulphonic acid	0.90	0.91	0.96	0.20
4-Hydroxyazobenzene	0.01	0.03	0.86	0.01

* n.d. = not determined.

changer changes from high values (curves 6, 7, 8 and 9) to small values (curve 1). With the exception of the phenols with an amine group (curves 8 and 9), the greatest change in the R_F values occurs at alcohol contents between 20 and 50%. With alcohol contents less than 20%, a series of straight lines is obtained, in accordance with the results observed by Sherma and Hood[2] for some phenols on layers of the same exchanger with water–methanol mixtures as eluents.

The trends of these curves can be explained by assuming that the addition of alcohol to the eluent causes, other than an increase in the solubility in the mobile phase, a decrease in the interactions between the phenols and the exchanger. Such interactions seem to be completely eliminated for alcohol contents in the eluent above 50%.

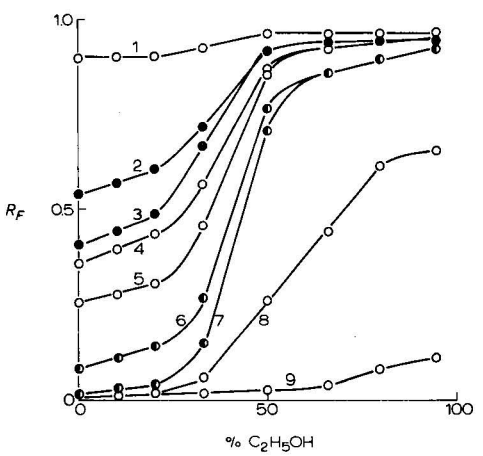

Fig. 1. R_F values of phenols on Dowex 50-X4 (H^+) thin layers *versus* ethanol content in the eluent. 1, Picric acid; 2, resorcinol; 3, orcinol; 4, *m*-cresol; 5, *m*-chlorophenol; 6, α-naphthol; 7, 4-hydroxyazobenzene; 8, 3-hydroxydiphenylamine; 9, 4-hydroxydiphenylamine.

The behaviour of the aminophenols (see curves 8 and 9) seems to contradict this assumption; it must be pointed out, however, that in this case the interactions between the amine group of these two compounds and the sulphonic group of the exchanger[3] play an important role. Such interactions are less affected by the increase in the alcohol content in the eluent than those between the phenols and the matrix of the exchanger.

Among the separations possible on the basis of the R_F values, we effected the following: picric acid, 2,6-dinitrophenol and 2,5-dinitrophenol (water); phenol and α-naphthol (4:1 water–ethanol); and 4-hydroxydiphenylamine, 3-hydroxydiphenylamine, 2,4-dinitro-4'-hydroxydiphenylamine-3'-sulphonic acid and 2,4-dinitro-4'-hydroxydiphenylamine (1:2 water–ethanol). It should be noted that the phenols with an amine group can be separated from all the others and, further, that some of these same phenols may be separated from each other under suitable elution conditions.

Rexyn 102 (H^+)

On this exchanger, there are no appreciable differences in the chromatographic behaviour of the phenols on elution with water and water–ethanol mixtures compared with that observed on Dowex 50-X4 (H^+) under the same conditions.

The use of acidic water–alcohol solutions as eluents does not affect the R_F values of the phenols without an amine group, whereas it causes a noticeable increase in the R_F values of the aminophenols, as the results in Table I show.

On the basis of the same behaviour of the phenols on Rexyn 102 (H^+) and Dowex 50-X4 (H^+), we can assume that the influence of the interactions between the two exchangers and the phenols is similar. As the interactions of the paraffinic matrix with the aromatic compounds are weaker than those of the polystyrene ones[4], the similar behaviour of the phenols must be ascribed to the different ionic environments in these two exchangers.

The following separations have been effected on Rexyn 102 (H^+) layers with 0.5 *M* hydrochloric acid in 4:1 water–ethanol as eluent: 4-hydroxydiphenylamine and 3-hydroxydiphenylamine; and α-naphthol, 2-hydroxy-1-naphthaldehyde and 1,5-naphthalenediol. In both instances the migration distance was 13 cm.

Dowex 50-X4 (Na^+)

On these layers, eluting with water and with water–alcohol mixtures, there are no appreciable differences in the chromatographic behaviour of the phenols compared with that observed on the same exchanger in the acidic form (even if the R_F values are generally lower), with the exception of the aminophenols, which are less retained on Dowex 50-X4 (Na^+) layers.

The behaviour of the phenols at different pH values and ionic strengths is particularly interesting. The R_F of the protonated form of the phenols is only slightly affected by a change in the ionic strength from 0.01 to 0.1, while that of the deprotonated form, depending on the substituent groups in the aromatic ring, may be considerably decreased, for instance for β-naphthol it decreases from 0.86 to 0.66 (see Table II).

On changing the ionic strength from 0.1 to 1 (see Table II), the decrease in the R_F of the deprotonated form is generally greater than that observed in the 0.01–0.1 range. The dependence of the R_F of the phenols on the ionic strength of the eluent

TABLE II

R_F VALUES OF PHENOLS ON DOWEX 50-X4 (Na^+) THIN LAYERS

Phenol	*Eluent*					
	0.1 M Acetate buffer	*0.1 M $NaHCO_3$*	*0.05 M Na_2CO_3*	*1 M NH_3*	*1 M NH_3 + 0.1 M CH_3COONa*	*1 M NH_3 + 1 M CH_3COONa*
Phenol	0.49	0.53	0.65	0.92	0.90	0.73
Guaiacol	0.38	0.41	0.58	0.92	0.89	0.56
Hydroquinone	0.43	n.d.*	n.d.	n.d.	n.d.	n.d.
Catechol	0.44	0.50	0.70	0.94	0.93	e.s.**
Resorcinol	0.41	0.49	0.72	0.94	0.94	0.83
Orcinol	0.33	0.38	0.63	0.94	0.92	0.73
Pyrogallol	0.46	0.71	0.88	0.95	0.95	n.d.
Phloroglucinol	0.39	0.80	0.93	0.95	0.95	0.88
Pyrocathechic acid	0.43	0.95	0.95	0.95	0.95	n.d.
Gallic acid	0.44	0.95	0.95	0.95	0.95	n.d.
o-Cresol	0.35	0.35	0.50	0.92	0.82	0.49
m-Cresol	0.35	0.36	0.53	0.92	0.88	0.56
2,6-Dimethylphenol	0.01	0.01	0.05	n.d.	n.d.	n.d.
2,3-Dimethylphenol	0.24	0.24	0.31	0.90	0.72	0.34
3,4-Dimethylphenol	0.25	0.25	0.41	0.91	0.78	0.39
3,5-Dimethylphenol	0.25	0.25	0.42	0.91	0.78	0.39
m-Nitrophenol	0.21	0.58	0.80	0.94	0.90	0.46
o-Nitrophenol	n.d.	0.91	0.92	0.96	0.92	0.65
p-Nitrophenol	0.18	0.81	0.84	0.94	0.84	0.44
2,5-Dinitrophenol	0.28	0.80	0.80	0.92	0.80	0.45
2,4-Dinitrophenol	0.50	0.72	0.72	0.88	0.72	0.26
2,6-Dinitrophenol	0.79	0.88	0.88	0.94	0.88	0.53
Picric acid	0.65	0.67	0.67	0.82	0.66	0.19
m-Aminophenol	0.06	0.53	0.68	0.94	0.88	0.71
o-Aminophenol	0.03	0.53	e.s.	e.s.	e.s.	e.s.
p-Aminophenol	0.00	e.s.	e.s.	e.s.	e.s.	e.s.
5-Aminosalicylic acid	0.47	0.91	0.91	0.94	0.90	0.65
4-Aminosalicylic acid	0.53	0.91	0.91	0.94	0.90	0.64
3-Hydroxyanthranilic acid	0.54	0.92	0.92	0.94	0.92	0.65
2-Aminophenol-4-sulphonic acid	0.96	0.96	0.96	0.96	0.96	0.96
4-Amino-2-nitrophenol	0.07	0.74	0.87	0.94	0.88	0.66
2-Amino-5-nitrophenol	0.09	0.24	0.65	0.87	0.76	0.49
2-Amino-4-nitrophenol	0.10	0.71	0.78	0.89	0.78	0.51
2-Amino-4,6-dinitrophenol	0.25	0.55	0.56	0.77	0.56	0.18
2-Amino-3,4,6-trichloro-phenol	0.02	0.54	0.63	0.84	0.63	0.20
p-Chlorophenol	0.19	0.24	0.65	0.94	0.89	0.53
m-Chlorophenol	0.20	0.31	0.72	0.94	0.90	0.66
o-Chlorophenol	n.d.	n.d.	0.86	0.96	0.95	0.78
p-Bromophenol	0.13	0.21	0.60	0.94	0.88	0.53
o-Bromophenol	0.21	0.51	0.84	0.96	0.94	0.74
3,4-Dichlorophenol	0.08	0.24	0.66	0.94	0.82	0.45
3,5-Dichlorophenol	0.08	0.36	0.75	0.94	0.84	0.50
2,4-Dichlorophenol	0.09	0.58	0.82	0.95	0.90	0.50
2,3-Dichlorophenol	0.09	0.66	0.84	0.95	0.91	0.56
2,5-Dichlorophenol	0.11	0.78	0.88	0.96	0.91	0.59

TABLE II *(continued)*

Phenol	*Eluent*					
	0.1 M Acetate buffer	*0.1 M $NaHCO_3$*	*0.05 M Na_2CO_3*	*1 M NH_3*	*1 M NH_3 + 0.1 M CH_3COONa*	*1 M NH_3 + 1 M CH_3COONa*
2,6-Dichlorophenol	0.12	0.94	0.95	0.96	0.95	0.65
β-Naphthol	0.04	0.05	0.29	0.86	0.66	0.31
α-Naphthol	0.04	0.06	0.35	0.92	0.75	0.34
1,5-Naphthalenediol	0.03	0.07	e.s.	e.s.	e.s.	e.s.
2-Hydroxy-1-naphthaldehyde	0.04	0.38	0.52	n.d.	n.d.	n.d.
7-Amino-2-naphthol	0.00	0.03	0.26	0.86	0.49	0.28
1-Amino-7-naphthol	0.00	0.05	0.35	0.86	0.61	0.34
5-Amino-1-naphthol	0.00	0.05	0.36	0.86	0.61	0.36
4-Hydroxydiphenylamine	0.00	0.00	0.12	0.78	0.51	0.20
3-Hydroxydiphenylamine	0.01	0.01	0.15	n.d.	0.30	0.10
2,4-Dinitro-4′-hydroxydiphenylamine	0.00	0.02	0.16	0.68	0.38	0.10
2,4-Dinitro-4′-hydroxydiphenylamine-3′-sulphonic acid	0.42	0.74	0.86	0.90	0.85	0.30
4-Hydroxyazobenzene	0.01	0.06	e.s.	0.78	0.45	0.13

* n.d. = not determined.
** e.s. = elongated spot.

can be used, in some instances, in order to obtain or to improve separations among phenols with similar pK_a values and must be taken into account in the determination of the R_F values on this exchanger.

As regards the influence of pH, it must be noted that the protonated form of the phenols exhibits a higher affinity towards the exchanger than the deprotonated form. Such behaviour is similar to that observed by Grieser and Pietrzyk[5] for some phenols on Amberlite XAD-2 columns with water–alcohol eluents.

In that paper, the following relationship was used:

$$K_D = \frac{[HA]_R + [A^-]_R}{[HA]_S + [A^-]_S} \cdot \frac{v}{w} \tag{1}$$

where K_D is the distribution coefficient, $[HA]_R$, $[A^-]_R$, $[HA]_S$ and $[A^-]_S$ are the concentrations of the protonated and deprotonated form of the phenol in the resin and in the solution, v is the volume of the solution and w is the weight of the resin. Introducing the K_a value into eqn. 1 and rearranging it in order to obtain the distribution coefficient as a function of the hydrogen ion concentration and of K_a, Grieser and Pietrzyk obtained log K_D *versus* pH curves with a shape similar to that of an acid–base titration curve. In these curves, the greatest change in log K_D is observed at a pH value about one unit higher than the pK_a value of the corresponding phenols.

In our case, using the equation

$$K_D = \left(\frac{1}{R_F} - 1\right)\frac{A_1}{A_s} = \frac{[HA]_R + [A^-]_R}{[HA]_S + [A^-]_S} \tag{2}$$

where A_l and A_s are the cross-sectional areas of the mobile and stationary phases, respectively, we obtained the relationship

$$\left(\frac{1}{R_F} - 1\right) = \left(\frac{1}{R_{F_{ac}}} - 1\right)\frac{[H^+]}{K_a + [H^+]} + \left(\frac{1}{R_{F_{alk}}} - 1\right)\frac{K_a}{[H^+] + K_a} \quad (3)$$

where $R_{F_{ac}}$ and $R_{F_{alk}}$ are the R_F values of the protonated and deprotonated form of the phenol obtained by eluting, with a low pH solution (*e.g.*, 1 *M* acetic acid or 0.1 *M* equimolecular acetate buffer) and a high pH solution (*e.g.*, 1 *M* ammonia + 0.1 *M* sodium acetate or 1 *M* ammonia + 0.1 *M* sodium hydroxide), respectively. Applying eqn. 3 to some phenols, the $[(1/R_F) - 1]$ *versus* pH curves reported in Fig. 2 were obtained. The good agreement between the theoretical curves and the experimental values in Table III supports the validity of such an equation in thin-layer chromatography.

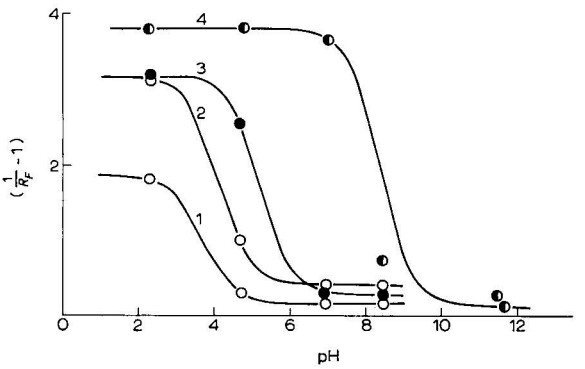

Fig. 2. $(1/R_F - 1)$ *versus* pH plots for phenols on Dowex 50-X4 (Na^+) thin layers. 1, 2,6-Dinitrophenol ($pK_a = 3.71$); 2, 2,4-dinitrophenol ($pK_a = 4.09$); 3, 2,5-dinitrophenol ($pK_a = 5.22$); 4, *m*-nitrophenol ($pK_a = 8.40$).

In these curves, the major change in $[(1/R_F) - 1]$ with pH occurs at pH = pK_a. In fact, differentiating eqn. 3 twice with respect to log $[H^+]$ and equating to zero the relationship obtained, it can be seen that the mean value of $[(1/R_{F_{ac}}) - 1]$ and $[(1/R_{F_{alk}}) - 1]$ is achieved at pH = pK_a.

As regards the application of eqn. 3, it should be noted that the $[(1/R_F) - 1]$ quantity, contrary to the case with R_F, is rarely used in thin-layer chromatography. We tried, therefore, to explain the R_F in eqn. 3. Fig. 3 shows the R_F *versus* pH curves of some phenols and, as in the case of the curves in Fig. 2, there is good agreement in most instances between the theoretical curves and the experimental values (see Table III). It should be noted in these curves that the mean R_F value (R_{F_m}) of $R_{F_{ac}}$ and $R_{F_{alk}}$ occurs at a pH value higher than that corresponding to the pK_a value. Differentiating R_F twice with respect to log $[H^+]$ and equating to zero, the following relationship is obtained:

$$[H^+] = K_a \cdot \frac{R_{F_{ac}}}{R_{F_{alk}}} \quad (4)$$

TABLE III

R_F AND $(1/R_F - 1)$ VALUES OF PHENOLS ON DOWEX 50-X4 (Na^+) THIN LAYERS OBTAINED WITH ELUENTS AT DIFFERENT pH VALUES

1 *M* acetic acid (pH = 2.35); 0.1 *M* acetic acid + 0.1 *M* sodium acetate (pH = 4.75); 0.05 *M* disodium phosphate (pH = 7.00); 0.1 *M* sodium hydrogen carbonate (pH = 8.50); 0.05 *M* sodium carbonate (pH = 11.50); 1 *M* ammonia + 0.1 *M* sodium acetate (pH = 11.70); 1 *M* ammonia + 0.1 *M* sodium hydroxide (pH = 13.00).

2,5-Dinitrophenol			*2,6-Dinitrophenol*			*2,4-Dinitrophenol*		
pH	R_F	$(1/R_F - 1)$	*pH*	R_F	$(1/R_F - 1)$	*pH*	R_F	$(1/R_F - 1)$
2.35	0.24	3.17	2.35	0.36	1.80	2.35	0.24	3.17
4.75	0.50	1.00	4.75	0.79	0.26	4.75	0.28	2.57
7.00	0.72	0.39	7.00	0.88	0.14	7.00	0.77	0.30
8.50	0.72	0.39	8.50	0.88	0.14	8.50	0.80	0.25
11.70	0.72	0.39	11.70	0.88	0.14	11.70	0.80	0.25

m-Nitrophenol			*Phloroglucinol*		*3,5-Dichlorophenol*		*α-Naphthol*	
pH	R_F	$(1/R_F - 1)$	*pH*	R_F	*pH*	R_F	*pH*	R_F
2.35	0.21	3.76	4.75	0.39	4.75	0.08	4.75	0.04
4.75	0.21	3.76	7.00	0.39	7.00	0.09	7.00	0.04
7.00	0.22	3.55	8.50	0.80	8.50	0.36	8.50	0.06
8.50	0.58	0.72	11.50	0.93	11.50	0.75	11.50	0.35
11.50	0.80	0.25	11.70	0.95	11.70	0.84	11.70	0.75
11.70	0.90	0.11					13.00	0.80

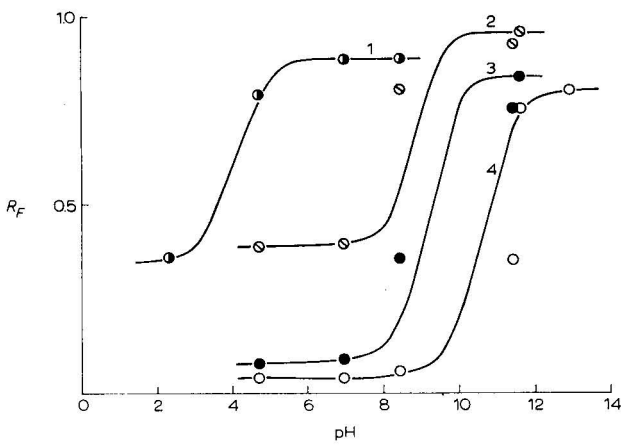

Fig. 3. R_F *versus* pH plots for phenols on Dowex 50-X4 (Na^+) thin layers. 1, 2,6-Dinitrophenol (pK_a = 3.71); 2, phloroglucinol (pK_a = 8.45); 3, 3,5-dichlorophenol (pK_a = 8.18); 4, α-naphthol (pK_a = 9.34).

On the basis of eqn. 4, we can predict that the R_{F_m} value will be shifted more with respect to the pH = pK_a value the lower is the $R_{F_{ac}}/R_{F_{alk}}$ ratio. The experimental points at pH 8.5 and 11.5, which do not fit the theoretical curves in Figs. 2 and 3, were obtained with 0.1 *M* sodium hydrogen carbonate and 0.05 *M* sodium carbonate solutions.

In the first case, R_F values higher than those predicted at pH 8.5 on the basis of the theoretical curves in Figs. 2 and 3 are obtained for *m*-nitrophenol, phloroglucinol and 3,5-dichlorophenol; with sodium carbonate, on the contrary, the above-mentioned phenols and α-naphthol exhibit lower R_F values than those predicted on the basis of the starting pH of the eluent. Such disagreement between theoretical and experimental values may be correlated with the different pH on the layer with respect to that of the eluent. In fact, with the method described in a previous paper[6], we have measured the pH on the layer and found pH values between 9.2 and 9.5 for sodium hydrogen carbonate and between 10.3 and 9.8 for sodium carbonate (see Table IV).

Referring the $[(1/R_F) - 1]$ and R_F values to the pH values reported in Table IV, there is good agreement in the curves in Figs. 2 and 3 between the theoretical and experimental results with these eluents.

TABLE IV

HYDROGEN ION CONCENTRATION GRADIENT ALONG THE LAYER OF DOWEX 50-X4 (Na^+) FOR 0.1 *M* $NaHCO_3$, 0.05 *M* Na_2CO_3 AND 0.05 *M* $NaHCO_3$ + 0.05 *M* Na_2CO_3 SOLUTIONS IN THE ELUENT

*Distance** (cm)	*pH*		
	0.1 M $NaHCO_3$	*0.05 M Na_2CO_3*	*0.05 M $NaHCO_3$ + 0.05 M Na_2CO_3*
0– 2	9.2	10.3	10.0
2– 4	9.3	10.2	9.9
4– 6	9.3	10.1	9.8
6– 8	9.4	10.0	9.8
8–10	9.5	9.8	9.7

* Distances of the front and rear limits of the band (2 × 10 cm) from the starting point.

The above effects, which might be a serious limitation in the use of eqn. 3, are, however, very important as they show a close correlation between the difference in the experimental R_F value compared with the theoretical value and that of the pH values on the layer and in the eluent. It follows, therefore, that, on the basis of eqn. 3, the pH on the layer can be determined from the R_F value.

Analytical applications

In Table V are reported the separations of some isomers eluted with solutions at different pH values. Such separations can all be predicted on the basis of the R_F *versus* pH curves with the exception of that of the aminonitrophenols, whose pK_a values are unknown. For chloro- and bromophenols, the eluent was a solution of sodium carbonate and hydrogen carbonate in order to have a pH about 9.8 on the layer because, on the basis of the R_F *versus* pH curves, at this pH there is the best resolution of the R_F values of different isomers. As shown by the results in Table IV, the carbonate–hydrogen carbonate mixture involves a pH between 10 and 9.7 on the layer. In this case, the use of the ammonium buffer must be avoided, as the pH on the layer changes owing to the exchange reaction between the ammonium ions and the sodium ions of the exchanger.

TABLE V

SEPARATIONS OBTAINED ON THIN LAYERS OF DOWEX 50-X4 (Na^+)

Migration distance 12.5 cm.

Mixture	*Eluent*	R_F *value*
o-Chlorophenol	0.05 *M* $NaHCO_3$ + 0.05 *M* Na_2CO_3	0.84
m-Chlorophenol		0.61
p-Chlorophenol		0.47
o-Bromophenol	0.05 *M* $NaHCO_3$ + 0.05 *M* Na_2CO_3	0.81
p-Bromophenol		0.40
2,6-Dichlorophenol	0.05 *M* $NaHCO_3$ + 0.05 *M* Na_2CO_3	0.95
2,3-Dichlorophenol		0.80
3,5-Dichlorophenol		0.64
3,4-Dichlorophenol		0.50
2,6-Dichlorophenol	0.1 *M* $NaHCO_3$	0.94
2,3-Dichlorophenol		0.62
3,5-Dichlorophenol		0.33
3,4-Dichlorophenol		0.21
o-Nitrophenol	0.1 *M* $NaHCO_3$	0.86
p-Nitrophenol		0.75
m-Nitrophenol		0.52
2,6-Dinitrophenol	0.1 *M* $NaHCO_3$	0.82
2,5-Dinitrophenol		0.73
2,4-Dinitrophenol		0.65
2,6-Dinitrophenol	0.1 *M* acetate buffer	0.69
2,4-Dinitrophenol		0.42
2,5-Dinitrophenol		0.24
4-Amino-2-nitrophenol	0.1 *M* Na_2CO_3	0.83
2-Amino-4-nitrophenol		0.70
2-Amino-5-nitrophenol		0.61
2-Amino-4,6-dinitrophenol		0.50

The dichlorophenols, on the contrary, may be better separated from each other with 0.1 *M* sodium hydrogen carbonate solution than with carbonate–hydrogen carbonate mixtures, as predicted from the R_F *versus* pH curves.

The nitrophenols were developed in sodium hydrogen carbonate solution, as *o*-nitrophenol cannot be detected with eluents of lower pH (*e.g.*, 0.05 *M* disodium hydrogen orthophosphate solution at pH 7.00).

As regards the dinitrophenols, a good separation of these isomers with both alkaline and acidic eluents can be usefully effected. The separation obtained in alkaline medium, that is, at a pH value at which the three isomers are completely deprotonated, is due to the different influence of the ionic strength of the solution on such isomers.

Rexyn 102 (Na^+)

On this exchanger, when eluting with water, most phenols run with the solvent front. Also with 0.1 *M* sodium hydrogen carbonate solution many phenols exhibit high R_F values ($\geqslant 0.90$), with the exception of most phenols with two aromatic nuclei

(R_F between 0.4 and 0.8), 2-amino-5-nitrophenol ($R_F = 0.81$), 2-amino-4,6-dinitrophenol ($R_F = 0.79$), 2-amino-3,4,6-trichlorophenol ($R_F = 0.84$), *p*-bromophenol ($R_F = 0.76$) and 3,4-dichlorophenol ($R_F = 0.85$). The different behaviour of the phenols with water and with sodium hydrogen carbonate can be ascribed to the different ionic strengths of the two solutions. The pH on the layer, in fact, is about 10 with both eluents and is determined by the alkaline reaction of the functional groups of the exchanger. On eluting with 0.1 *M* acetate buffer, a decrease in the R_F values is observed only for those phenols which are the most retained with sodium hydrogen carbonate. Such behaviour is correlated with the pH on the layer (between 8.4 and 10) less in this case than with the two previous eluents. The results achieved with 0.1 *M* sodium hydrogen carbonate are interesting from an analytical standpoint, as the following separations can be effected: 7-amino-2-naphthol and 5-amino-1-naphthol (migration distance 12 cm); and α-naphthol and β-naphthol (migration distance 14 cm).

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GEL FILTRATION CHROMATOGRAPHY OF PETROLEUM SULFONATES

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SUMMARY

Sulfonated fractions of partially refined petroleum were chromatographed on Sephadex beads in water–95% ethanol (9:1). Each petroleum sulfonate was separated into two portions, a polymeric colloid fraction and an association colloid fraction. The composition of each sample with respect to these two fractions was calculated on a dry weight basis. For each sample, the initial micelle concentration of the association colloid fraction was determined from surface tension measurements and from gel filtration data. A comparison of the results showed that the two techniques yield critical micelle concentrations which differ by as much as a factor of four.

INTRODUCTION

Gel filtration chromatography of micellar solutions on Sephadex beads has been reported by Nakagawa and Jizomoto[1], Suzuki and Sasaki[2], and Coll[3]. These articles stress the theoretical aspects of the transport of a micellar solution on a gel filtration matrix. For the most part, the experimental work reported by these workers consisted of analyzing purified laboratory surfactants. Experiments with sodium dodecyl sulfate demonstrated that the transport of a micellar solution proceeds in the following manner[2]. The micelles, which are colloidal-sized species, are excluded from the beads and elute at the void volume. A plateau, the concentration of which equals the critical micelle concentration, trails the micelle peak. This situation arises because of the equilibrium between micelles and monomers. Micelles move faster down the column than monomers, therefore the micelles must dissociate to regenerate the equilibrium concentration of monomer (*i.e.*, the critical micelle concentration) during the transport process.

The chromatography of a surfactant mixture on a gel filtration matrix has been described by Nakagawa and Jizomoto[4]. In this latter paper, also, the authors' major concern was the theoretical aspects of the system. Nonetheless, interesting

observations of a practical nature were reported. In particular, Nakagawa and Jizomoto[4] showed that if a property common to each surfactant in a mixture (*e.g.*, conductivity) were plotted as a function of eluted volume, the resultant elution profile would have a more or less characteristic shape as a consequence of the different physical properties of the constituent surfactants. We became interested in determining whether useful qualitative information about the surfactant composition of petroleum sulfonates could be obtained by this technique. In addition, we wanted to determine whether the critical micelle concentration of a petroleum sulfonate measured by this technique was essentially the same as that determined by a more classical procedure, *e.g.* surface tension measurements. Petroleum sulfonates have recently become popular as inexpensive surfactants for use in tertiary oil recovery systems[5,6].

EXPERIMENTAL

A 3:2 mixture of Sephadex G-25 and Sephadex G-50 was prepared on a dry weight basis and allowed to hydrate in a large excess of twice distilled water for 24 h. Fines were removed by decanting. The slurry was poured into a column to form a bed 2.8 × 44 cm. Subsequently, the bed was equilibrated with the solvent used for all of the studies described in this paper, water–95% ethanol (9:1).

Sulfonated fractions of partially refined petroleum were prepared as previously described[7]. Prior to analysis by gel filtration chromatography, each petroleum sulfonate was subjected to a solvent extraction procedure to remove inorganic salts and unsulfonated organic material[8]. Samples were prepared for chromatography by dissolving 40 mg of the active petroleum sulfonate in 2.0 ml of the solvent. Gentle heating with live steam and frequent swirling on a Vortex mixer were used to speed up the dissolving process. After a sample had cooled to ambient temperature, it was carefully placed on the bed of Sephadex beads. Eluent fractions of 5 ml were obtained with an automatic fraction collector.

The eluted fractions were assayed for conductivity with a YSI Model 31 conductivity bridge equipped with a microprobe. Dry weight was determined by pouring a 5-ml fraction into a pre-weighed aluminum weighing dish, drying at 65° for at least 36 h, and weighing the sample plus dish on a Mettler H-20 balance.

Surface tension measurements were made with a Cahn electrobalance, Model RM-2, equipped with a platinum/iridium ring. The solvent was water–95% ethanol (9:1).

Sulfur analyses were performed by Huffman Labs. (Wheat Ridge, Colo., U.S.A.).

RESULTS AND DISCUSSION

Composition of petroleum sulfonates

Profiles of conductivity *versus* elution volume are shown in Fig. 1 for three different petroleum sulfonates. For these surfactant mixtures, such profiles are much more complex than the analogous profile of a pure surfactant (see ref. 2). Also shown in Fig. 1 are profiles of dry weight *versus* elution volume. The trough in these profiles at about fraction 33 suggests that the material which elutes at the void volume is not

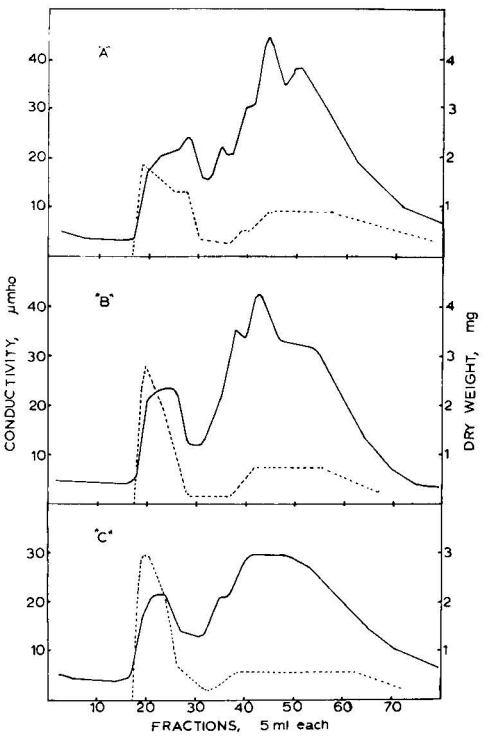

Fig. 1. Elution profiles for three different petroleum sulfonates. Conductivity (solid line) and dry weight (broken line) are plotted *versus* eluted volume in each case. Total bed volume = 270 ml.

in equilibrium with the material eluting later. Presumably, a significant portion of each different petroleum sulfonate is composed of relatively large, polysulfonated compounds, *e.g.* polysulfonated asphaltines. These materials, due to their large size and, possibly, due to charge repulsion effects, elute at or near the void volume. For convenience, we have labelled this peak (*i.e.*, fractions $\approx$ 19–35) the "polymeric colloid fraction". The pigments peculiar to petroleum, if present, always elute in this fraction. When isolated and re-chromatographed under the same conditions, the polymeric colloid fraction continues to elute at the void volume, *i.e.* as if it consisted of compounds large enough to be completely excluded from the Sephadex beads.

The second portion of the pronounced two-peak profiles (Fig. 1) has been labelled the "association colloid fraction" (*i.e.* fractions $\approx$ 40–70). On the basis of UV, IR, and NMR spectra this material appears to be composed of monosulfonated compounds of the alkylaryl sulfonate class[9]. When the association colloid fraction is isolated and re-chromatographed under the same conditions, the elution behavior is strikingly similar to that of a pure surfactant. Such an observation suggests that this fraction is composed of a mixture of surfactants possessing similar physical properties. The compounds in the association colloid fraction appear to be strongly adsorbed by Sephadex, since solute elution extends significantly beyond the total bed volume.

The composition of each petroleum sulfonate with respect to the polymeric colloid fraction and the association colloid fraction has been calculated from the dry

TABLE I

DISTRIBUTION OF PETROLEUM SULFONATE INTO POLYMERIC COLLOID AND ASSOCIATION COLLOID FRACTIONS AND THE EQUIVALENT WEIGHT OF EACH FRACTION

Petroleum sulfonate	*Polymeric colloid*		*Association colloid*	
	Percentage of sample	*Equivalent weight**	*Percentage of sample*	*Equivalent weight**
"A"	43	400	57	280
"B"	54	480	46	265
"C"	56	430	44	275

* Based on sulfur content.

weight profiles of Fig. 1. The data are shown in Table I. Equivalent weights based on sulfur content are also shown in Table I. While the polymeric colloid fraction has the higher equivalent weight of each pair, the equivalent weights for the two fractions are not dramatically different.

We conclude that gel filtration chromatography of petroleum sulfonates does yield limited but useful qualitative information about the sample composition. In particular, it is possible to estimate the association colloid content of a sample by this technique. It seems reasonable to suggest that petroleum sulfonates with the greater association colloid content would be more effective, on a weight basis, in forming micellar solutions, stabilizing microemulsions, and so on. The polymeric colloid fraction should also be surface active, in the same sense that other polyelectrolytes (*e.g.* proteins) are. However, these large species diffuse to interfaces relatively slowly and once at the interface tend not to leave. Thus, the polymeric colloid fraction probably does not contribute to the general detergent properties of a solution containing petroleum sulfonates.

Critical micelle concentrations of petroleum sulfonates

The critical micelle concentration for the association colloid fraction of each different petroleum sulfonate was estimated from the plateau of constant dry weight per fraction observed in each experiment (Fig. 1). The critical micelle concentration was calculated by dividing the concentration in the plateau region (in g/l) by the equivalent weight of the association colloid fraction (from Table I). As a comparison, the critical micelle concentration for each petroleum sulfonate was determined by

TABLE II

COMPARISON OF CRITICAL MICELLE CONCENTRATIONS DETERMINED BY SURFACE TENSION MEASUREMENTS AND GEL FILTRATION CHROMATOGRAPHY

Petroleum sulfonate	*Surface tension (mequiv./l)*	*Gel filtration (mequiv./l)*
"A"	0.15	0.64
"B"	0.88	0.53
"C"	0.33	0.44

surface tension measurements. For this technique, the critical micelle concentration was obtained by multiplying the measured value (in g/l) for the petroleum sulfonate by the percentage of the sample attributed to the association colloid fraction, and then dividing by the equivalent weight of the association colloid fraction. The critical micelle concentrations determined by the two procedures are listed in Table II. A comparison of the data in Table II reveals that the two procedures yield critical micelle concentrations which differ by as much as a factor of four. However, as noted above, adsorption of the association colloid fraction onto Sephadex appears to be a significant factor in the transport process. The monomer concentration in the plateau of the elution profiles (Fig. 1) presumably arises from an equilibrium with both adsorption sites and micelles. Thus, the relatively large differences in critical micelle concentrations measured by the two techniques is not an unexpected result.

Concluding comments

A gel filtration column composed of only Sephadex G-50 gives a much better resolution of the two fractions present in petroleum sulfonates than that shown in Fig. 1. However, with the system described in Experimental, it is most difficult to estimate the critical micelle concentration with Sephadex G-50 because the number of fractions constituting the plateau is very small. On the other hand, a gel filtration column composed of only Sephadex G-25 does not resolve the petroleum sulfonate into polymeric colloid and association colloid fractions. But, in this case, the plateau includes slightly more fractions than are present in the data shown in Fig. 1. By empirical testing, a gel filtration column composed of a 3:2 mixture of Sephadex G-25 and G-50 was selected, so that both sample composition and critical micelle concentration could be estimated in one experiment.

ACKNOWLEDGEMENT

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CHROM. 8246

APPLICATION OF DENSITOMETRY TO THE QUALITATIVE AND QUANTITATIVE EVALUATION OF PHARMACEUTICAL COLOURANTS

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SUMMARY

Samples from all the British manufacturers of food grade Amaranth and Sunset Yellow FCF were examined by a densitometric thin-layer chromatographic procedure which provides a rapid and convenient method for producing a qualitative and quantitative statement on the impurity profiles of dye materials. Wide variations in the impurities were found which could account for variations in toxicological results. The method could be incorporated into official specifications to control more precisely the quality of food grade dyes.

INTRODUCTION

As a result of the evidence of possible detrimental effects of food additives strict regulations are in force governing the use of colourants in foods and pharmaceuticals. The dyes permitted vary markedly from country to country and Table I indicates the international acceptability of ten common materials. Only Amaranth is acceptable in all 65 countries whose regulations were examined. Coloured impurities are usually present in food grade dyes and the nature and proportions of these are specified in the official standards. In certain cases these coloured subsidiaries are dyes in their own right, as defined by the Colour Index, and as such are controlled. For example Amaranth may contain four subsidiary dyes which are structurally related to the main component (Fig. 1). Crystal Ponceau, which is no longer acceptable in any of the countries whose regulations were examined, Ponceau 6R, which is acceptable in only 14, Fast Red E, which is acceptable in 24, and Ponceau 4R, which is acceptable in 45. Sunset Yellow FCF contains only one subsidiary, Orange II, which is a Colour Index dye. This is no longer permitted for use in any of the 65 countries examined, although it was previously used in the U.S. as D & C Orange No. 4.

TABLE I

RANKED LIST OF THE FREQUENCY OF ACCEPTANCE FOR USE BY COUNTRIES OF FOOD AND DRUG DYES

Regulations of 65 countries were examined.

Dye	*Colour Index (1956) No.*	*Colour*	*Number of countries accepting for use*
Amaranth	16185	red	65
Indigo Carmine	73015	blue	62
Tartrazine	19140	yellow	60
Erythrosine	45430	red	56
Sunset Yellow FCF	15985	orange	53
Ponceau 4R	16255	red	47
Azorubine	14720	red	39
Black PN	29440	blue	34
Patent Blue V	42051	blue	32
Fast Red E	16045	red	28

Pharmaceuticals are frequently coloured and mixtures of dyes are often used to obtain a particular shade. However, the differences in quantities of components used to produce a particular shade may differ by a hundredfold and therefore the situation can arise where the concentration of an impurity in a major component can

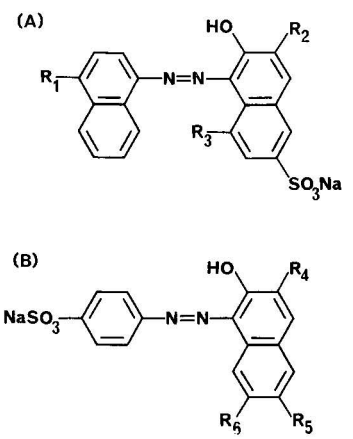


Fig. 1. Relationship between structures of (A) Amaranth and (B) Sunset Yellow FCF and their subsidiary dyes.

	R_1	R_2	R_3
Amaranth	$-SO_3Na$	$-SO_3Na$	$-H$
Ponceau 4R	$-SO_3Na$	$-H$	$-SO_3Na$
Fast Red E	$-SO_3Na$	$-H$	$-H$
Crystal Ponceau	$-H$	$-H$	$-SO_3Na$

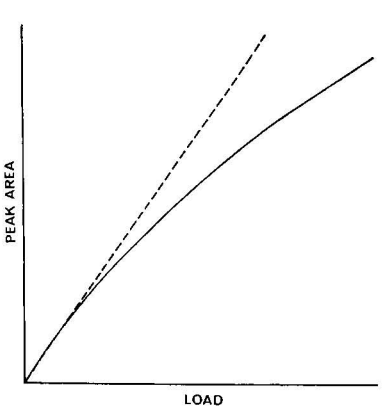
	R_4	R_5	R_6
Sunset Yellow FCF	$-H$	$-SO_3Na$	$-H$
Orange II	$-H$	$-H$	$-H$
R salt derivative	$-SO_3Na$	$-SO_3Na$	$-H$
G salt derivative	$-H$	$-SO_3Na$	$-SO_3Na$

exceed the total concentration of a minor component. If such an impurity is a dye which is specifically excluded in a particular country an interesting legal anomaly arises. If a naturally impure sample of the major dye is taken which contains the required proportion of minor subsidiaries to give the desired shade, then this is acceptable, provided that the level of subsidiaries is below the level of impurities laid down in the specification for the major dye. On the other hand, if the same mixture is artificially created by mixing clean major dye with the specific subsidiary dye required, then this is not acceptable, as the subsidiary dye must be considered as an individual component of the product rather than a minor impurity, and therefore this addition constitutes adulteration. This latter situation is further complicated by the variations in permitted dyes which can necessitate the use of different combinations to obtain the same hue for different countries. The main objective of this present investigation was to determine the quality and quantity of coloured impurities present in a range of samples of commercial dyes so that "reasonable" levels might be suggested to which subsidiary dyes could be reduced or, alternatively, levels to which they might be included.

Samples of the dyes Amaranth and Sunset Yellow FCF, permitted in Great Britain, were requested from all the British manufacturers of food grade dyestuffs, and in addition French National Standard samples of these were obtained from the Laboratoire National de la Santé Publique in Paris. France is the only country which will provide a national standard sample. Details of the test procedure and results are given below.

TEST PROCEDURE

We have previously reported briefly[1] a method for the determination of the impurity profiles of pharmaceutical colourants by densitometry of thin-layer separations. Although this gave a complete pattern of the distribution of material on the



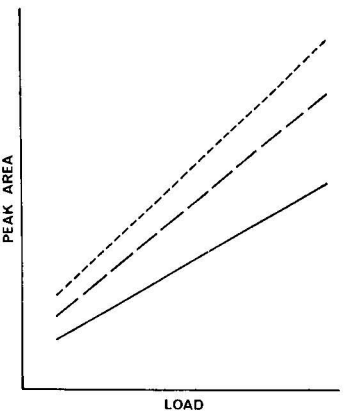


Fig. 2. Deviation of the absorption of light by Amaranth spots on cellulose plates from the Lambert–Beer relationship over the range 0–30 μg. – – –, Lambert–Beer relationship; ———, observed values.

Fig. 3. Response curves for Amaranth subsidiaries from 0.1–0.4 μg. ·····, Crystal Ponceau; – – –, Ponceau 4R; ———, Fast Red E.

chromatogram, it tended to overemphasise the proportions of impurities as, when the subsidiary peaks were sufficiently high to allow their estimation, the major component was oversaturated and therefore the Lambert–Beer law was not obeyed (Fig. 2). In addition there are variations in response factors between individual subsidiaries (Fig. 3). To obtain an accurate estimation of the levels of these impurities, it is therefore necessary to compare the test sample with standards of the subsidiary dyes of approximately equal, and accurately known, concentration, and to relate this determination to the load applied to indicate the percentage of each impurity.

$$\frac{\text{impurity peak area in test sample}}{\text{standard peak area}} \times \text{standard load} = \text{impurity load}$$

$$\frac{\text{impurity load in test sample}}{\text{total test load}} \times 100 = \text{percentage impurity}$$

1% (w/v) solutions of each of the twelve commercial British samples of Amaranth and of Amaranth LNSP, and 0.01% (w/v) and 0.02% (w/v) solutions of each of the three subsidiary dyes, Fast Red E, Crystal Ponceau, and Ponceau 4R (Solmedia, London, Great Britain), were prepared. The following volumes were applied to a 20 × 20-cm prepared thin-layer plate (MN CEL 300/254, Machery, Nagel & Co., Düren, G.F.R.) using Microcaps: for each subsidiary dye 3 × 1 μl of 0.01% (0.1 μg), 3 × 1 μl of 0.02% (0.2 μg), and 3 × 2 μl of 0.02% (0.4 μg), and for each of the 1% commercial Amaranth sample solutions, and for Amaranth LNSP, 1 × 2 μl (20 μg). Plates were prepared in triplicate, developed in the solvent system butanone–acetone–water (7:3:3)[2] for 10 cm, allowed to air dry, and scanned in transmission on a Vitatron TLD 100 flying spot densitometer in the log mode, with a scan speed of 1 cm/min, an aperture of 0.25 mm, a strike length of 14 mm, a filter of 525 nm, and a span of 970. Peak areas were estimated directly from the integrating recorder after correction for baseline drift.

Essentially the same procedure was used for the samples of Sunset Yellow FCF except that the plates were developed in butanone–acetone–ammonia (0.880)–water (7:3:0.02:3)[3] for 16 cm. Whilst the subsidiary Orange II was available (Solmedia) it was necessary to synthesise the higher sulphonated R and G salt derivatives[4].

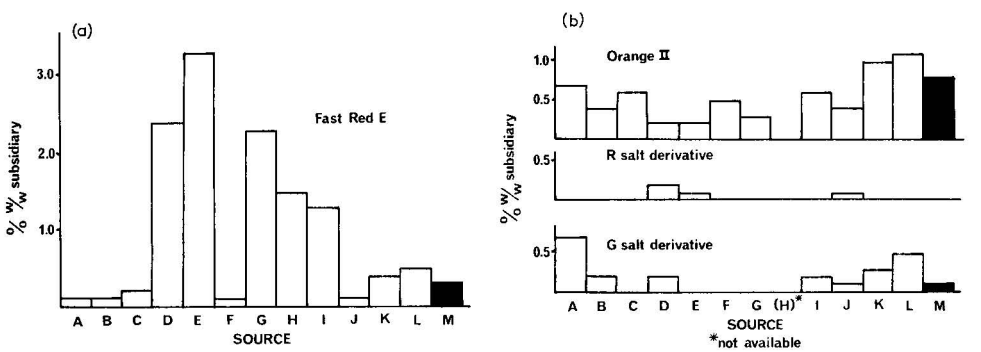


Fig. 4. Subsidiary dye content of commercial samples (A–L) and French National Standard Samples (M) of Amaranth (a) and of Sunset Yellow FCF (b).

RESULTS

The results for both Amaranth and Sunset Yellow FCF samples are given in Fig. 4. In the Amaranth samples the only subsidiary present in measurable quantities (greater than 0.05%, w/w) was Fast Red E. This appeared in all samples, being below 1% (w/w) in eight, between 1 and 2% in two, and above 2% in the last three. Traces of Crystal Ponceau were detected in three samples (B, H, and M) and of Ponceau 4R in one (E). Ponceau 6R was not detected in any samples in contrast to reports[5] of its presence in U.S. samples of Amaranth. Sunset Yellow FCF showed a more complex pattern of impurities in that whilst all contained Orange II and nine of the twelve samples the G salt derivative, the R salt derivative was found in only three cases. At the 0.5% (w/v) subsidiary level the reproducibility of the estimations was $\pm$ 7.8% of the mean value, *i.e.* 0.5 $\pm$ 0.04% (w/v). All the samples except specimen E of Amaranth fell within the 3% subsidiary dye limit set by the appropriate British Standard[2,3]. The samples designated M which were French National Standard samples contained considerable subsidiary dye.

DISCUSSION

There is clearly wide variation in the subsidiary dyes present in samples from different manufacturers of the same food grade dyes. In the case of Amaranth this is essentially a quantitative variation as Fast Red E is the only subsidiary present at appreciable concentration. With Sunset Yellow FCF more subsidiaries are found, although Orange II is always the major component. It is well recognised that it is impractical to produce a commercial dye of food grade which is 100% pure, but on the other hand the results from the Amaranth samples suggest that certain manufacturers do not find it too difficult to reduce subsidiaries to a low level.

Workers in different countries have often obtained different results in toxicological studies on colourants[6] and this present report emphasises the need for most careful characterisation of samples for toxicity testing[7], in particular with respect to their subsidiary dye content. It is unrealistic to test 100% pure dye as any results obtained will not apply to impure commercial material. Toxicity may well be due to the minor components and it must be remembered that it is usual to carry out toxicity testing at very high dose levels where the absolute level of subsidiaries is high relative to any normal intake pattern.

The procedure described here is a rapid and convenient method for making a qualitative and quantitative statement of the impurity profiles of dyes and it is suggested that it could be used to lay down standards where the subsidiary dye content was set at levels which prudent manufacturers did not find too onerous. We would tentatively suggest that perhaps 0.5% (w/w) of Fast Red E in Amaranth would be a reasonable example, although it must be emphasised that only samples from British manufacturers have been investigated and that there appear to be differences between countries[5] in the subsidiaries present, presumably due to alternative methods of manufacture. On the other hand, it could be argued that many of the impurities have not been satisfactorily proven to be toxic and that, since commercial samples containing relatively high concentrations of these have not produced obvious toxicity in use, a reasonable level would be that of the highest routinely found concentration.

Although this latter thesis is less satisfactory in some ways than the former low level designation, it could be developed to allow the use of synthetic mixtures, provided that the levels of subsidiaries in these mixtures did not exceed the maximum level specified.

There is certainly scope here for detailed coordinated studies on the toxicity and impurity profiles of different samples of the same food grade dyes.

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CHROM. 8233

Note

A procedure for boron trifluoride-catalyzed esterification suitable for use in gas chromatographic analysis

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Treatment with an alcohol and a Lewis acid is the commonly used esterification procedure in the gas chromatographic analysis of organic acids in biological materials[1]. When methanol is used as the alcohol[2,3], difficulties are encountered owing to the volatility of the corresponding esters, *i.e.*, losses during the concentration step and interference with solvents in the gas chromatographic analysis, while propanol[4] and butanol[5] produce more stable and less volatile esters.

Boron trifluoride has been shown[6] to be the most efficient catalyst as it produces pure esters in high yields in the shortest time. However, the reaction mixture containing boron trifluoride cannot be injected directly into the gas chromatographic column because the boron trifluoride may alter the packing and produce secondary reactions[1]. Therefore, at the end of the esterification reaction, the alcohol–boron trifluoride reagent is usually decomposed by adding water or a salt solution, and the esters are then extracted into an organic solvent. By this procedure, the recoveries of minimal amounts of acids do not seem to be quantitative; in fact, in the extraction step, the alcohol may assist in transferring the ester between water and the organic solvent.

In this paper, we describe an esterification procedure in which the extraction step is no longer required. We have applied this method to fumaric, benzoic and stearic acids, as models, employing isopropanol–boron trifluoride and *n*-butanol–boron trifluoride as reagents; before the gas chromatography stage, the boron trifluoride is completely neutralized by adding an organic base (triethylamine or pyridine), thus forming a well known complex[7] that is insoluble in non-polar solvents. The gas chromatographic analysis of isopropyl and *n*-butyl esters gives better recoveries than those obtained by employing the water extraction procedure.

EXPERIMENTAL

Reagents

Boron trifluoride was purchased from J. T. Baker, Phillipsburgh, N.J., U.S.A. All solvents and other compounds were obtained from Carlo Erba, Milan, Italy. Isopropanol and *n*-butanol were refluxed over calcium chloride and re-distilled before use.

The alcohol–boron trifluoride reagent was prepared by bubbling boron tri-

fluoride gas into the alcohol at 0° until a concentration of 2 mequiv./ml was attained. The boron trifluoride content was determined by adding a known excess of pyridine and titrating the base with 0.1 *N* hydrochloric acid to pH 3.

Apparatus

A Fractovap GV gas chromatograph (Carlo Erba) equipped with a flame ionization detector was used. The instrument contained two U-shaped columns (2 m × 2.5 mm): one column was packed with 3% OV-17 on Gas-Chrom Q, 100–120 mesh (Applied Science Labs., State College, Pa., U.S.A.) and the other with 2% neopentyl glycol succinate (NPGS) (LAC 767) on Gas-Chrom P, 100–120 mesh (Carlo Erba). Nitrogen was used as the carrier gas at a flow-rate of 30 ml/min; the flow-rates of hydrogen and air were 33 and 400 ml/min, respectively. The injector and detector temperatures were 200° and 250°, respectively. The column temperature was kept at 80° for 1 min after the injection, then programmed at 20°/min to 210° and kept at 210° for 5 min.

Procedure

Two equal aliquots of each acid (25–100 μg) were placed in two screw-capped stoppered 3-ml tubes. Alcohol–boron trifluoride reagent (0.15 ml) was added and the reaction was carried out at 100° for 20 min. To one sample, anhydrous diethyl ether (0.2 ml), *n*-pentane (0.2 ml) and a mixture (1:1, v/v) of *n*-pentane and triethylamine (or pyridine) (0.15 ml) were added with shaking in an ice-bath (the neutralization is exothermic) and the suspension was centrifuged. The second sample was added with 1 ml of water and extracted three times with 0.4 ml of chloroform for 2 min. The combined extracts were transferred into a tube containing a few grains of anhydrous sodium sulphate. After adding to both samples 50 μg of phenanthrene (as internal standard) in diethyl ether (0.1 ml), 1 μl of each supernatant was injected into the gas chromatograph.

Quantitative analysis

Peak areas were calculated as peak height times the width at half height and corrected against the peak area of the internal standard (phenanthrene). The corrected peak areas of the esters from the two neutralization methods were compared. The values obtained by the decomposition with water are reported as a percentage of those obtained by our method. For the calibration graphs, 5, 10, 20 and 30 μg of each acid were placed in different 1-ml tubes and made to react with the alcohol–boron trifluoride reagent (100 μl) as previously described. In order to precipitate boron trifluoride, 40 μl each of diethyl ether and *n*-pentane and 50 μl of a mixture of *n*-pentane + pyridine or triethylamine were used; 10 μg of phenanthrene in diethyl ether (40 μl) were finally added.

RESULTS AND DISCUSSION

The acids examined were chosen from those of biological interest but with different chemical features (one dicarboxylic, one aromatic and one fatty acid) in order to verify the suitability of the method. For the same purpose, two different alcohols, isopropanol and butanol, were used.

The neutralization of boron trifluoride by the addition of an organic base in a non-polar solvent permits the complex base–boron trifluoride to be precipitated and a supernatant containing the esters without boron trifluoride to be obtained; this solution can be injected directly into the gas chromatographic columns.

As the solvent for the precipitation, *n*-pentane seemed to be ideal because of its volatility and non-polarity, but, as it is not soluble in the alcohol–boron trifluoride reagent, the added organic base agglomerates the reaction mixture. Hence the prior addition of diethyl ether to the reaction mixture is necessary in order to make the solution homogeneous during precipitation. The precipitates obtained with triethylamine were transparent and gel-like, while those obtained with pyridine were white, flaky and easily separated. Therefore, pyridine was the base of choice except for the esters that gave peaks which interfered with the pyridine tailed peak. Both stationary phases used (NPGS and OV-17) were found to be efficient; the retention times of the isopropyl and *n*-butyl esters analyzed are reported in Table I.

TABLE I

RETENTION TIMES RELATIVE TO PHENANTHRENE (1.00) OF ISOPROPYL AND *n*-BUTYL ESTERS ANALYZED ON OV-17 AND NPGS

Compound	*OV-17*	*NPGS*
Isopropyl fumarate	0.46	0.37
Isopropyl benzoate	0.53	0.34
Isopropyl stearate	1.27	0.89
n-Butyl fumarate	0.59	0.80
n-Butyl benzoate	0.69	0.62
n-Butyl stearate	1.69	1.33

In order to compare our method of neutralization of boron trifluoride with that used previously, we studied the corresponding recoveries by gas chromatographic analysis. The analysis was carried out on NPGS, precipitating boron trifluoride with triethylamine for isopropyl esters and with pyridine for *n*-butyl esters. The results are reported in Tables II and III.

TABLE II

RECOVERY OF ISOPROPYL ESTERS OBTAINED BY THE TWO DIFFERENT METHODS OF BORON TRIFLUORIDE NEUTRALIZATION

Results given are percentages of isopropyl esters recovered after neutralization with water relative to the same ester recovered after neutralization with triethylamine. Single values are means from five independent experiments, and the ranges are given in parentheses.

Acid	*Amount of acid esterified (μg)*			
	25	*50*	*75*	*100*
Fumaric	70 (63–75)	84 (81–88)	86 (82–91)	98 (92–102)
Benzoic	71 (65–76)	79 (75–82)	88 (83–92)	96 (90–99)
Stearic	76 (69–81)	85 (80–88)	87 (81–92)	99 (96–103)

TABLE III

RECOVERY OF *n*-BUTYL ESTERS OBTAINED BY THE TWO DIFFERENT METHODS OF BORON TRIFLUORIDE NEUTRALIZATION

Results given are percentages of *n*-butyl esters recovered after neutralization with water relative to the same ester recovered after neutralization with pyridine. Single values are means from five independent experiments, and the ranges are given in parentheses.

Acid	*Amount of acid esterified (μg)*			
	25	*50*	*75*	*100*
Fumaric	83 (78–87)	86 (81–89)	92 (87–95)	101 (96–105)
Benzoic	78 (73–82)	91 (87–94)	94 (90–98)	100 (94–103)
Stearic	80 (74–84)	82 (78–86)	88 (83–92)	101 (96–104)

It was found that the results obtained on OV-17 and using both bases for both esters were comparable with those reported earlier. With large amounts of acids (1–10 mg), the recoveries proved to be similar using both methods. With small amounts (below 100 μg), our method gave better recoveries. The calibration graphs for esters of fumaric, benzoic and stearic acids (Fig. 1) show a linear response up to 50 μg.

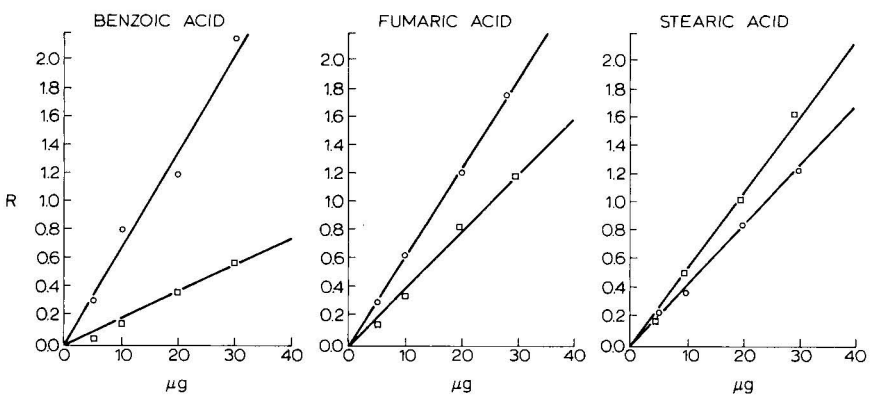

Fig. 1. Calibration graphs for the isopropyl (□) and *n*-butyl (○) esters analyzed. On the abscissa are recorded the amounts of acid and on the ordinate the ratios (R) between areas of esters and area of phenanthrene (10 μg).

We can therefore state that the method described here, in comparison with the usual techniques, simplifies the esterification procedure and improves the product recoveries.

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CHROM. 8251

Note

A simple semi-micro gas–liquid chromatography sample trap for aerosol-forming substances

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The trapping of material from a gas–liquid chromatographic (GLC) column is frequently complicated by the formation of aerosols (especially for compounds with boiling points above 175° at 760 mm). Several methods have been used in order to overcome this problem. A thermal gradient, either longitudinal[1,2] or radial[3], is useful in breaking down these aerosols. For instance, a high-efficiency trap suitable for large-scale work (although of fairly complicated construction) that uses a radial thermal gradient has been described by Teranishi *et al.*[3]. High-tension electrostatic traps (Cottrell-type precipitators) have been used[2] but the inconveniences (electric shock, ignition) of such devices are obvious.

This paper describes a simple trap system for the 1–100 mg range using combined longitudinal and radial thermal gradients. It is made up of a simple glass collector tube, an electrically heated glass insert, a copper sleeve that penetrates the wall of the GLC oven and a simple splitter at the column outlet. Substances from the chromatographic column are collected by pushing the collector tube (with heater insert fitted) through the copper tube against the rubber septum (Fig. 1, component 9) on the splitter. The copper tube is mounted on a copper plate inside the oven and provides a longitudinal temperature gradient; the heater insert provides a radial gradient.

The trap system was tested using a number of compounds that fumed heavily by injection of accurately measured amounts of pure material and re-collection of the material in weighed traps. The recovery of material varied markedly with the heater insert temperature (Fig. 2). When the insert was not heated, heavy white fumes appeared, but at the maximum recovery almost no fumes could be seen. No condensation could be detected on the insert when it was heated.

The collector tubes were usually air-cooled. When a small water cooling jacket was fitted to the collector tube (rubber stoppers), there was a slight improvement in yield (see curves 5 and 6 in Fig. 2).

The material in the tubes could be distilled out or sealed off in the narrow part of the tube after collection by centrifugation. Alternatively, the material could be washed out, for instance directly into an NMR tube. Samples of about 30 mg can be readily collected from two or three injections of moderately pure material.

A slightly modified Varian 600 chromatograph was used. A 13-mm hole was

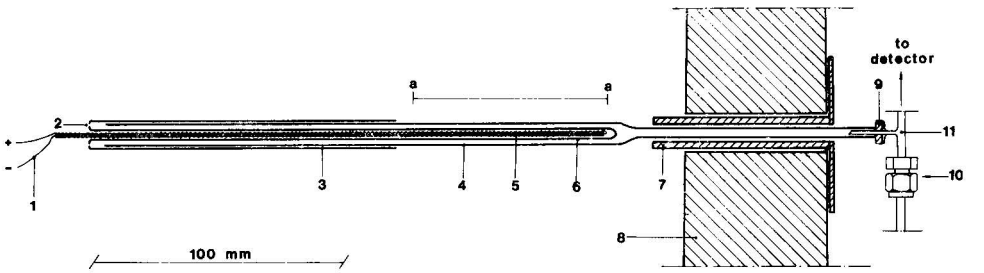

Fig. 1. GLC trap with heated insert. 1, Kanthal wire, 0.2 mm, total resistance 50 Ω; 2, pressure release vents; 3, guide tube to center the heater insert; 4, collector tube; 5, glass capillary with heater winding; 6, glass envelope of heater insert; 7, copper tube; 8, gas chromatograph oven wall; 9, rubber septum; 10, Swagelok connection; 11, splitter.

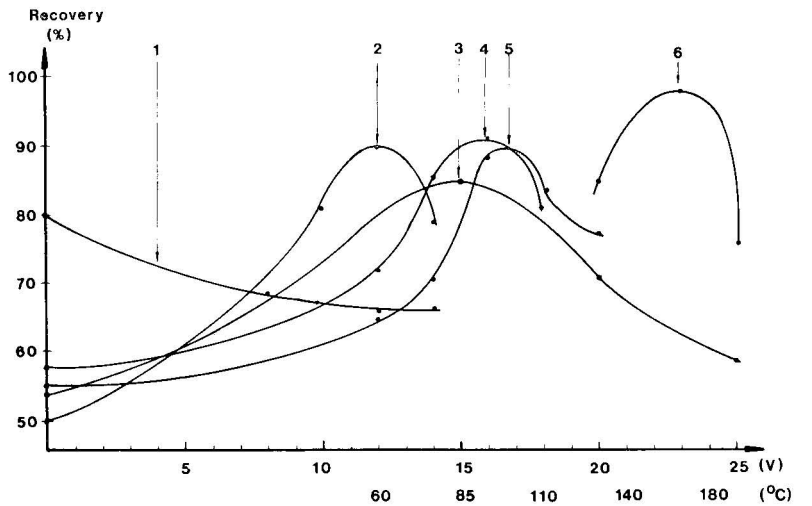

Fig. 2. Recovery *versus* heater insert voltage (or approximate temperature). Column, 2.5 m × 4 mm I.D.; 20% DC-550 on Chromosorb A, 45–60 mesh; oven temperature, 220°. 1, *n*-Nonane (b.p. 151°); 2, *n*-dodecane (b.p. 216°); 3, quinoline (b.p. 237°); 4, *n*-tetradecane (b.p. 254°); 5, *n*-hexadecane (b.p. 287°); 6, *n*-hexadecane collected using water cooling on the section a–a in Fig. 1.

drilled in the oven wall for mounting the copper tube and a simple splitter (*ca.* 100:1) was fitted between the flame detector and the end of the column.

The trapping system described here has given ideal performance in routine collection of small samples for NMR and other forms of spectroscopy.

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CHROM. 8257

Note

Analysis of data from amino acid and other automated analyses

III. A magnetic tape cassette data logging system for gas chromatographs

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A system utilizing a magnetic tape cassette data logger for the analysis of data from an amino acid analyser has already been described[1]. The basic requirements of a data logger for use with a gas chromatograph are somewhat different in that there is a large linear range over which a flame ionization detector equipped with a suitable amplifier can operate, and the short duration of many peaks. For this reason the logger must be capable of digitizing and recording voltages over a wide range without loss of accuracy, and at a rate sufficient to record an adequate number of data points from the shortest peaks normally encountered.

DESIGN OF THE DATA LOGGING SYSTEM

The gas chromatograph to be provided with a data logger was a Pye 104 Model 64 equipped with flame ionization detectors and a wide-range amplifier. The latter has a separate integrator output, not affected by the attenuation setting, which gives an output of up to 10 V. At an attenuation setting $\times$ 1, full scale deflection on the potentiometric recorder corresponds to an integrator output of 10 mV. Thus a digitizing device with a resolution of 10 μV would ideally give an accuracy of 0.1% on the most sensitive range of the gas chromatograph. In practice, stability of the digitizing device and of the gas chromatographic system makes this absolute limit of accuracy and sensitivity of limited practical value, but assures more than adequate accuracy under normal operating conditions. The digitizing system selected on this basis was the Advance DVM5P autorange digital voltmeter with printer output option (Advance Instruments, Bishops Stortford, Great Britain). The output consists of five binary coded decimal (BCD) numbers corresponding to the displayed voltage, one BCD number for range (automatically selected), and a single output for sign. All outputs are positive logic at TTL levels (logic 0 $<$ 0.5 V, logic 1 $>$ + 2.4 V and $<$ + 5 V).

The input terminals of the digital voltmeter were connected to the integrator output of the ionization amplifier. This system displays the output from the amplifier continuously, new measurements being made at the maximum operating speed of the digital voltmeter (5 readings per sec, approximately). The reading rate, and hence the command for the data logger to record the data, must be controlled to make

voltage measurements and record this data at a rate which can be selected to suit the chromatographic data being logged. This requires a 5-V pulse of duration greater than 200 msec to be applied, at the selected intervals, to the digitize input on the printer output socket of the digital voltmeter (voltmeter in the hold mode). A simple timer, based on a 555 integrated circuit was designed to perform this function, and the circuit is shown in Fig. 1. The time intervals provided are 0.5, 1, 2 and 4 sec, and the pulse length is approximately 250 msec. The unit was conveniently enclosed in a Type A instrument case together with a 5-V regulated power supply (RS Components, London, Great Britain). The switch for the end-of-run indicator described below was also included in this unit.

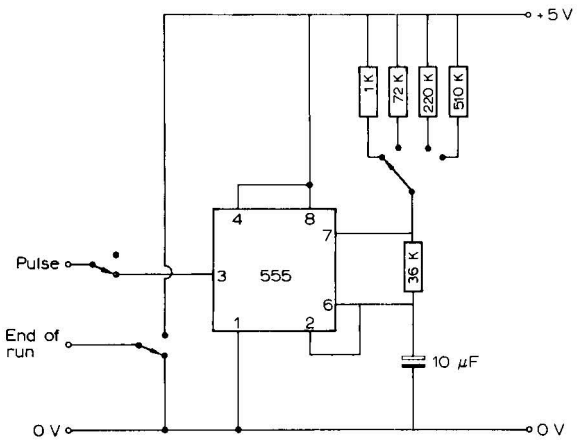

Fig. 1. Timer unit and end-of-run switch. Power supply omitted.

The recording device chosen was a DL1B magnetic tape cassette data logger (Digitronix, Milton Keynes, Great Britain). This will record up to 32 bits of parallel data, *i.e.*, 8 four-bit words[2]. Words 0 and 4 do not give rise to numeric characters on replay[3], and were therefore used for the beginning of record character and the sign. They were connected to produce * and + or − when extended to 8 bit data on normal replay. Of the remaining six words, word 1 was used to record the automatically selected range, and words 2, 3, 4, 6 and 7 the displayed voltage. The recorded data were thus of the form *RDD ± DDD where R is the range and D are the digits displayed on the digital voltmeter. The print command output of the digital voltmeter was connected to the data-true input on the data logger to complete the logging sequence initiated by the pulse from the timer.

When the data from more than one chromatogram are recorded on one cassette some means of including an end-of-run signal in the logged data is required. Although all the available words of the logger are used for each data point, the value of R never exceeds 2 because the maximum output of the ionization amplifier is approximately 10 V. Thus only the first two bits of word 1 need be used (1 and 2 of the 1248 BCD). Thus by providing a facility for changing the third bit from logic 0 to logic 1 by means of a switch mounted in the timer unit, R can be made equal to or greater than 4 whenever an end-of-run signal is required. The interconnections between the three units comprising the data logging system are shown in Fig. 2.

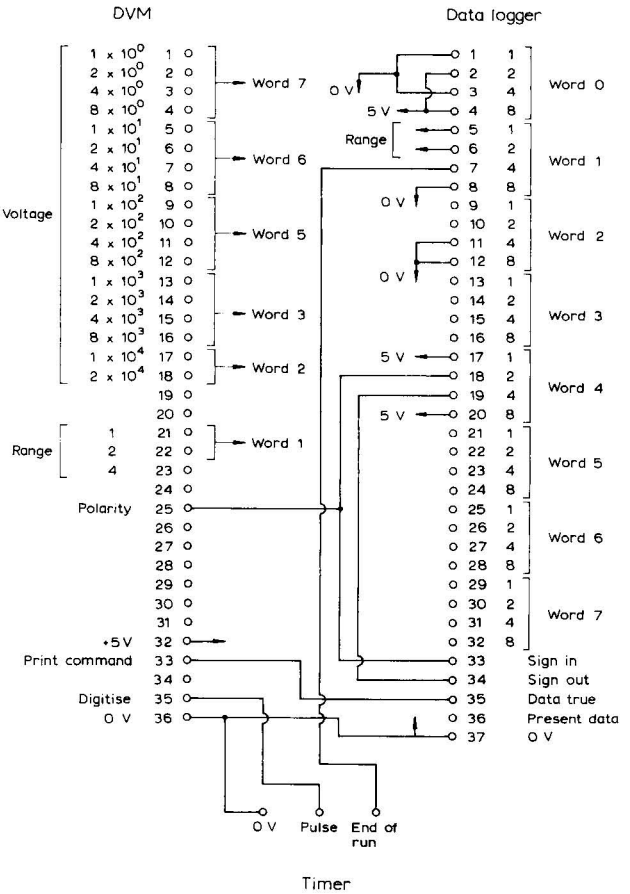

Fig. 2. Interconnections between timer, digital voltmeter (DVM) and data logger.

The data were transcribed to paper tape using the modified replay unit described previously[1]. During the process the * is changed into a new-line character to make the format of the logged data compatible with the computer we are using. This transcription will no longer be necessary when cassette replay facilities become available at the computer.

THE PROGRAM

The program is a modified version of an existing general-purpose integration program[4]. The following major changes were made:

(*a*) The READ and associated statements were changed to take account of the unusual data format dictated by the hardware.

(*b*) The end-of-run signal was taken as two consecutive range values equal to or greater than 4. After the output of results from one run the integration of the next run was commenced, and this process was continued until the raw data file was exhausted (END = label in all READ statements).

(*c*) The first peak with maximum greater than 4 V after the start of a run was assumed to be the solvent peak and all retention times were calculated from the start of this peak. Also the area of this peak was not taken into account in percentage calculations. The value of the total peak area excluding the solvent peak was output. This could prove useful by providing a measure of the total weight of a heterogenous mixture injected, *e.g.*, total fatty acids from a lipid sample and hence a measure of the weight of lipid.

(*d*) An error trap routine was incorporated to prevent the program from being halted if a data error occurs[5]. The subroutine called by the error trap assigns values of zero to the range and voltage reading. This data point will subsequently be rejected by the program as a noise spike if it does not relate to the values of the adjacent data points.

DISCUSSION AND CONCLUSIONS

The system described has all the advantages of the data logging system for amino acid analyses described previously[1], namely low price, compactness, and silent operation. Data recorded on cassettes may be conveniently handled as input into a number of programmable calculators for analysis. The digital voltmeter used can measure wide ranges of current and resistance, in addition to voltage and this makes the system suitable for logging data from a wide range of instruments.

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CHROM. 8264

Note

The use of Sephadex LH-20 to separate dodecyl sulphate and buffer salts from denatured proteins

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Chromatography or electrophoresis under denaturing conditions (*e.g.*, in the presence of sodium dodecyl sulphate, urea or guanidine hydrochloride) is increasingly being used to separate complex mixtures of proteins. However, dodecyl sulphate is reported to interfere with the action of trypsin[1], and urea interferes with amino acid analysis. Dodecyl sulphate may be removed by prolonged dialysis[1,2] and urea by gel filtration in 50% acetic acid using Sephadex G-25[3]. However, dialysis requires several days, and the gel filtration is not applicable to proteins insoluble in 50% acetic acid. Anion exchange resins may be used to separate dodecyl sulphate from denatured proteins, but recovery of protein is poor on occasions[4], or requires the presence of 6 *M* urea which then must be removed[5]; moreover, these resins fail to remove the cationic components of buffer salts.

This paper describes a general procedure using Sephadex LH-20 for separating buffer salts (including dodecyl sulphate and non-covalently bound protein stains) from denatured proteins by gel filtration in a volatile solvent.

MATERIALS AND METHODS

Formic acid (AR, 98–100%) was obtained from Fisons (Loughborough, Great Britain) and L-leucine from Roche (Welwyn Garden City, Great Britain). Other reagents (which were, with the exception of β-mercaptoethanol, AR grade) were obtained from British Drug Houses, Poole, Great Britain. Radioisotopes were obtained from the Radiochemical Centre, Amersham, Great Britain. Salt-free cytochrome *c* (horse-heart) was obtained from Boehringer (Mannheim, G.F.R.) and calf serum from Biocult Labs. (Glasgow, Great Britain).

Denaturation of protein

To remove low-molecular-weight material absorbing at 280 nm, calf serum (5 ml) was dialyzed with one change against 500 ml phosphate-buffered saline (Dulbecco's A[11] containing 0.05 % NaN_3) for two days, followed by 1000 ml 0.05% NaN_3

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for one day, at 22° after which it was lyophilized. Of this, 20 mg was mixed with 1.5 mg cytochrome *c*, 30 mg sodium dodecyl sulphate, and 2 ml distilled water; an aliquot of sodium dodecyl [^{35}S]sulphate was added, and the protein solution held at 100° for two min. A sample was removed for analysis, and the remainder dialyzed for 24 h at 37° against 5 l of toluene-saturated distilled water containing 0.5 ml β-mercaptoethanol, followed by 500 ml of buffer (3 mg/ml L-leucine and 3 mg/ml Na_2HPO_4, pH 8.3) for 16 h at 37°. To this dialyzed material (after samples were taken for analysis) aliquots of L-leucine-4,5-[^{3}H], [^{32}P]-phosphate and dodecyl [^{35}S]-sulphate were added, together with 5 mg sodium dodecyl sulphate. The mixture was lyophilized and dissolved in 1.5 ml 70% (v/v) formic acid.

Chromatography

Gel filtration was effected at 4° in 70% (v/v) formic acid on a column of Sephadex LH-20 (39 × 1.6 cm) using a solvent flow-rate of 12 ml/h. The column effluent was monitored with an LKB Uvicord operating at 280 nm. Fractions were assayed for absorbance at 394 nm, and for radioactivity.

Radioassay

Samples (0.05 ml) were mixed with 15 ml of scintillant consisting of 3.5 vols. Triton X-100 plus 6.5 vols. of toluene containing 7 g PPO and 0.3 g POPOP per litre. Counts were not corrected for quenching.

RESULTS

It is evident (Table I) that 99% of the dodecyl sulphate was removed by the dialysis procedure described above. However, most of the cytochrome *c* was also lost during the dialysis. Thus, although dodecyl sulphate may be removed by dialysis, the procedure is protracted and may be accompanied by loss of low-molecular-weight proteins.

In contrast, the gel filtration procedure effectively separates both the serum proteins and the cytochrome *c* from the buffer salts (Fig. 1). The protein eluted from

TABLE I

REMOVAL OF DODECYL[^{35}S]SULPHATE FROM DENATURED SERUM AND CYTOCHROME *c* BY DIALYSIS, AND BY GEL FILTRATION ON SEPHADEX LH-20

N.T. = not tested; N.S. = not significant.

Condition	*Optical density*			*Radioactivity (cpm)*
	Wavelength			
	280 nm	*409 nm*	*394 nm*	
Before dialysis	18.2	11.7	N.T.	292,000
After dialysis	17.6	1.5	N.T.	3,200
Recovery	96.7%	12.8%	–	1.1%
Applied to Sephadex column	17.5	N.T.	1.97	198,000
Recovered from Sephadex column (Fractions 13–17 incl., see Fig. 1)	17.3	N.T.	1.985	N.S.
Recovery	99.0%	–	100.8%	0%

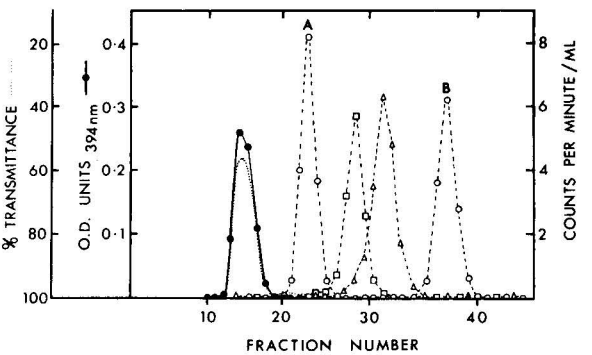

Fig. 1. The use of Sephadex LH-20 to separate protein from buffer salts by chromatography in 70% (v/v) formic acid. The transmission of the column effluent was monitored at 280 nm (···) and the optical density of fractions measured at 394 nm (●—●). Isotopes used were ^{35}S (as sodium dodecyl sulphate, ○---○, c.p.m./ml $\times 10^{-3.3}$), ^{32}P (as phosphate, □---□, c.p.m./ml $\times 10^{-3}$) and ^{3}H (as L-leucine-4,5-^{3}H, △---△, c.p.m./ml $\times 10^{-5}$).

the column in five fractions (13–17 inclusive) within five hours. None of the phosphate and dodecyl sulphate anions and the leucine zwitterion eluted with these fractions. Recovery of the protein and haem was essentially quantitative as measured by absorption (see Table I), protein recovery on a dry weight basis being 98%. The radioisotope ^{35}S eluted as two peaks. In other experiments free sulphate was found to elute in the position of peak A and [^{14}C]-lauryl alcohol in the position of peak B. As solutions of dodecyl sulphate hydrolyze on storage[6] it is likely that the sodium dodecyl [^{35}S]-sulphate used here was partially hydrolyzed.

It is clear from these results that a complex mixture of denatured proteins may be separated from low-molecular-weight compounds such as leucine, dodecyl sulphate and phosphate. In addition to these compounds, it was possible to separate proteins from glucose, fucose, lauryl alcohol, uridine, UTP, GTP, mixed amino acids, iodide, sulphate, phenol red, calcium, glucosamine and Coomassie Brilliant Blue R250.

DISCUSSION

Anhydrous formic acid is a satisfactory solvent for many proteins[7]. However, attempts to use formic acid alone as the solvent for the gel filtration procedure as described failed, as both phenol red and mixed amino acids eluted over a broad band which encompassed the protein peak; this did not occur when 70% formic acid was used. In addition to improving the solvating power of the formic acid, the presence of water prevents the formic acid freezing at 4°.

In the course of many hours at room temperature, anhydrous formic acid will formylate proteins[8]. However, proteins are routinely exposed for hours to 70% aqueous formic acid at room temperature during the cyanogen bromide cleavage procedure[9] and to formic acid buffer at pH 1.9 during peptide mapping[2], and exposure of proteins to 70% formic acid for a few hours at 4° is unlikely to alter them chemically.

Proteins may be recovered from the column effluent by precipitating them with diethyl ether or by removing the solvent by lyophilization. However, proteins recovered in these ways, although readily soluble in 6 *N* HCl, are generally insoluble

in aqueous solution near neutrality and are thus digested with difficulty by trypsin. This problem may be overcome by removing the formic acid by dialysis against water followed by 0.5% ammonium bicarbonate. This frequently leaves proteins as a fine suspension which is rapidly digested by trypsin[10].

Protein may be eluted from stained acrylamide gels stored dry by soaking the latter in 70% formic acid. This protein is available for further analysis after removal of stain by the procedure described in this work. Although not tested, it is probable that other small molecules, such as urea, guanidine hydrochloride and iodoacetamide may be separated from denatured protein by the method described here, which seems to have the advantage over other desalting procedures in being quite rapid, and of general application.

ACKNOWLEDGEMENTS

I wish to thank Dr. Gordon Koch for helpful discussions and a gift of sodium dodecyl [^{35}S]sulphate, Dr. R. D. Barry for advice and encouragement, and the Wellcome Trust for generous financial support.

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CHROM. 8277

Note

Esterification and etherification by silver oxide–organic halide reaction gas chromatography

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Fatty acids are usually esterified prior to their separation by gas chromatography (GC) because of difficulties associated with adsorption of the acids on the chromatographic support[1]. Also, GC separation of esters can be performed on liquid phases of a wider range than can be used for the separation of the free acids. Long-chain fatty acids are usually converted to their methyl esters[1] while the corresponding short-chain acids may be esterified with propanol[2] or other higher alcohols[3–7].

As part of a programme on mutton flavour research[8], we had the problem of obtaining the chemical identity of the unusual medium-chain (C_6–C_{12}) volatile fatty acids of mutton fat after GC separation and sensory evaluation. A method was required to isolate the individual compounds, convert them into methyl esters and re-chromatograph the esters for characterisation by GC–mass spectrometry (MS). Because of the submicrogram quantities involved, an "on-column" technique in which the operations of trapping, esterification and introduction for re-chromatography could be combined in the same apparatus was mandatory.

The reactions of methyl iodide with silver salts of acids and of methyl iodide–silver oxide with alcohols are used as standard methods for the preparation of methyl esters and methyl ethers, respectively[9,10]. Based on these reactions, the present paper describes a technique in which a silver oxide-packed tube was used for trapping fatty acids after their separation by GC and methylation was carried out *in situ* by subsequent introduction of methyl iodide. The tube was then used for introduction of the sample into a second gas chromatograph or into a GC–MS system. The same tube could also be used for "on-column" methylation of the original mixture of acids, with subsequent analysis of the ester derivatives by GC. The effect of the silver oxide–methyl iodide combination on certain hydroxy compounds was also studied.

EXPERIMENTAL

Methyl iodide was distilled in a fume cupboard before use (the reagent is carcinogenic[11]). Silver oxide was deposited by precipitation from silver nitrate and an excess of sodium hydroxide onto 40–60 mesh Celite to give 10% (w/w) of the oxide. The coated material was washed with distilled water until the washings were neutral, and dried overnight at 100°.

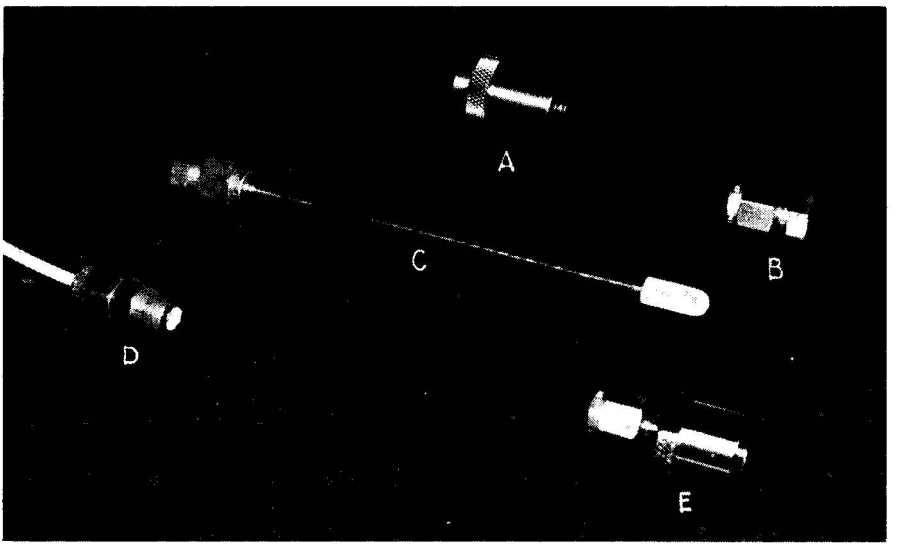

Fig. 1. Modified septum holder and associated equipment for transfer of samples from traps onto a GC column for the Hewlett-Packard chromatograph. A = Original septum holder; B = Swagelok cap and nut with PTFE ferrules ($\frac{1}{8}$ in.); C = trap with Swagelok cap ($\frac{1}{8}$ in., metal ferrules) and partially drilled PTFE rod (for storage of samples) D = external gas supply; E = modified septum holder with Swagelok nut ($\frac{1}{8}$ in.).

The traps were constructed of 150 × 3.2 mm O.D. stainless-steel tubes packed 25 mm of their length at the collection end with the silver oxide reagent. The other end of the tube was fitted with an $\frac{1}{8}$-in. Swagelok cap and ferrules (Fig. 1C). Before use, the packed traps were heated to 200° for 1 h with a slow passage of nitrogen.

Steam volatile fatty acids were isolated from mutton fat as described previously[8]. The acids were separated on a 2.5 m × 3.2 mm O.D. stainless-steel column containing 10% (w/w) stabilised polyethylene glycol adipate (EGA; Analabs, North Haven, Conn., U.S.A.) on 100–120 mesh Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.) in a Hewlett-Packard Model 7620A gas chromatograph. The exit of the column was attached to a 5:1 splitter connected to a heated outlet (five parts) maintained at 200°. The acids were collected at room temperature from the gas chromatograph in the silver oxide-packed tubes so that the acids condensed on the oxide.

Esterification of trapped acids was accomplished by injecting 1 μl methyl iodide into the silver oxide reagent followed by heating the reagent-filled portion on a hot-plate at 100° for 2 min after the trap had been sealed at both ends by Swagelok caps. For the "on-column" methylation of a mixture of acids in solution, an aliquot of the solution was injected onto the reagent, followed by the methyl iodide. A new, freshly prepared trap was required for each sample because no reaction took place when a trap was used for a second collection–esterification.

The esterified contents of the traps, after cooling to dry-ice temperature, were injected onto a GC column in the Hewlett-Packard instrument through a modified septum retainer having a Swagelok thread attached (Fig. 1E). The holder was drilled to allow the trap to pass through it into a wide-bore column insert liner (that is normally used with the pyrolysis or solid sample injection units) in the injection port (at

200°) of the gas chromatograph. The trap was held in place by an $\frac{1}{8}$-in. Swagelok nut with PTFE ferrules on the holder. An external gas supply (*ca.* 20 lbs./sq.in., to yield a flow-rate of 45 ml/min through the column at room temperature) was connected to the trap to flush its contents onto the column, which was cooled 5–10 cm of its length at the injection port with dry ice. During connection of the trap to the gas chromatograph, the external gas supply was regulated to give a flow-rate of 10 ml/min through the cooled trap. Absence of this gas flow resulted in loss of sample, probably by blow-back into the cold part of the external gas line. Normal flow-rate of carrier gas through the column during the transfer of sample was reduced to 10 ml/min. The transfer of sample was allowed to proceed for 2.5 min, after which the normal carrier gas flow was established, the external gas supply was shut off and the oven of the gas chromatograph was rapidly heated to 50° for initiation of the temperature programme. Chromatography of the esters was carried out on the EGA column, or on a similar column containing silicone OV-101, with a standard temperature programme of 50°–220° at 2°/min. Similar equipment and procedures were used for transfer of samples to a Pye 104 gas chromatograph coupled by a silicone elastomer membrane interface to an AEI MS-30 mass spectrometer. Helium (40 ml/min) was used as the carrier gas for the mass spectral runs and nitrogen (50 ml/min) for all others.

RESULTS

Typical gas chromatograms of a mixture of standard *n*-fatty acids and of the methyl esters formed from them by the silver oxide–methyl iodide method are shown in Fig. 2.

The reaction appeared to go to completion under the described conditions,

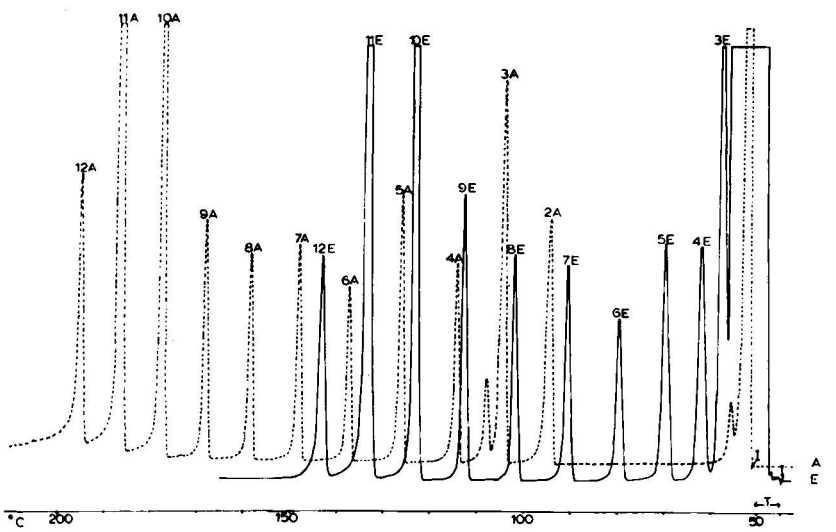

Fig. 2. Composite gas chromatograms on the EGA column of standard normal fatty acids (A) and their methyl esters (E) formed by the silver oxide–methyl iodide esterification of the acids. Peak numbers refer to the chain length of the acids. T = Transfer period; I = injection point.

acids were not apparent in the ester chromatograms and the relative peak sizes within the chromatograms were similar. Also, no degradation was apparent since chromatograms of esterified single acids did not contain peaks of lower homologues. The first peak to appear on the "solvent" tail on either the polar or the non-polar column was methyl propionate or methyl butyrate. Methyl formate (if it was formed) and methyl acetate were "lost" under the "solvent" peak. This "solvent" peak could be reduced by passing carrier gas through the trap at 10 ml/min at dry-ice temperatures for up to 1 min, but various amounts of the low-molecular-weight esters up to methyl caproate were lost at the same time.

An example of the use of the technique for the collection–esterification–rechromatography of fatty acids from the mutton flavour study is shown in Figs. 3 and 4. Acids of sensory interest, corresponding to small areas of the chromatogram on EGA (numbered in Fig. 3), were collected on the silver oxide reagent, esterified and introduced into the GC–MS system. Tracings of the esters from the GC detector and the total ion monitor of the mass spectrometer closely followed each other (Fig. 4).

The effect of the silver oxide–methyl iodide reagent on a limited number of hydroxy compounds was studied. Octanol was converted by the reagent to methyl octyl ether and 2-octanol was similarly converted to 2-methoxyoctane without formation of the corresponding fatty acid methyl ester, aldehyde or ketone. Identity of the methyl esters was confirmed by their mass spectral patterns. Both β-hydroxybutyric acid and methyl β-hydroxybutyrates were converted to methyl β-methoxybutyrate by the reagent, with a trace of methyl crotonate produced by dehydration of the hydroxy compounds (Table I). Butyrolactone was converted to methyl γ-methoxybutyrate by the reagent.

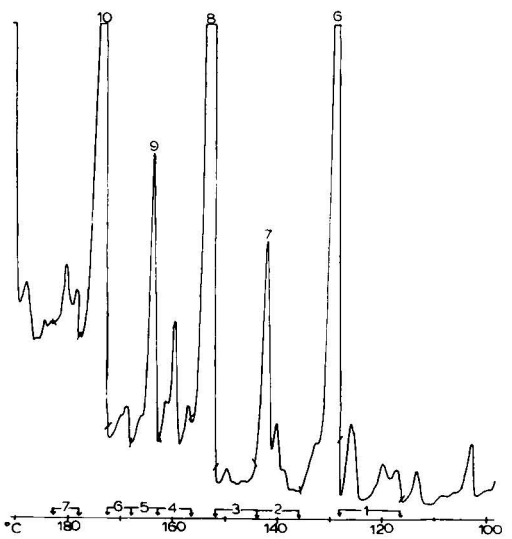

Fig. 3. Partial gas chromatogram on the EGA column of steam volatile fatty acids from a mutton mince cook-up showing areas collected in silver oxide traps. Peak numbers refer to the chain-lengths of the normal fatty acids.

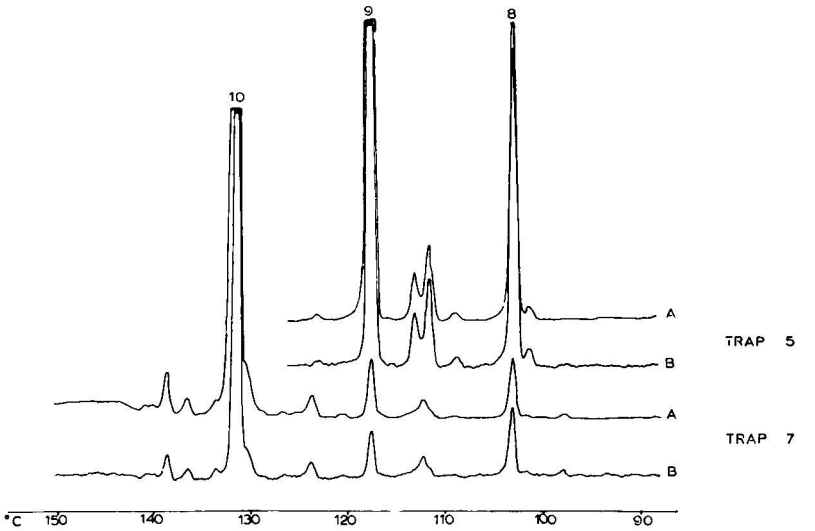

Fig. 4. Partial gas chromatograms on the OV-101 column of methyl esters from acids in traps 5 and 7 (Fig. 3). (A) Trace from total ion monitor of the mass spectrometer. (B) Trace from the flame ionisation detector of the gas chromatograph. Peak numbers refer to the chain-lengths of the normal fatty acids.

TABLE I

GC AND MS DATA FOR HYDROXYBUTYRATE DERIVATIVES AND THEIR Ag_2O/CH_3I REACTION PRODUCTS

Compound	*ECL (on EGA)*[16]	*Major MS peaks (relative intensities in parentheses)*
Butyrolactone	10.6	86 (53), 67 (38), 55 (67), 53 (100)
Methyl γ-methoxybutyrate	7.5	117 (4), 101 (54), 87 (5), 85 (7), 74 (100)
Methyl β-hydroxybutyrate	8.7	117 (0.5), 103 (8), 100 (4), 87 (9), 85 (5), 75 (2), 74 (32)*, 45 (44), 43 (100)
Methyl β-methoxybutyrate	6.8	131 (0.2), 117 (6), 102 (7), 101 (6), 87 (3), 85 (6), 75 (23)*, 74 (0.9), 59 (100)
Methyl crotonate	5.2	100 (90), 85 (100), 59 (32)

* Ref. 17.

DISCUSSION

GC separation and structural analysis of fatty acids by mass spectrometry is facilitated by conversion of the acids into the corresponding esters[1,12]. Acids are poorly transmitted (compared with the esters) through the elastomer interface of the GC–MS system. Extensive MS data have been recorded on methyl esters of fatty acids[12]. Reaction of methyl iodide with silver salts of fatty acids has been recommended for samples containing a wide range of acids, *e.g.* milk fats[13]. This method, suitably modified, proved useful here for the derivatisation of acids collected from the GC column and for other small quantities of acids or alcohols. The reagent, when used under the conditions described, did not appear to affect the basic structure of normal,

branched-chain and unsaturated fatty acids, but opening of a γ-lactone ring to yield a methoxy ester was shown to take place. Dehydration of hydroxy compounds, where the result was a conjugated system of double bonds, also took place to a limited extent.

The apparatus and procedures described in this work can be recommended as a general and useful technique for the "on-line" esterification and etherification of sub-microgram quantities of fatty acids and hydroxy compounds as a prelude to their GC analysis. The use of the system for both trapping and sample introduction, as illustrated by the mutton flavour work, should find other applications in situations where trace components in complex mixtures of these compounds need to be concentrated and further separated prior to their characterisation by GC or GC–MS means. Similar types of apparatus to that described here have been developed for small-scale hydrogenation[14,15] and ozonolyses[15].

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Note

Gibberelline

XXXIV. Mitt.* Beitrag zur Gaschromatographie von Gibberellinen und Gibberellin-O-glucosiden—N,O-Bis(trimethylsilyl)acetamid als Silylierungsreagens

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(Eingegangen am 18. Februar 1975)

Für gaschromatographische Untersuchungen von Gibberellinen (GA), die mehrfach beschrieben worden sind[2,3], werden überwiegend zwei Derivate eingesetzt: (1) die Methylester (GA-Me), die aus den freien Gibberellinen mit Diazomethan leicht darstellbar sind[4,5] und (2) die Trimethylsilyläther der Methylester (TMS-GA-Me), die aus den Methylester mit Hexamethyldisilazan (HMDS) und Trimethylchlorsilan (TMClS) zu gewinnen sind[6]. Die Herstellung der TMS-GA-Me aus den freien Gibberellinen erfordert somit eine Zweistufenreaktion, bietet aber bei Vorhandensein freier Hydroxylgruppen die Möglichkeit, von Gibberellinen nacheinander die GA-Me und die TMS-GA-Me gaschromatographisch untersuchen zu können.

Für unser Problem, nach chemisch-präparativer Entmethylierung von GA-Me nach Bartlett und Johnson[7] neben freier Säure Reste von Methylestern gaschromatographisch nachzuweisen[8], sind weder die GA-Me noch die TMS-GA-Me geeignet. Deshalb versuchten wir, die erstmals von Davis *et al.*[9] für Phytohormone (Kinetin, Auxin und GA_3) angewendete Silylierungsmethode mit N,O-Bis(trimethylsilyl)acetamid (BSA) zu standardisieren. Da BSA in der Lage ist, sowohl Hydroxylgruppen als auch Carboxylgruppen zu silylieren, sind aus einem Gemisch von GA und GA-Me die Trimethylsilyläther der Gibberellin(trimethylsilylester) (TMS-GA-TMS) und die Trimethylsilyläther der Gibberellinmethylester (TMS-GA-Me) zu erwarten. Für deren gaschromatographische Trennung waren geeignete Bedingungen auszuarbeiten.

Weiterhin sollte die Anwendbarkeit der "BSA-Silylierung" für GA-O-glucoside[10,11] geprüft werden, da deren Umsetzung zu den Methylestern oftmals unvollständig ist[8].

EXPERIMENTELLES

Silylierung

Für die Silylierung benutzten wir BSA bzw. ein Gemisch von BSA mit 5–10% TMClS. Die getrockneten Proben der Gibberelline, GA-Me bzw. GA-O-glucoside

* XXXIII. Mitt., s. Lit. 1.

(0.1–1.0 mg) wurden entweder direkt mit BSA oder BSA + 10% TMCIS (200 µl) bzw. nach Lösen in Acetonitril, Benzol oder wenig Pyridin bei Raumtemperatur unter Feuchtigkeitsausschluss umgesetzt. Von diesem Reaktionsgemisch war 1 µl für eine Analyse ausreichend.

Säulenfüllung

Es wurden ausschliesslich silanisierte Glassäulen benutzt, die bei 4 mm Innendurchmesser eine Länge von 1.5 m aufwiesen. Als stationäre Phasen wurden 3% QF-1 bzw. 2% SE-33 an Gas-Chrom Q (125–160 µm) eingesetzt.

Analysenbedingungen

Die Analysen wurden mit einem Pye-Gerät Modell "Panchromatograph" mit Flammenionisationsdetektor durchgeführt. Die Ofentemperatur (Einlass, Säule und Detektor) betrug für die GA-Derivate bei isothermem Betrieb für SE-33 210° bei 165 ml Stickstoff/min und für QF-1 195° bei 150 ml Stickstoff/min. Die Derivate der GA-O-glucoside wurden an QF-1 isotherm bei 245° und 175 ml Stickstoff/min chromatographiert; für die Trennung von TMS-GA- und TMS-(GA-O-glucosid)-Derivaten war ein Temperaturprogramm von 195–245° bei 4°/min Heizrate und 140 ml Stickstoff/min (205°) geeignet.

TABELLE I

RETENTIONSZEITEN (t_r), RELATIVE RETENTIONEN (r) UND TRENNSTUFENZAHL (n) DER TMS-DERIVATE VON GIBBERELLINEN, GIBBERELLINMETHYLESTERN UND VERTRETERN WEITERER PHYTOHORMONGRUPPEN

Analysenbedingungen, s. Experimentelles.

TMS-Derivat von	*2% SE-33*						*3% QF-1*					
	Freien Säuren (TMS-GA-TMS)			*Methylestern (TMS-GA-Me)*			*Freien Säuren (TMS-GA-TMS)*			*Methylestern (TMS-GA-Me)*		
	t_r	r	n	t_r	r	n	t_r	r	n	t_r	r	n
GA_1	29.5	7.8	870	23.0	6.1	900	14.6	3.5	680	13.4	3.2	860
GA_2	40.5	10.7	890	28.5	7.6	810	22.5	5.4	960	18.8	4.5	940
GA_3	27.0	7.1	480	22.5	5.9	680	13.8	3.3	460	12.1	2.9	620
GA_4	19.0	5.0	980	14.0	3.7	1100	10.9	2.6	800	9.6	2.3	1080
GA_5	16.7	4.4	1080	12.9	3.4	1080	10.4	2.5	800	9.2	2.2	950
GA_6	22.5	5.9	880	17.8	4.7	1080	17.2	4.1	810	16.3	3.9	1020
GA_7	18.2	4.8	670	14.0	3.7	990	12.1	2.9	820	11.3	2.7	800
GA_8	46.5	12.3	850	35.0	9.4	810	20.0	4.8	720	16.7	4.0	870
GA_9	10.6	2.8	940	8.0	2.1	1320	7.1	1.7	910	6.3	1.5	930
GA_{13}	29.0	7.7	970	21.1	5.3	1030	4.2	1.0	670	5.0	1.2	780
5α-Cholestan	34.0	8.9	1200	34.0	8.9	1200	4.2	1.0	700	4.2	1.0	800
Stearinsäure-methylester	3.8	1.0	1400	3.8	1.0	1430	—	—	—	—	—	—
cis–trans-Abscisinsäure	4.9	1.3	1000	4.2	1.1	940	1.2	0.3	670	2.9	0.7	800
3-Indolyl-essigsäure	1.9	0.5	960	1.4	0.4	1200	0.8	0.2	470	0.8	0.2	600
6-(Furfuryl-amino)-purin (Kinetin)	5.3	1.4	700	—	—	—	2.1	0.5	670	—	—	—

Als Standardsubstanzen benutzten wir je nach Trennproblem 5α-Cholestan, Stearinsäuremethylester oder Progesteron.

Bei voller Empfindlichkeit des Gerätes ergaben 0.5 μg TMS-GA_4-TMS bzw. 1.0 μg TMS-GA_3-TMS 20% Zeigerausschlag.

ERGEBNISSE UND DISKUSSION

Die durchgeführten Silylierungsversuche zeigten, dass BSA gut geeignet ist, nicht nur Hydroxylgruppen, sondern auch Carboxylgruppen der Gibberelline glatt zu silylieren. Die Geschwindigkeit der unkatalysierten Reaktion ist bei Raumtemperatur gering, kann aber durch Zusatz von 10% TMCIS beschleunigt werden. Eine rasche Reaktion erfolgt, wenn die GA-Probe in Pyridin gelöst mit BSA/10% TMCIS umgesetzt wird. Weitere mögliche Lösungsmittel sind u.a. Acetonitril und Benzol.

Wie aus Tabelle I hervorgeht, ergeben sowohl die TMS-Derivate der freien Gibberelline (TMS-GA-TMS) als auch die der Methylester (TMS-GA-Me) an QF-1 und SE-33 distinkte und scharfe Peaks. Die gefundenen Retentionen der TMS-GA-Me stimmen mit Ausnahme von TMS-GA_3-Me gut mit den Literaturwerten[6] überein. Der Peak von TMS-GA_3-Me erscheint unter unseren Standardbedingungen mit geringerer Retention als in der Literatur[6] beschrieben. Einen zusätzlichen Peak, der mit dem Literaturwert der Retention für TMS-GA_3-Me übereinstimmt, finden wir, wenn bei der Silylierung extreme Bedingungen, wie z.B. GA_3-Me-Überschuss bzw. Silylieren ohne Pyridin angewendet werden oder die Chromatographie in Stahlsäulen durchgeführt wird. Dieser Befund sowie die verringerte Trennstufenzahl *n* für TMS-GA_3-Derivate (vgl. Tabelle I) deuten auf Zersetzung oder Umlagerung unter harten Analysenbedingungen hin. Da bei GA_7 ähnliche Tendenzen zu beobachten sind, dürfte die besondere Struktur des Ringes A beider Gibberelline dafür verantwortlich sein.

Ein Vergleich der Retentionen zeigt, dass mit Ausnahme von GA_{13} die TMS-GA-TMS-Derivate regelmässig höhere Werte besitzen als die entsprechenden TMS-GA-Me-Derivate. Der Nachweis und die Trennung von Methylester und freier Säure

TABELLE II

RETENTIONSZEITEN (t_r) UND RELATIVE RETENTIONEN (*r*) DER TMS-DERIVATE VON GIBBERELLIN-O-GLUCOSIDEN UND DEREN METHYLESTERN AN 3% QF-1 UNTER ISOTHERMEN BEDINGUNGEN

Analysenbedingungen, s. Experimentelles.

TMS-Derivat von	*Freien Säuren (TMS-GA-glucosid-TMS)*		*Methylestern (TMS-GA-glucosid-Me)*	
	t_r *(min)*	*r*	t_r *(min)*	*r*
GA_1-O(3)-glucosid	15.9	2.49	15.6	2.45
GA_1-O(13)-glucosid	15.4	2.41	15.1	2.37
GA_3-O(3)-glucosid	12.8	2.01	14.0	2.20
GA_3-O(13)-glucosid	10.0	1.57	11.3	1.76
GA_8-O(2)-glucosid	12.1	1.90	12.8	2.00
Allogibberinsäure-O(13)-glucosid	3.25	0.51	3.20	0.50
Progesteron	6.4	1.00	1.00	6.4
5α-Cholestan	0.9	0.14		

gelingen an SE-33. An QF-1 sind die Unterschiede der Retentionen beider Derivate relativ gering.

Tabelle II enthält die Ergebnisse der Gaschromatographie von TMS-Derivaten nativer und synthetisierter GA-O-glucoside, sowie ihrer Methylester an QF-1. Bei diesen GA-Konjugaten konnte im Gegensatz zu den freien Gibberellinen kein einheitlicher Einfluss der Methylestergruppe auf die Retention festgestellt werden. Bemerkenswert ist der analytisch wichtige Unterschied zwischen den Retentionen der TMS-Derivate von GA_3-O(3)-glucosid und dem neu synthetisierten GA_3-O(13)-glucosid bzw. ihren Methylestern[11].

Eine Charakterisierung von GA-O-glucosiden und den entsprechenden Agluca in einem Chromatogramm gelingt, wenn man dem sehr unterschiedlichen gaschromatographischen Verhalten ihrer TMS-Derivate durch ein Temperaturprogramm Rechnung trägt (Tabelle III).

TABELLE III

RETENTIONSZEITEN (t_r) UND RELATIVE RETENTIONEN (r) DER TMS-DERIVATE VON GIBBERELLIN-O-GLUCOSIDEN AN 3% QF-1 UNTER PROGRAMMIERTEN TEMPERATURBEDINGUNGEN (195–250°) MIT 4°/MIN HEIZRATE

Analysenbedingungen, s. Experimentelles.

TMS-Derivat von	t_r *(min)*	r
GA_1	10.0	1.90
GA_3	10.0	1.90
GA_8	10.4	1.95
GA_1-O(3)-glucosid	22.7	4.25
GA_3-O(3)-glucosid	21.5	4.04
GA_8-O(2)-glucosid	20.7	3.90
5α-Cholestan	5.3	1.00

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Note

Liquid chromatographic method for the determination of phthalate esters

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Phthalate esters are probably one of the most common plasticizers employed in the plastics industry. They are added frequently to paints, lacquers and plastic materials to reduce rigidity and impart flexibility to solids. Particular attention has been paid recently to their role as plasticizers in plastics used for packaging food and storing blood. It has been reported that in some of these applications, phthalates have found their way into the human body by the leaching of plastic materials, notably polyvinyl chloride (PVC). Jaeger and Rubin[1] have shown that blood packaged in PVC units can, in a few months time, accumulate plasticizer levels of up to 5–7 mg per 100 ml blood. An editorial article in the British Medical Journal[2] has also reviewed the biological effects of phthalate plasticizers. Recently Vessman and Rietz[3] have developed a method to determine nanogram levels of phthalates in plasma and fractionated plasma proteins. The increasingly widespread use of these compounds has resulted in the development of IR, gas chromatographic and, more recently, liquid chromatographic (LC) methods for their determination.

IR methods of analysis are not specific enough to distinguish between closely related members of a phthalate series. Furthermore, these methods cannot be used for the analyses of mixtures and lack the sensitivity necessary for trace analysis[4].

Gas–liquid chromatography has been described by Esposito[5] and also by Krishen[6] in their determination of plasticizers. Temperature-programmed gas chromatography was used after methylation and hydrolysis of some of the plasticizers found in polymeric materials. Vessman and Rietz[3] used an electron capture detector for the trace analysis of phthalates.

Lately, LC has received a great deal of attention in the analysis of relatively high-molecular-weight compounds. This technique, however, cannot easily resolve neighboring members of the homologous series of both high- and low-molecular-weight phthalates without using gradient elution in a reversed-phase system or employing a series of different solvents in a normal phase separation[7].

This note reports a LC procedure for the determination of phthalates found in industrial and biological samples. The method is simple, rapid and does not require gradient elution or solvent change during separation in the analyses of mixtures containing both high- and low-molecular-weight phthalates.

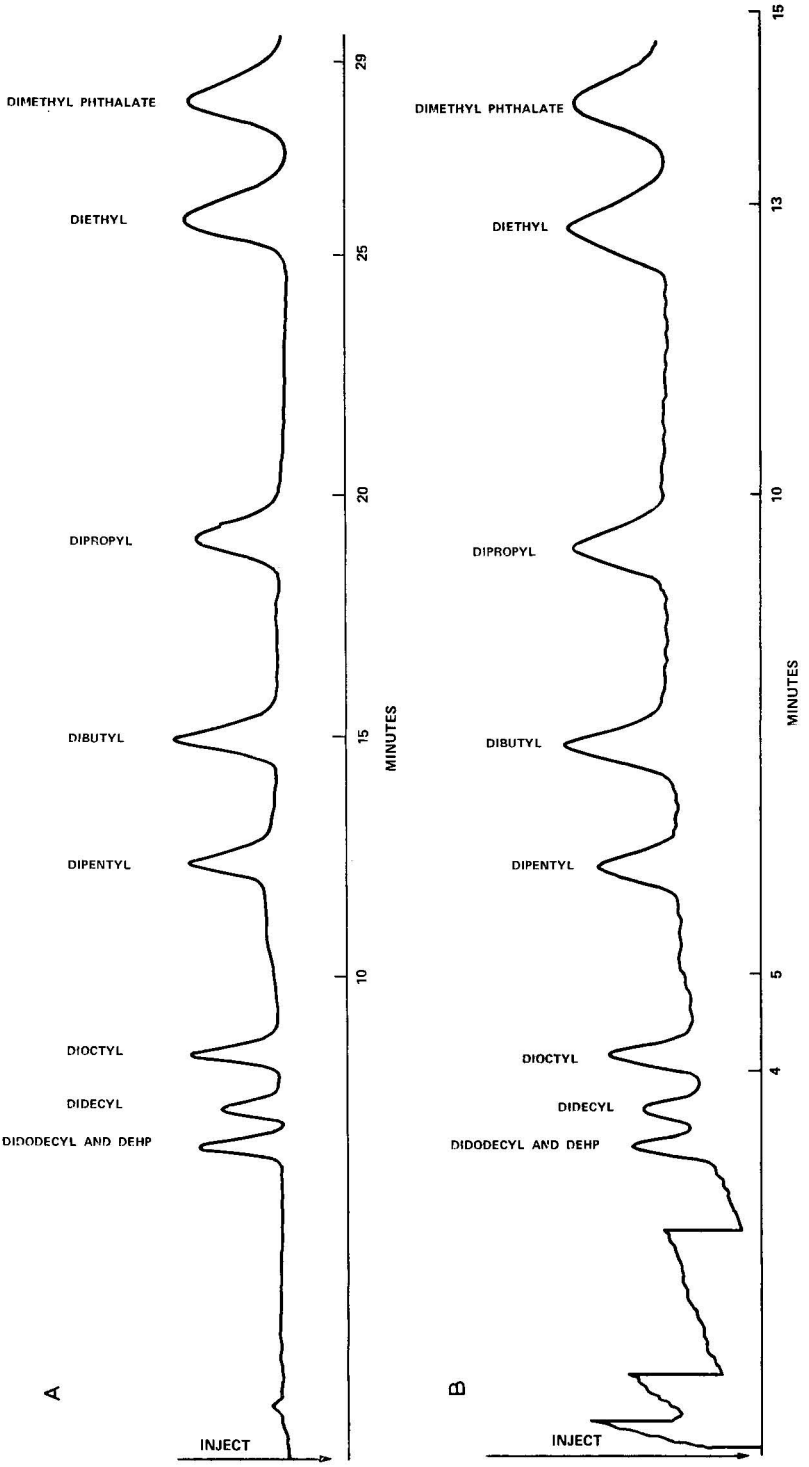

Fig. 1. Typical chromatograms of a mixture of phthalates. Flow-rates: (A) 3 ml/min; (B) 6 ml/min.

EXPERIMENTAL

Apparatus and reagents

A Waters Associates Model 202 liquid chromatograph equipped with a Model 6000 high-pressure pulseless liquid pump and a UV detector absorbing at 254 nm was used. Analyses were carried out on a 1-ft.-long, 10-μm Porasil polar column. Normal phase separation was used with 50% methylene chloride–50% isooctane mobile phase. The solvents were glass-distilled reagents obtained from Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.). A flow-rate of 3 ml/min was maintained with a column pressure up to 1500 lb./sq.in. Reagent-grade phthalates (Eastman-Kodak, Rochester, N.Y., U.S.A., and Fisher Scientific, Pittsburgh, Pa., U.S.A.) were used as standards. Septum injections of 1 μl were made with a high-pressure injection syringe manufactured by Scientific Glass Engineering Ltd. Standards were made up to 0.1% concentration using eluent solvent. Three-millilitre fractions of LC effluent were collected in a 5-ml vial containing 25 mg of KBr. The solvent content of the vial was evaporated using dry nitrogen. The residue consisting of KBr and sample was pressed into a micropellet. Spectra were recorded with a Perkin-Elmer 621 IR spectrophotometer using a Perkin-Elmer Refractive Beam Condenser.

Sample preparation

Lacquers consisting of a cellulose-type polymer (nitrocellulose or ethylcellulose), a low-molecular-weight phthalate and a mixture of solvents were analyzed. Excess methanol was first added to the lacquer solution to precipitate the cellulose material. After centrifugation, the supernatant liquid was separated and its solvent content evaporated. The residue was redissolved in the methylene chloride–isooctane solvent mixture which had been drawn from the reservoir of the liquid chromatograph and brought up to 10-ml volume.

Solid plastics were extracted with methanol at room temperature for several hours. The mixture was centrifuged and the supernatant liquid evaporated with a stream of dry nitrogen. The residue was redissolved in the eluent solvent.

Blood samples of 10 ml were taken from a standard PVC blood packaging unit and extracted with 20 ml of toluene three times. The organic layer was separated by centrifugation and the supernatant toluene layer removed and evaporated to dryness under a dry nitrogen stream. The residue was redissolved in 1 ml of the methylene chloride–isooctane eluent.

The buffer solution present in the blood packaging bags was also examined for phthalate contamination using a similar extraction and analysis procedure.

RESULTS AND DISCUSSION

A typical chromatogram shown in Fig. 1A, was run at a flow-rate of 3 ml/min and required about 30 min to complete. The same separation, using a flow-rate of 6 ml/min, took only 14 min and is shown in Fig. 1B. The solvent system used provides good resolution from C_1–C_{12} without resorting to gradient elution or to the use of a series of solvents. Of the two lacquer solutions analyzed, one was found to contain 3% dimethyl phthalate, the other 1% dibutyl phthalate. These results were also checked by IR analyses and confirmed gravimetrically. The calibration curve constructed

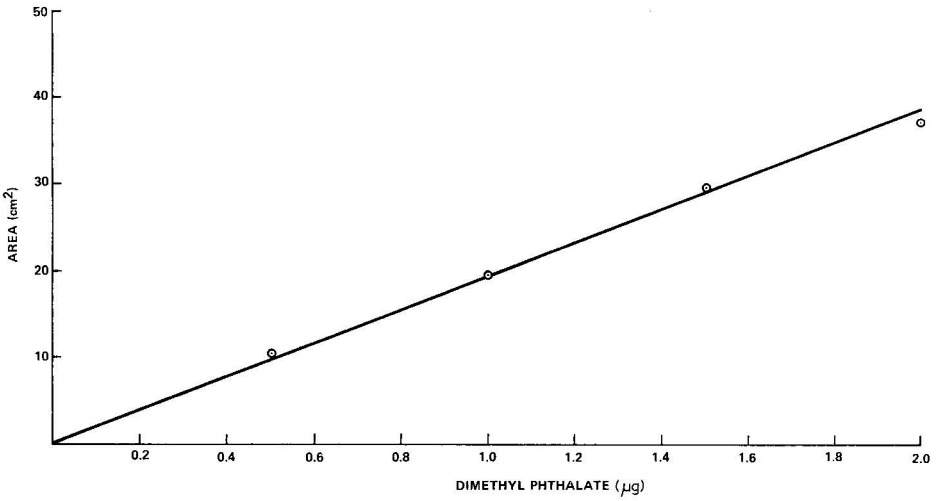

Fig. 2. Calibration curve for the determination of dimethyl phthalate.

for the determination of dimethyl phthalate is shown in Fig. 2. This curve projects a limit of detection of 0.1 μg of dimethyl phthalate. Under the conditions used in this work, the low-molecular-weight phthalates elute last from the column and increasing band broadening is observed. As a result, lowest sensitivity can be expected for low-molecular-weight phthalates (C_1–C_5) while higher-molecular-weight phthalates (C_6–C_{12}) with much shorter retention times will exhibit higher sensitivities, with the limit of detection approaching 0.05 μg of phthalate.

PVC used in the fabrication of a blood packaging unit was analyzed for the qualitative identification of the plasticizer added. Analysis by high resolution LC identified the plasticizer as di-2-ethylhexyl phthalate (DEHP). It was found that since

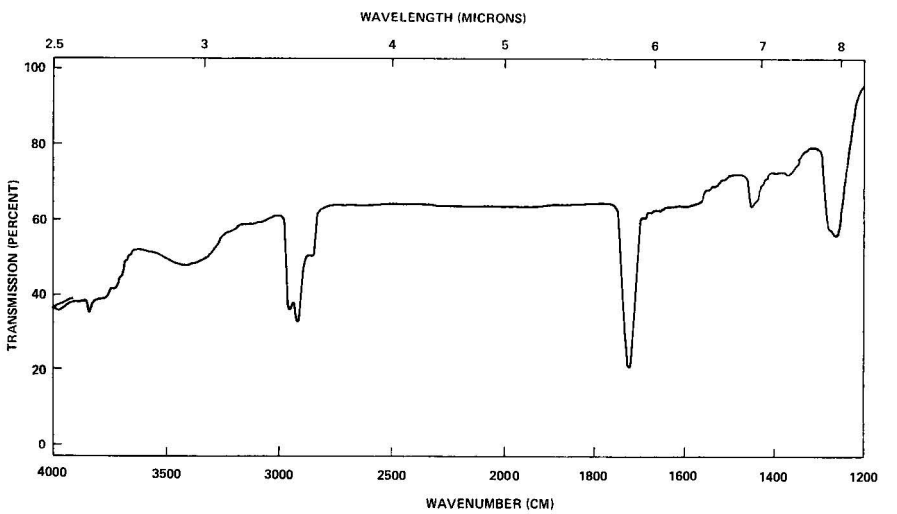

Fig. 3. IR spectrum of an LC fraction (DEHP).

didodecyl phthalate has a retention time close to DEHP, it was necessary to distinguish the plasticizer found in the PVC bag from didodecyl phthalate. This was accomplished by IR spectroscopy.

Eluent fractions from LC were collected and dispersed in KBr. Micropellets were prepared and the spectrum obtained (Fig. 3) showed that the plasticizer was DEHP.

The buffer solution present in the blood packaging unit was analyzed by LC and no phthalate was detected. Blood serum was contacted for 24 h with strips of the plasticized PVC taken from a blood packaging unit. At the end of this period, the test serum was analyzed and found to be contaminated with DEHP. Whole blood was then removed from a packaging unit with a recently expired shelf life (blood in contact with plastic bag for four months) and an extraction performed. Analysis of these samples showed an average of 5 mg of phthalate per 10 ml of whole blood. These results tend to confirm and extend previously reported findings[1,2], namely, the buffer solution does not extract plasticizer from a PVC blood unit, while stored whole blood can become contaminated and accumulate relatively high amounts of phthalate.

In summary, the method described avoids the cumbersome use of gradient elution and variation of types of solvents. It is simple to use and rapid requiring very little sample preparation and instrument time. By using high pressure solvent delivery (*ca.* 3000 lbs./sq. in.), elution times are shortened and analyses can be completed in about 15 min. without a substantial loss of resolution.

ACKNOWLEDGEMENTS

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Note

Säulenchromatographische Trennung von Methyl- und *n*-Octylzinnchloriden

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Über die schichtchromatographische Trennung von Alkylzinnverbindungen ist schon mehrfach berichtet worden[1–5]. Dagegen sind uns säulenchromatographische Verfahren zur Trennung von Alkylzinnchloriden bzw. der Alkylzinnreste solcher Organozinnverbindungen, die sich leicht in die Chloride überführen lassen, aus der Literatur nicht bekannt.

Nachfolgend sollen nun zwei säulenchromatographische Trennsysteme vorgestellt werden, mit deren Hilfe Methylzinn- bzw. *n*-Octylzinnchloride in analytischem und präparativem Massstab aufgetrennt und isoliert werden können.

EXPERIMENTELLER TEIL

Trennung der Methylzinnchloride

Füllen des Chromatographierohres. Das Chromatographierohr hat eine Länge von 50 cm und einen Innendurchmesser von 2.5 cm (z.B. ein verkürztes Whatman-Präzisionschromatographierohr Typ MS-PC 2500); das Adsorbens ist 75 g Kieselgel 60, Korngrösse 0.063–0.2 mm (Merck-Art. 7734); als Elutionsmittel dient 1.0 gew.-%ige ätherische Chlorwasserstofflösung–Petroleumbenzin (Siedebereich 40–60°) (8:2).

Das Adsorbens wird zunächst in 300 ml und danach noch zweimal in je 100 ml Elutionsmittel jeweils 1 min geschüttelt und nach dem Dekantieren der letzten Flüssigkeitsmenge mit weiteren 80 ml Elutionsmittel in das Chromatographierohr übergeführt. Danach wird die Säule mit weiteren 300 ml Elutionsmittel ausgewaschen.

Durchführung der Trennung. 20–250 mg des zu trennenden Gemisches aus Methylzinnchloriden, gelöst in wenig Elutionsmittel oder Diäthyläther, werden auf die Säule gegeben und bei einem Elutionsmitteldurchsatz von *ca.* 1 ml/min eluiert. Das Säuleneluat wird in Fraktionen aufgefangen und dünnschichtchromatographisch überprüft[1,5]. Zur schnelleren Kontrolle der Fraktionen auf Methylzinnchloride genügen das Tüpfeln und die anschliessende Anfärbung nach Literaturverfahren[1,5].

Zur Überprüfung der Methode diente ein Gemisch von ^{14}C-markierten Methylzinnchloriden folgender Zusammensetzung: 4.6 mg (85.47 μCi) Monomethylzinntri-, 18.0 mg (121.10 μCi) Dimethylzinndi- und 0.2 mg (111.38 μCi) Trimethylzinnmonochlorid. Das Eluat wurde in 5- und 10-ml-Fraktionen aufgefangen, die Radioaktivität in den Einzelfraktionen durch Flüssigszintillationsmessung bestimmt

und in Abhängigkeit vom Gesamtelutionsvolumen graphisch dargestellt (vgl. Fig. 1). 96.4% der eingesetzten Radioaktivität wurden im Eluat wiedergefunden.

Trennung eines Gemisches von n-Octylzinnchloriden und Tetra-n-octylzinn

Füllen des Chromatographierohres. Das Chromatographierohr hat eine Länge von 70 cm und einen Innendurchmesser von 2.0 cm; das Adsorbens ist 80 g Kieselgel 60 silanisiert, Korngrösse 0.063–0.2 mm (Merck-Art. 7719); die Elutionsmittel sind: (I) Methanol–Tetrahydrofuran–2 *N* wässr. HCl (3:1:1) und (II) Äthanol–Diisopropyläther–Isoamylalkohol–2 *N* wässr. HCl (3:1:1:1).

Das Adsorbens wird 2 h in 300 ml Elutionsmittel I geschüttelt, danach in das Chromatographierohr übergeführt und mit weiteren 1500 ml Elutionsmittel I ausgewaschen.

Durchführung der Trennung. Bis zu 1.5 g* des zu trennenden, in wenig Elutionsmittel oder Methanol gelösten Gemisches aus Tetra-*n*-octylzinn und den *n*-Octylzinnchloriden werden auf die Säule gegeben. Zur Auftrennung der Mono- und Di-*n*-octylzinnchloride wird das Elutionsmittel I und zur anschliessenden Fraktionierung von Tri-*n*-octylzinnchlorid und von Tetra-*n*-octylzinn das Elutionsmittel II verwendet. Das Eluat wird in Fraktionen geeigneter Grösse aufgefangen und dünnschichtchromatographisch überprüft[1]. Zur schnelleren Kontrolle der Fraktionen auf *n*-Octylzinnchloride bzw. Tetra-*n*-octylzinn kann getüpfelt und nach Literaturverfahren[1,5] angefärbt werden.

Die Methode wurde mit einem Gemisch aus je *ca.* 400 mg Mono-*n*-octylzinntri-, Di-*n*-octylzinndi-, Tri-*n*-octylzinnmonochlorid und Tetra-*n*-octylzinn sowie 250 mg Zinntetrachlorid überprüft. Die Einzelfraktionen wurden eingedampft und die Gewichte der Rückstände graphisch als Funktion des Gesamtelutionsvolumens dargestellt (vgl. Fig. 2).

ERGEBNISSE UND DISKUSSION

Es wird gezeigt, dass Gemische der Methyl- bzw. *n*-Octylzinnchloride in präparativem Massstab an Kieselgelsäulen mit Hilfe chlorwasserstoffhaltiger Elutionsmittel aufgetrennt werden können.

Das Ergebnis der Trennung eines Gemisches von ^{14}C-markierten Methylzinnchloriden mit einem Elutionsmittel aus ätherischer Chlorwasserstofflösung und Petroleumbenzin ist in Fig. 1 wiedergegeben. Bei der Trennung von 20 mg eines Gemisches aus 19 Mol-% Monomethylzinntri-, 80 Mol-% Dimethylzinndi- und *ca.* 1 Mol-% Trimethylzinnmonochlorid auf einer Säule aus 75 g Kieselgel 60 treten zwischen den einzelnen Verbindungen Leerfraktionen auf. Auch bei einer Steigerung der aufgetragenen Menge auf *ca.* 250 mg Methylzinnchloride wird noch eine einwandfreie Trennung erzielt. Da das verwendete Elutionsmittel leicht flüchtig ist, können die Säuleneluate trotz verhältnismässig hohen Dampfdrucks der Methylzinnchloride —insbesondere trotz der hohen Flüchtigkeit des Trimethylzinnchlorids— ohne wesentliche Substanzverluste eingeengt werden (vgl. auch Lit. 6).

Mit Hilfe des verwendeten chlorwasserstoffhaltigen Elutionsmittels lassen sich auch die Methylzinnreste solcher Methylzinnverbindungen trennen, die durch Chlor-

* Bei ungefähr gleichen Gewichtsanteilen der Einzelkomponenten im Gemisch.

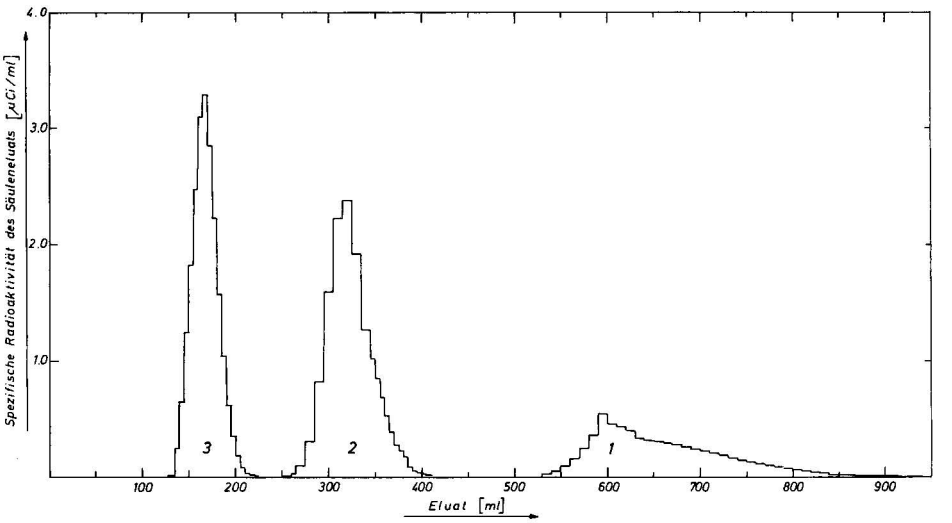

Fig. 1. Säulenchromatographische Trennung von [^{14}C]Monomethylzinntri- (1), [^{14}C]Dimethylzinndi- (2) und [^{14}C]Trimethylzinnmonochlorid (3).

wasserstoff leicht in die entsprechenden Methylzinnchloride übergeführt und dadurch als solche eluiert werden (vgl. auch Lit. 6).

Das gleiche gilt für die Chromatographie von *n*-Octylzinnverbindungen, die an silanisiertem Kieselgel ebenfalls mit chlorwasserstoffhaltigen Elutionsmitteln als *n*-Octylzinnchloride getrennt werden können. Dabei sind die Elutionsgeschwindigkeiten nur vom Alkylierungsgrad des Zinns und der Grösse des Alkylrestes am Zinn abhängig, nicht jedoch von der Art der durch die Cl-Ionen des Elutionsmittels er-

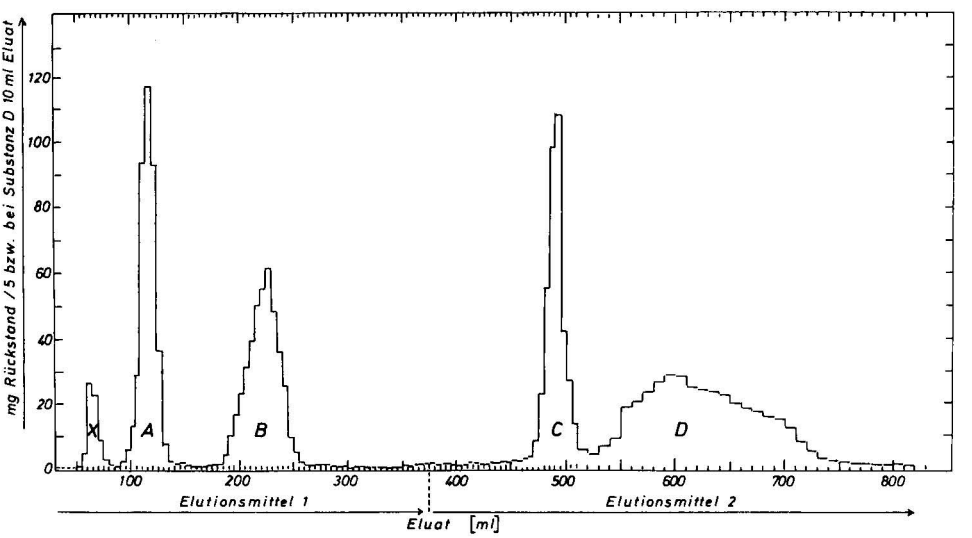

Fig. 2. Säulenchromatographische Trennung eines Gemisches von Mono-*n*-octylzinntri- (A), Di-*n*-octylzinndi- (B) und Tri-*n*-octylzinnmonochlorid (C), Tetra-*n*-octylzinn (D) und Zinntetrachlorid (X).

setzten Substituenten, wie z.B. vom Thioglykolsäureesterrest in Organozinnstabilisatoren[1].

Das Ergebnis der Trennung eines Gemisches aus Tetra-*n*-octylzinn und den *n*-Octylzinnchloriden an silanisiertem Kieselgel mit den chlorwasserstoffhaltigen Elutionsmitteln I und II ist in Fig. 2 wiedergegeben. Die Alkylzinnchloride werden bei der Chromatographie an silanisiertem Kieselgel im Vergleich zu der an normalem Kieselgel in umgekehrter Reihenfolge eluiert. Dieses Verhalten ist aufgrund der unterschiedlichen Aussenphasen des Adsorbens verständlich ("Phasenumkehrchromatographie"). Mit diesem Verfahren lässt sich eine eindeutige Auftrennung in die einzelnen *n*-Octylzinnchloride erzielen, so dass die reinen Verbindungen isoliert werden können; anorganische Zinnverbindungen, wie z.B. Zinnbutter $SnCl_4 \cdot 5\,H_2O$, werden noch vor den Alkylzinnchloriden eluiert.

DANK

Für die Durchführung der Chromatographie danken wir Frau H. Lubba und Herrn H.-P. Voss.

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Note

Separation of mono-, di- and tri-L-leucylglycine by droplet countercurrent chromatography

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A new technique of countercurrent chromatography, namely droplet countercurrent chromatography (DCCC), was introduced by Tanimura *et al.*[1], who demonstrated that a mixture of dinitrophenylamino acids was separated efficiently into its components by this technique. Okamoto *et al.*[2] demonstrated further that pure constituent peptides were obtained from a mixture of gramicidins and of tyrocidines by DCCC. In this paper we report the separation of a mixture of the repeated sequential peptides such as H(L-Leu–Gly)$_n$OH (n = 1, 2 and 3) and discuss the effectiveness of the two techniques DCCC and gel chromatography with Sephadex G-10 for the separation of these peptide mixtures.

EXPERIMENTAL

Three model peptides, H(L-Leu–Gly)$_n$OH (n = 1, 2 and 3), were synthesized by conventional methods. *tert.*-Butyloxycarbonyl-L-leucylglycine benzyl ester was deacylated with hydrogen chloride in dioxane and coupled with benzyloxycarbonyl-L-leucylglycine azide, which was derived from the corresponding hydrazide. A portion of the protected tetrapeptide ester obtained was catalytically hydrogenolyzed to give H(L-Leu–Gly)$_2$OH. Another portion of the tetrapeptide ester was converted into the tetrapeptide hydrazide and the azide derived from this hydrazide was coupled with L-leucylglycine benzyl ester to give the protected hexapeptide ester, which was finally hydrogenolyzed to afford H(L-Leu–Gly)$_3$OH. H(L-Leu–Gly)OH was obtained by hydrogenation of benzyloxycarbonyl-L-leucylglycine benzyl ester, which had been prepared by a known procedure. Details of the synthetic procedure and physical constants of the prepared peptide derivatives will be reported elswhere[3]. All of the peptides that are used for the separation by DCCC and by Sephadex G-10 gel chromatography were obtained as analytically pure compounds. The purity of each compound was verified by the results of elemental analyses and paper and thin-layer chromatography. The R_F values of the synthesized peptides and solvent systems used are summarized in Table I.

DCCC was carried out with an apparatus made by Seikagaku Kogyo Co. (Tokyo, Japan). It consists of 150 column units (mounted perpendicularly) of glass tubing (0.6 mm wall thickness and 2.4 mm I.D.), 60 cm long and connected by PTFE tubing (0.5 mm I.D.). All experiments were carried out at room temperature. The

TABLE I

R_F VALUES OF H(L-LEU-GLY)$_n$OH (n = 1, 2 AND 3)

Solvent system used in thin layer chromatography (TLC), n-butanol–acetic acid–water (4:1:5, upper phase); in paper chromatography (PC), n-butanol–acetic acid–pyridine–water (15:3:10:12).

Method	*H(L-Leu–Gly)OH*	*H(L-Leu–Gly)$_2$OH*	*H(L-Leu–Gly)$_3$OH*
TLC	0.46	0.53	0.86
PC	0.11	0.47	0.53

solvent mixture, n-butanol–acetic acid–water (4:1:5), was allowed to equilibrate in a separating funnel and the upper phase was then loaded into the required units of the glass columns as the stationary phase. A sample dissolved in the lower phase (2 ml) was placed at the top of the first column, and the lower phase was pumped as the mobile phase by nitrogen pressure (2 atm) through the top of this column at a flow-rate of 7 ml/h. Fractions from the last column were collected in fractions of 4 ml each, and the peptide content in each fraction was determined by the method described by Yemm and Cocking[4].

In Sephadex G-10 gel chromatography, a sample dissolved in water (0.5 ml) was applied to a column (100 × 1.3 cm) and development was continued with water. Elution was carried out at room temperature at a flow-rate of 13 ml/h, and 2-ml fractions were collected. The peptide content in each fraction was determined by the method described above. Fractionated peptides were identified by comparison of their R_F values with those of authentic samples.

RESULTS

A mixture of H(L-Leu–Gly)$_n$OH (n = 1, 2 and 3) (5 mg each) was used for the separation experiment by DCCC as described above. The mobile phase was collected up to fraction number 177, and the stationary phase was then ejected from the last column by nitrogen pressure (2 atm). As shown in Fig. 1, the mixture of the model peptides was completely fractionated into three components. The components of peaks

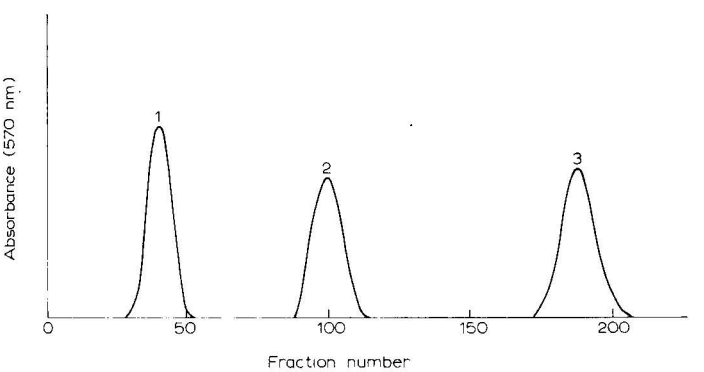

Fig. 1. Droplet countercurrent chromatogram of mono-, di- and tri-L-leucylglycine. Solvent, n-butanol–acetic acid–water (4:1:5). Each fraction is 4 ml. 1 = Mono-L-leucylglycine; 2 = di-L-leucyl glycine; 3 = tri-L-leucylglycine.

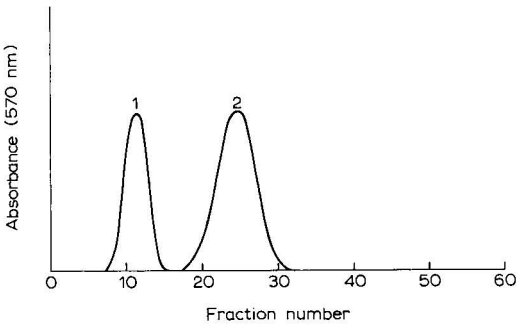

Fig. 2. Chromatography on a 100 × 1.3 cm column of Sephadex G-10 of mono-, di- and tri-L-leucylglycine. Solvent, water; flow-rate, 13 ml/h. Each fraction is 2 ml. 1 = Tri-L-leucylglycine; 2 = mixture of mono- and di-L-leucylglycine.

1, 2 and 3 were identified as H(L-Leu–Gly)OH, H(L-Leu–Gly)$_2$OH and H(L-Leu–Gly)$_3$OH, respectively.

In a separate experiment, a mixture of the three model peptides (1 mg each) was chromatographed on a Sephadex G-10 column. As shown in Fig. 2, two peaks were obtained. Peak 1 was identified as H(L-Leu–Gly)$_3$OH, but peak 2 was found to be a mixture of H(L-Leu–Gly)$_2$OH and H(L-Leu–Gly)OH. This result showed that the complete separation of the oligopeptides with a repeated sequence could not be obtained under these conditions.

It was concluded that, in comparison with gel chromatography with Sephadex G-10, DCCC was a more effective technique for the separation of these similar peptides. It appears to be suitable for the separation of synthetic oligopeptides.

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Note

Reaktionschromatographischer Nachweis einiger N-Nitrosamine der Tabakalkaloide

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Im Rahmen unserer Arbeiten über die Nitrosamine der Tabakalkaloide[1,2] erwies es sich als notwendig, ein dünnschichtchromatographisches Trenn- und Identifizierungsverfahren für diese Verbindungsgruppe auszuarbeiten. Mit dieser Methode sollte ihr Nachweis auch in Gegenwart von Tabakalkaloidspuren mit Sicherheit zu führen sein.

Die Auftrennung der Tabakalkaloide mit Hilfe der zweidimensionalen Dünnschichtchromatographie wurde bereits vor Jahren von Hodgson *et al.*[3] beschrieben. Auch bei unseren Arbeiten hat sich dieses chromatographische System bewährt. Die Trennung von N-Nitrosonornikotin, N-Nitrosoanabasin und N-Nitrosoanatabin stösst hingegen auf Schwierigkeiten. Diese drei Verbindungen zeigen bei den von Hodgson angegebenen Bedingungen fast idente R_F-Werte, ausserdem liegt auch der des Nikotins im gleichen Bereich. Überführt man hingegen die N-Nitrosoverbindungen nach der ersten dünnschichtchromatographischen Auftrennung durch Besprühen mit saurer $TiCl_3$-Lösung in die entsprechenden sekundären Tabakalkaloide und entwickelt die Platte anschliessend in der zweiten Richtung, so ist die Identifizierung der Nitrosamine und ihre Abtrennung vom Nikotin möglich.

Kürzlich wurde von Schütz $TiCl_3$ für den reaktionschromatographischen Nachweis von Nitrazepam vorgeschlagen[4]. Die von ihm angewandte Methodik entspricht teilweise auch der von uns eingesetzten.

MATERIAL UND METHODEN

Alle verwendeten Reagenzien und Lösungsmittel, ausser der $TiCl_3$-Lösung, waren p.A.-Ware. Die $TiCl_3$-Lösung (15%ig; Merck, Darmstadt, B.R.D.) wurde mit dem gleichen Volumen konzentrierter Salzsäure verdünnt. Die N-Nitrosamine wurden nach bekannten Verfahren aus den entsprechenden Basen dargestellt. Nornikotin und Anatabin gewannen wir aus einer nebenalkaloidreichen Tabakvarietät, Anabasin und Nikotin waren in den Beständen unseres Labors vorhanden. Die beiden letzteren Alkaloide wurden vor ihrer Verwendung im Vakuum unter Stickstoff destilliert.

Dünnschichtchromatographische Bedingungen

Kieselgel Fertigplatten HF_{254} (Merck), Schichtdicke 0.25 mm, 10 × 10 cm, wurden verwendet. Das Laufmittel war Chloroform–Methanol–Ammoniak (60:10:1),

Kammersättigung. Die Sichtbarmachung der Alkaloide erfolgte durch Besprühen mit einer Mischung aus gleichen Volumina 2%iger äthanolischer *p*-Aminobenzoesäure und Phosphatpuffer, pH 7, und anschliessendem Bedampfen mit BrCN.

Arbeitsvorschrift

Die Probenlösung wird, wie für die zweidimensionale Dünnschichtchromatographie üblich, auf die Platte aufgebracht. Nach dem Entwickeln in der ersten Laufrichtung wird die Dünnschichtplatte so abgedeckt, dass nur mehr die den Nitrosaminen entsprechende Zone freibleibt. Nun besprüht man diese Zone mit HCl-saurer $TiCl_3$-Lösung, trocknet kurz bei 120° und setzt die gebildeten Alkaloide durch Bedampfen mit NH_3 aus ihren Hydrochloriden frei. Anschliessend wird das Chromatogramm in der zweiten Richtung mit dem gleichen Laufmittel entwickelt.

ERGEBNISSE

Durch das Besprühen mit HCl-saurer $TiCl_3$-Lösung werden die N-Nitrosamine quantitativ in die entsprechenden Alkaloide überführt. Wie man aus den unten angeführten R_F-Werten sieht, können diese im Gegensatz zu den Nitrosaminen an Kieselgel getrennt werden. Die Nachweisgrenzen sind durch die Empfindlichkeit der Farbreaktion gegeben und liegen bei 0.5 μg pro Fleck.

Als R_F-Werte für die Nitrosamine wurden gefunden: N-Nitrosonornikotin, 0.60; N-Nitrosoanabasin, 0.64; N-Nitrosoanatabin, 0.64. Als R_F-Werte für die entsprechenden Alkaloide und das Nikotin wurde gefunden: Nornikotin, 0.37; Anabasin, 0.42; Anatabin, 0.50; Nikotin, 0.58.

Aromatische Nitrosamine, wie das N-Nitroso-N-Methylanilin, bilden durch Behandeln mit $TiCl_3$ auf der Dünnschichtplatte ebenfalls die entsprechenden Basen. Hingegen konnten mit flüchtigen Nitrosaminen, wie dem N-Nitrosopyrrolidin, dem N-Nitrosopiperidin, usw. diese guten Ergebnisse nicht erhalten werden. Der Umsatz lag bei diesen Verbindungen bei maximal 30%. Die freigesetzten sekundären Amine wurden hier durch Besprühen mit 1% 7-Chlor-4-nitrobenzofurazan in Methanol sichtbar gemacht.

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Note

New solvent systems for the separation of free and conjugated bile acids

II*. Separation of free bile acids as a group

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The separation of free (unconjugated) bile acids from their glycine (G) and taurine (T) conjugates is of importance in biological investigation because free bile acids possess different physiological activities[2–4]. For example, free bile acids have been found to play a role in the genesis of acquired monosaccharide intolerance in infants[5], and in several other clinical entities associated with overgrowth of bacteria in the small intestine such as Kwashiorkor[6], and stagnant-loop syndrome[7]. A number of thin-layer chromatographic procedures for bile acids have been suggested[8–21] but only a few of them[8–13] were able to achieve some degree of separation of free bile acids from conjugates based upon the assumption that glycomonohydroxy acids such as glycolithocholic acid (GLC) were present in negligible amounts in biological samples. This assumption appears to be invalid by the observation that GLC may constitute 1–17% of the total bile acids in clinical specimens[22,23]. In this report we propose a new solvent system, H, which gives better separation of free bile acids from their G and T conjugates, including GLC, than previously published procedures using an unidirectional single development system.

MATERIALS AND METHODS

Free and conjugated bile acid standards were obtained from Steraloids (Pawling, N.Y., U.S.A.), Applied Science Labs. (State College, Pa., U.S.A.), Supelco (Bellefonte, Pa., U.S.A.), and Calbiochem (La Jolla, Calif., U.S.A.). [^{3}H]-Cholic acid was purchased from New England Nuclear (Boston, Mass., U.S.A.) with a specific activity of 3.8 Ci/mmole and was found to be 98% pure by thin-layer chromatography. All solvents used were of reagent grade and obtained from Aldrich (Milwaukee, Wisc., U.S.A.) and Mallinckrodt (St. Louis, Mo., U.S.A.). Glass plates (20 × 20 cm), precoated with silica gel G to a thickness of 250 μm were purchased from Brinkmann (Westbury, N.Y., U.S.A.).

The present system (H) was made up of chloroform–methanol–water (70:25:3).

* Part I, see ref. 1.

TABLE I

COMPARISON OF VARIOUS SOLVENT SYSTEMS FOR SEPARATION OF FREE BILE ACIDS BY THIN-LAYER CHROMATOGRAPHY*

Abbreviations: LC = lithocholic acid; DOC = deoxycholic acid; CDC = chenodeoxycholic acid; C = cholic acid; UrsoDOC = ursodeoxycholic acid; 7-ketoDoc = 7-ketodeoxycholic acid; HyoC = hyocholic acid; 12-keto LC = 12-ketolithocholic acid. For conjugated bile acids: GLC = glycolithocholic acid; GDOC = glycodeoxycholic acid; GCDC = glycochenodeoxycholic acid; GC = glycocholic acid; TLC = taurolithocholic acid; TDOC = taurodeoxycholic acid; TCDC = taurochenodeoxycholic acid; TC = taurocholic acid.

System	*Ref.*	*Composition***	*Ratio (v/v)*
H§	Present work	$CHCl_3$–methanol–H_2O	70:25:3
1	8	isooctane–isopropanol–HAC	60:40:1
2	9	*n*-butanol–HAc–H_2O	100:7:5
3	10	isooctane–isopropyl ether–HAc	50:25:40
4	11	toluene–HAc–H_2O	50:50:10
5	12	isooctane–HAc–isopropyl ether–isopropanol	10:6:5:1
6	13	HAc–CCl_4–isopropyl ether–isoamyl acetate–*n*-propanol–benzene	5:20:30:40:10:10
7	14	ethyl acetate–methanol–HAc	70:20:10
8	15	isooctane–isopropyl ether–HAc	100:50:70
9	16	isooctane–ethylene chloride–HAc	60:30:30
10	17	isooctane–isopropyl ether–HAc–isopropanol	2:1:1:1
11	18	isooctane–ethyl acetate–HAc–*n*-butanol	20:10:3:3
12	19	isooctane–isopropyl ether–isopropanol–HAc	1:1:1:1
13	20	isooctane–ethyl acetate–HAc	5:5:1
14	21	$CHCl_3$–ethyl acetate–HAc	45:45:10

* Solvent systems giving inferior separation of GLC, GDOC and C within the same reference are not listed here. References giving negative ΔR_M values for both GLC–C and GDOC–C were omitted also.

** HAc = glacial acetic acid; adsorbent, silica gel G.

*** $\Delta R_M = R_{M,\text{GLC or GDOC}} - R_{M,C}$ where $R_M = \log [(1/R_F) - 1]$.

§ $R_F \times 100$ values for other free bile acids are: UrsoDOC, 59; 7-ketoDoc, 52; HyoC, 47; 12-Keto LC, 59.

All thin-layer chromatographic runs were carried out by applying 20–40 μg of the sample in 20–40 μl of ethanol–methanol (95:5) to the plate with a micropipette or by a sample streaker (Applied Science Labs.), allowed to dry, placed in a rectangular glass tank (10 × 30 × 25 cm) and developed by the ascending technique at room temperature (23–25°). The detailed procedure of thin-layer chromatography has been described in a previous report by the present workers[1].

In the recovery experiments, the silica gel was scraped off after the run at the zone corresponding to the R_F values of the free and conjugated bile acids. The remaining zones were also removed and examined for radioactivity. Solutions for counting were prepared by dissolving the bile acid in 8 ml of liquid scintillation solution, 12.8 ml of Spectrafluor PPO–POPOP concentrated liquid scintillator (Amersham/Searle, Arlington Heights, Ill., U.S.A.) in 200 ml of toluene. The solution was then transferred to a low-potassium liquid scintillation vial. Each flask was rinsed twice with 5 ml of the solution to effect complete transfer of the bile acid. Counting was done on a Packard Tri-Carb 3003 liquid scintillation spectrometer. Efficiencies were determined by external standardization using acetone-quenched standards (Packard) and results are reported as disintegrations per minute.

$R_F \times 100$ values												$\Delta R_M \times 100$ values***	
LC	*DOC*	*CDC*	*C*	*GLC*	*GDOC*	*GCDC*	*GC*	*TLC*	*TDOC*	*TCDC*	*TC*	*GLC–C*	*GDOC–C*
75	86	53	38	18	12	12	5	18	12	12	8	45	65
59	54	49	39	32	20	20	15	8	5	5	1	13	41
67	64	64	62	61	57	56	44	34	27	26	16	2	9
52	38	33	16	22	9	9	2	0	0	0	0	−17	29
38	29	23	11	16	6	6	1	0	0	0	0	−19	29
54	37	31	13	21	8	7	2	1	0	0	0	−25	24
64	43	35	10	30	6	5	1	0	0	0	0	−59	24
88	85	81	73	74	63	63	44	29	19	19	9	− 2	20
51	33	27	9	17	6	6	1	0	0	0	0	−32	19
43	26	20	6	12	4	4	1	0	0	0	0	−33	19
77	63	55	33	47	25	24	9	6	2	2	0	−26	17
52	36	30	10	26	7	7	0	0	0	0	0	−50	17
74	66	61	46	60	41	41	18	12	6	6	1	−25	9
46	29	22	6	17	5	5	0	0	0	0	0	−51	8
53	39	31	9	30	8	8	1	0	0	0	0	−64	6

RESULTS AND DISCUSSION

Table I gives a comparison of various solvent systems for the separation of free bile acids from G and T conjugates tested under identical conditions, and Fig. 1 shows the positions of different free and conjugated bile acids on thin-layer plates after development in system H and in systems 1–6 of Table I. It is clear from Table I that the resolution between free and conjugated bile acids is better in system H (relative mobility, $\Delta R_M = 45$ and 65 for GLC–C* and GDOC–C, respectively) than in any other system tested regardless of the presence of GLC. This is an advantage over other published systems because quantitative determination of total free bile acids as a group are possible even in the presence of glycomonohydroxy isomers. The results of the run using 2,4-[^{3}H]-cholic acid as a radioactive marker are shown in Table II. The recovery of radioactive cholic acid is nearly complete in the zone corresponding to the R_F values of the free bile acids (*ca.* 96%). This indicates the validity of system H as a tool for satisfactory isolation of free bile acids.

* For abbreviations, see Table I.

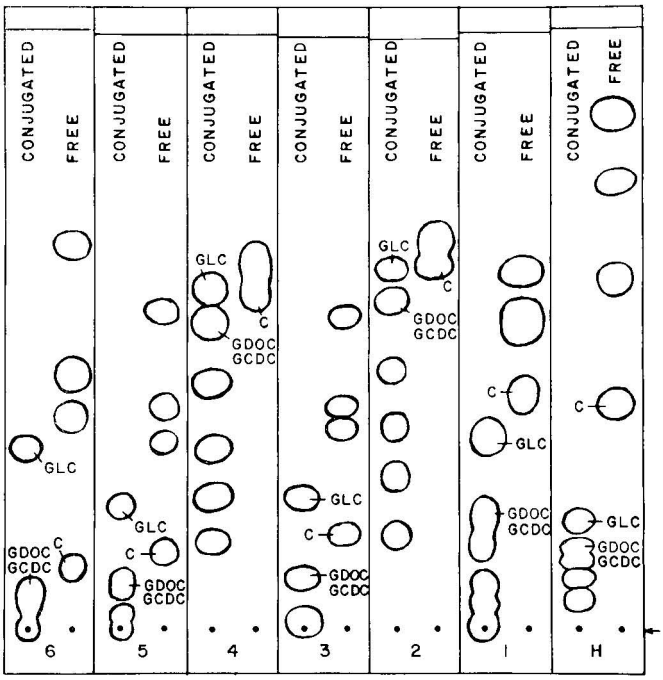

Fig. 1. Thin-layer chromatograms developed in various solvent systems under identical conditions. For abbreviations of compounds see Table I. On the left, from the bottom up, are TC, TCDC and TDOC (overlapping), TLC, GC, GCDC and GDOC (overlapping) and GLC for systems 2 and 4. Taurine-conjugated bile acids and GC are not resolved in systems 1, 3, 5, 6 and system H. On the right, from the bottom up, are C, CDC, LC and DOC for system H; C, CDC, DOC and LC for systems 3, 5 and 6. Free bile acids are not completely resolved in systems 1, 2 and 4.

TABLE II

CHROMATOGRAPHIC DISTRIBUTION OF RADIOACTIVITY OF 2,4-[^{3}H]-CHOLIC ACID AS RUN IN SYSTEM H

Region of plate	*Total Activity (%)*
Above cholic acid	2.9
Cholic acid zone	93.2
GLC zone*	2.3
Below GLC	1.6
Total	100.0

* Based on 10 determinations; average activity found in conjugated bile acid region (GLC and below) was 3.8 ± 1.2%.

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Note

Rapid gas chromatographic determination of disopyramide in serum using a nitrogen detector

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Disopyramide is a new antiarrhythmic agent that seems to be a drug with a greater therapeutic index than quinidine. Pharmacokinetic studies in man have indicated that disopyramide phosphate taken orally is absorbed rapidly and almost completely from the gut. The plasma half-life of disopyramide is about 7 h and 60% of the disopyramide is excreted unchanged in the urine within 48 h (ref. 1).

Quantitation of disopyramide has been performed by using a spectrofluorimetric method based on the fluorescence of disopyramide in 50% sulphuric acid[1]. However, this method is non-specific because the N-monodealkylated metabolite has the same fluorescence characteristics as the parent compound. Studies on the biotransformation and pharmacokinetics of disopyramide require a more sophisticated method, in which it is possible to quantitate disopyramide without interference by the metabolite. A gas chromatographic separation and quantitation of the drug is the most suitable method. Because of the interference by impurities from extraction solvents and serum, the use of a flame ionization detector necessitates a time-consuming clean-up procedure. The use of a nitrogen detector, a sensitive and selective detection system, combined with a simple extraction procedure, is described here.

EXPERIMENTAL

Apparatus

The gas chromatograph used was a Pye Unicam G.C.V. (Philips Nederland) equipped with a nitrogen detector. The column was a Pyrex glass coil of length 3 ft and I.D. 2 mm.

Operating conditions

Gas chromatography was carried out under the following conditions: stationary phase, 3% OV-17 on Gas-Chrom Q, 100–120 mesh; carrier gas, nitrogen at 30 ml/min; hydrogen flow-rate, 30 ml/min; air flow-rate, 300 ml/min; oven temperature, 255°; injector temperature, 275°; detector temperature, 275°.

Extraction procedure

Pipette into a glass-stoppered separating funnel 1.0 ml of serum, 2.0 ml of 0.1 *N* sodium hydroxide solution, 100 μl of internal standard solution (*p*-chlorodiso-

pyramide, 100 μg per solution in ethanol) and 10 ml of chloroform. Shake well for 30 sec, dry the chloroform layer with 1 g of anhydrous sodium sulphate and filter. Extract the aqueous layer with 10 ml of chloroform for 15 sec, dry and filter off the chloroform phase. Evaporate the pooled chloroform extracts to dryness on a water-bath at 50° under a stream of nitrogen. Transfer the dried residue with small portions of chloroform into a 3-ml glass tube, evaporate the chloroform under a stream of nitrogen and dissolve the residue in 25 μl of ethanol. Inject 1 μl of the final solution into the gas chromatograph.

RESULTS AND DISCUSSION

A calibration graph was prepared by adding known amounts of disopyramide phosphate to pooled blank serum, resulting in the following standards: 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0 and 10.0 μg disopyramide per ml. The standards were analyzed in triplicate by the extraction procedure described above. The ratio of the peak area of disopyramide to that of internal standard was plotted against the concentration of the standards and Fig. 1 shows a typical standard graph.

The extraction efficiency was measured by extracting the 10.0 μg/ml standard. After extraction, *p*-chlorodisopyramide was added, and the relative peak area was calculated and compared with the peak area of the same amounts of disopyramide

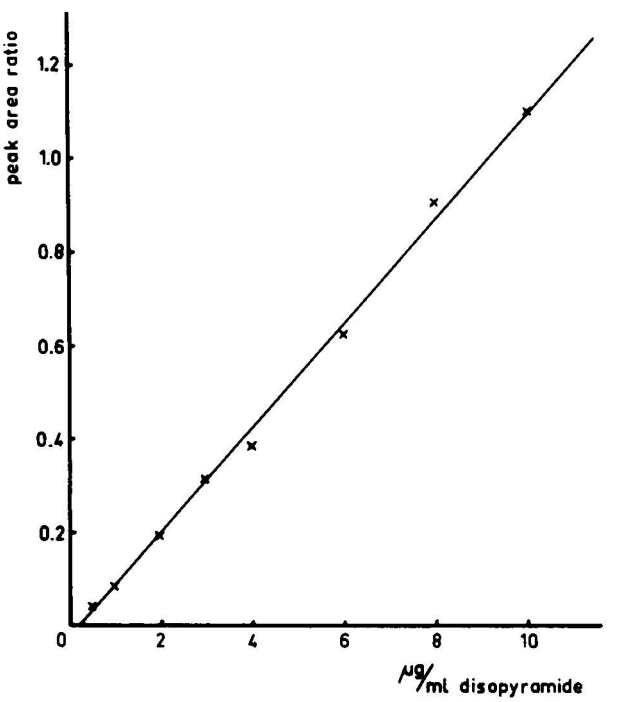

Fig. 1. Typical standard graph in which the peak area ratio of disopyramide to *p*-chlorodisopyramide is plotted against the concentration of disopyramide in serum.

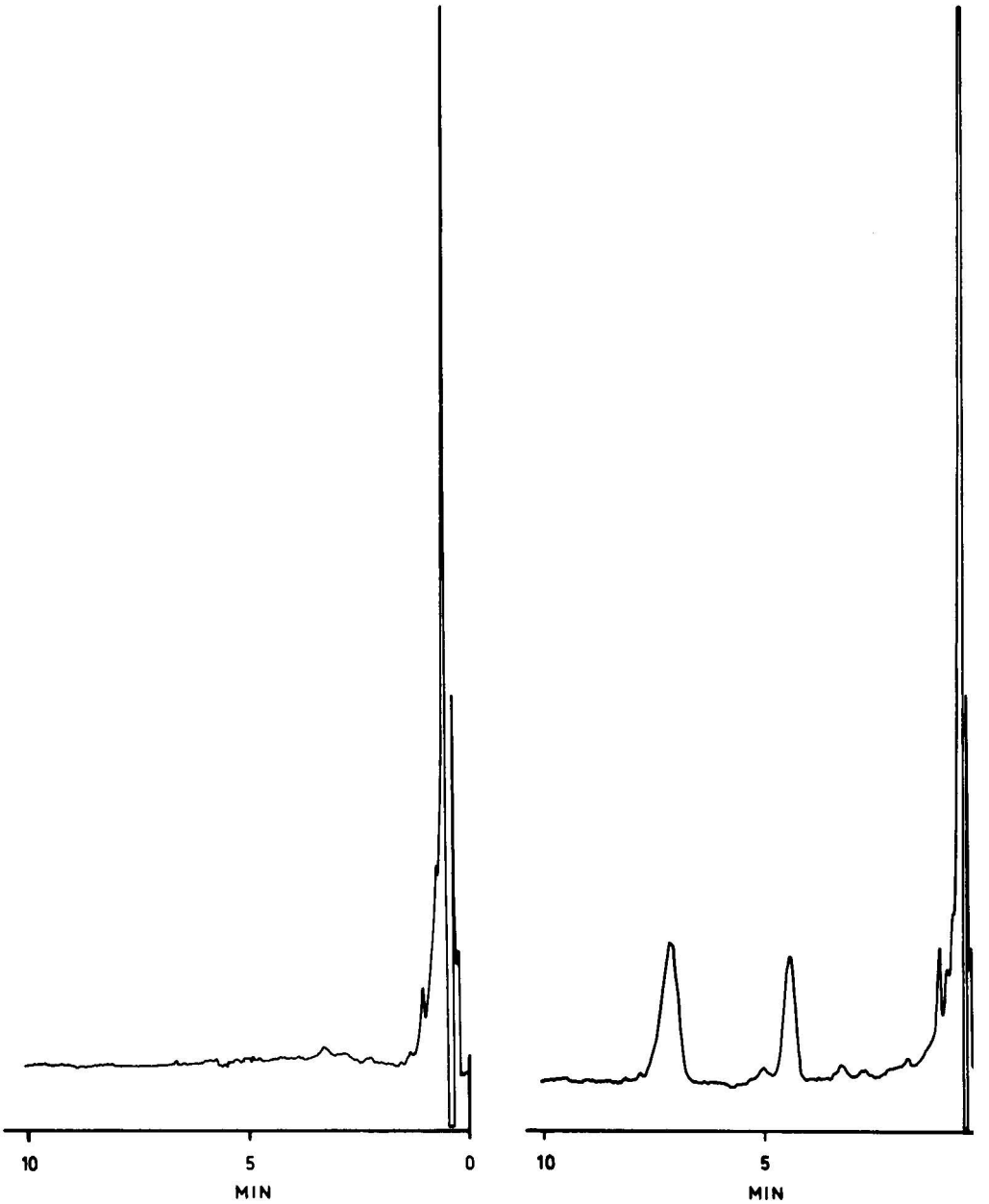

Fig. 2. Chromatogram of the blank serum extract. Attenuation: 512 × 1.

Fig. 3. Chromatogram of the standard containing 10.0 μg disopyramide per ml. Attenuation: 512 × 1.

and *p*-chlorodisopyramide. The extraction efficiency of disopyramide was 88.6% with a standard deviation of 2.7% ($n = 5$).

After the extraction of disopyramide and *p*-chlorodisopyramide together, comparison of the peak area ratio with the ratio of the known amounts of disopyramide and *p*-chlorodisopyramide showed (after extraction) a calculated amount of disopyramide of 102.5% with a standard deviation of 2.0% ($n = 5$).

These results indicate that in this assay *p*-chlorodisopyramide is a good internal standard to compensate for the extraction efficiency of disopyramide. Using a flame ionization detector there is a serum peak in the chromatogram that interferes with the peak of *p*-chlorodisopyramide. When using the nitrogen detector, this serum peak is not detected. The high sensitivity and selectivity of the nitrogen detector results in a very small solvent peak and a nearly horizontal base line when a blank serum extract is injected (see Fig. 2). A time-consuming clean-up procedure is not necessary when using the nitrogen detector (see Fig. 3).

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Note

Quantitative aspects of urinary indole-3-acetic acid and 5-hydroxyindole-3-acetic acid excretion

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In a recent paper the thin-layer separation of a large number of indolic tryptophan metabolites from human urine was described[1]. Some of these metabolites were quantitated by *in situ* photometry. The results for indican and indole-3-acetic acid (IAA) excretion were some 10–20 times lower than previously accepted values and these discrepancies were attributed by the authors to the greater specificity of the thin-layer method. 5-Hydroxyindole-3-acetic acid (5-HIAA) excretion was also apparently much lower than revealed in previous studies, averaging only 0.1 mg per 24 h (6 subjects, 16 collections, 10 after tryptophan load). A considerable proportion of the 24-h urine specimens contained less than 50 μg of 5-HIAA.

These findings are particularly crucial to the field of biochemical psychiatry, where a large number of studies (for example ref. 2) of the excretion of IAA and 5-HIAA under various conditions have been undertaken. 5-HIAA is an end metabolite of 5-hydroxytryptamine (serotonin), a neurotransmitter, though probably about 80% of urinary 5-HIAA is of extraneuronal origin. If the currently accepted methods for measuring IAA and 5-HIAA were indeed grossly erroneous, many basic concepts of human indole metabolism would need re-examination.

We are currently studying human tryptophan metabolism using stable isotopic tracers and have available a specific and accurate method for measuring both IAA and 5-HIAA using gas chromatography–mass spectrometry (GC–MS) with internal isotopic standards. The principle of this method is quite different from that used by Byrd and coworkers[1] and from other colorimetric or fluorimetric methods. Hence it seemed an appropriate technique to resolve this conflict.

MATERIALS AND METHOD

Synthesis of indole-3-acetic acid-(methylene-$^2H_2$)

Indole-3-acetic acid (100 mg) in ^2H_2O (1.2 ml) containing 10% (w/v) of NaO^2H was heated for 3 h at 120°. Acidification with 2 *N* hydrochloric acid gave a product with 92% $^2H_2$ and 8% $^2H_1$, which was used without further purification.

Synthesis of 5-hydroxyindole-3-acetic acid-(methylene-$^2H_2$)

5-Benzyloxyindole-3-acetic acid (Sigma, St. Louis, Mo., U.S.A.) (100 mg)

dissolved in 1.7 ml ^2H_2O containing 10% NaO^2H was heated for 6 h at 120°. After acidification with 2 *N* hydrochloric acid a 2.5-ml methanolic solution of the product was debenzylated by catalytic hydrogenation for 2 h at room temperature and atmospheric pressure using palladium on asbestos (75 mg)[3]. The catalyst was removed by filtration, washed with 5 ml methanol and the combined filtrate and washings treated with 200 mg neutral alumina (Camag MFC). Filtration and evaporation of the solvent gave 5-HIAA containing 85% $^2H_2$, 6% $^2H_1$, and 9% $^2H_0$ as an oil which crystallized on standing.

Urine analysis

Complete 24-h urine collections were obtained from 19 individuals, age range 5–50 years (av. 26 years). Strict dietary control was not observed but bananas were avoided[4].

For the estimations standardized solutions of the deuterated internal standards (0.5-ml aliquots, each containing about 25 μg of compound) were added to 10-ml aliquots of the urines. The mixture, adjusted to pH 4 with 2 *N* hydrochloric acid, was saturated with sodium chloride and then extracted with 3 × 10 ml ether. The ethereal extract after drying with sodium sulphate was evaporated under reduced pressure and the residue treated with bis-trimethylsilyltrifluoroacetamide containing 1% of chlorotrimethylsilane (1 part) and pyridine (1 part).

GC–MS was carried out as previously described[5,6] on a Perkin-Elmer 270 gas chromatograph–mass spectrometer, using both OV-101 and OV-17 stationary phases. IAA was measured by repetitive scanning of the molecular ions at *m/e* 319 and 321 for the natural compound and the isotopic standard, respectively, and the 5-HIAA was measured using the molecular ion minus $CO_2Si(CH_3)_3$ peaks at *m/e* 290 and 292. The deuterated standards were calibrated using authentic unlabelled IAA and 5-HIAA correcting for the contribution of the small amount of undeuterated material in the standard. With IAA the $^2H_0$ content was too small to detect, but with 5-HIAA it contributed the equivalent of 0.2 mg/l to the ratio readings.

RESULTS AND DISCUSSION

GC–MS has previously been used for the determination of 5-HIAA[7] and IAA[8] in cerebrospinal fluid though by methods that differed from those used here. The combination of the advantages of gas chromatographic resolution with the selective detection afforded by the mass spectrometer gives a high degree of specificity. In particular, indole carboxylic acid which Byrd and coworkers[1] regarded as a possible interfering substance in the colorimetric determination of IAA will not contribute to the values obtained by GC–MS. The internal isotopic standard compensates for losses and instrumental variability and gives a check on the gas chromatographic retention time of the natural compound to within a few seconds. On the OV-101 column, replicate analyses of the nineteen samples gave a standard deviation (S.D.) of 2.0% for the IAA and 2.3% for the 5-HIAA. The excretion of IAA ranged from 0.46–6.20 (2.55 ± 1.54 S.D.) mg per 24 h and that of 5-HIAA ranged from 1.66–5.01 (3.36 ± 0.80 S.D.) mg per 24 h. These results are for the free acids. Those samples examined also on the OV-17 column gave similar results.

The excretion of IAA in our subjects is somewhat lower than the values usually

obtained by solvent extraction and colorimetry[1]. IAA is a variable component of urine, the output being influenced by the state of the gut flora and by the pH of the urine[9]. Nevertheless, our values are roughly 10 times those obtained by Byrd *et al.*[1]. The excretion of 5-HIAA was much more consistent in spite of the varied food intake of our subjects and our values are very little lower than those obtained by previous workers using non-specific extraction methods followed by colorimetry. Brown *et al.*[10] have compared urinary 5-HIAA values obtained by extraction and colorimetry using 1-nitroso-2-naphthol with those given by chromatography on a short column and continuous monitoring of the effluent by fluorimetry using *o*-phthalaldehyde. Except in cases with drug interference, the more specific method of column chromatography gave only slightly lower results, these being in excellent agreement with ours.

The discrepancy between our results and those of most other workers on the one hand, and those of Byrd *et al.* on the other, is not easy to explain. Dietary differences and the small number of subjects studied by Byrd *et al.* may be contributing factors. Nevertheless, the general agreement between the colorimetric and fluorimetric methods and our GC–MS method, which is based on entirely different principles, is striking. At least as far as IAA and 5-HIAA are concerned, this study does not support the idea that the commonly used colorimetric methods are erroneous by a factor of 10 or more.

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Journal of Chromatography, 109 (1975) 439–440

CHROM. 8228

Note

A simple, sensitive determination and identification of vinyl chloride by gas chromatography with a Hall detector

G. F. ERNST and J. B. H. VAN LIEROP
Food Inspection Service, Nyenoord 6, Utrecht (The Netherlands)
(Received January 9th, 1975)

Recent publications have emphasized the toxicity of vinyl chloride monomer (VCM)[1]. In The Netherlands the Public Health Authorities have ordered a limit to the amount of VCM in food and this method for the determination of VCM was therefore developed in order to monitor VCM in food products.

VCM may be present in polyvinyl chloride packaging materials, for instance bottles, and may migrate into the contents, such as wine, vinegar and soft drinks. The VCM can be removed from the food by extraction with *m*-xylene and this extract can be analyzed by gas chromatography.

Methods involving many different types of columns and detectors have been described. However, these methods use the retention time as the only proof of identity. Even the use of two different columns does not give sufficient evidence, because compounds extracted with *m*-xylene from wine, for instance, can have the same gas chromatographic behaviour as VCM.

A more specific detector than a flame ionization or thermal conductivity detector was needed. Williams and Umstead[2] used a Dohrmann microcoulometer for the determination of halogenated hydrocarbons. The selective microelectrolytic conductivity detector according to Hall[3] is specific for halogens. We analyzed *m*-xylene extracts from foods on apolar columns with this detector and could identify and determine VCM even in nanogram amounts. The sample, having passed through the column, is reduced with hydrogen in a quartz tube and the conductivity of the hydrogen chloride formed is measured.

EXPERIMENTAL AND RESULTS

A Hewlett Packard 5750A gas chromatograph was equipped with a 4 m × 1/8 in. O.D. stainless-steel column, filled with 5% SE-30 on 80–100 mesh Chromosorb G (AW, DMCS). The Tracor 310 detector according to Hall[3] was connected to the gas chromatograph by a quartz tube of length 15 cm and I.D. 2 mm. The carrier gas (argon) had a flow-rate of *ca.* 5.5 ml/min, and hydrogen was passed into the quartz tube in an oven at 820° at an inlet pressure of 10 p.s.i.

Samples of 2.5 μl were injected at 90° isothermally. The sensitivity for 0.5 full-scale deflection was 4.0 ng of VCM, using a 1-mV recorder with a speed of 5 min/in. at a detector sensitivity setting of 10, attenuation 1.

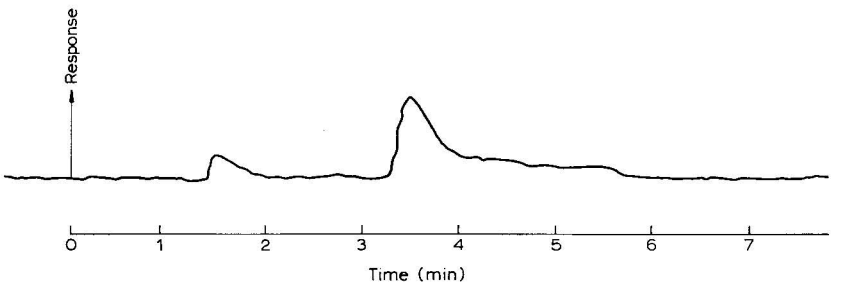

Fig. 1. Chromatogram of *m*-xylene.

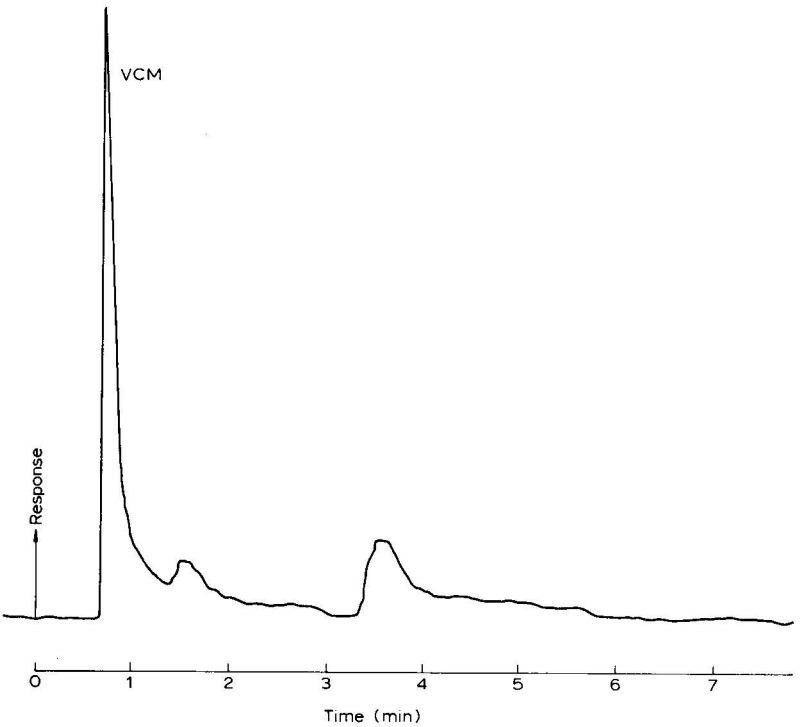

Fig. 2. Chromatogram of *m*-xylene containing 2.6 ppm (w/v) VCM.

Chromatograms of *m*-xylene and of *m*-xylene containing 2.6 ppm of VCM are shown in Figs 1 and 2.

The method was tested on *m*-xylene extracts from wine containing 0.1 ppm of VCM and the results will be published in this journal.

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Journal of Chromatography, 109 (1975) 441–442

CHROM. 8209

Note

High-speed liquid chromatographic separation of some *Strychnos* alkaloids

ROBERT VERPOORTE and A. BAERHEIM SVENDSEN

Department of Pharmacognosy, University of Leyden, Gorlaeus Laboratories, Leyden (The Netherlands)

(Received January 27th, 1975)

Bisset and his co-workers have studied the separation of some *Strychnos* alkaloids by means of thin-layer chromatography (TLC)[1] and gas–liquid chromatography (GLC)[2]. Since we had obtained a fairly good separation of a series of alkaloids by high-performance liquid chromatography (HPLC)[3,4], we found it of interest to try this technique for the separation of a number of alkaloids related to strychnine.

EXPERIMENTAL

The analyses were carried out on a Packard Model 8200 liquid chromatograph equipped with a UV detector (the wavelength 254 nm was used) and a stainless-steel column (30 cm × 2 mm I.D.) filled with Merckosorb Si 60 (5 μm); the balanced-density slurry technique was used for filling the column. The column temperature was maintained at 20°. The solvents used (diethyl ether, methanol and diethylamine) were *pro analysi* grade (Baker). The analyses were carried out at a flow-rate of 2.00

TABLE 1

RETENTION TIMES OF SOME *STRYCHNOS* ALKALOIDS

Column conditions as specified in Experimental.

Alkaloid	*Retention time (min) in solvent system*	
	I	*II*
Icajine	4.2	2.6
Vomicine	4.6	1.6
Pseudostrychnine	6.8	
Strychnine	7.2	12.4
4-Hydroxystrychnine	7.6	
α-Colubrine	8.8	14.3
Spermostrychnine	9.8	
β-Colubrine	10.3	10.2
Diaboline	16.0	10.9
Brucine	18.4	17.6
Serpentine	>20	
Alstonine	>20	

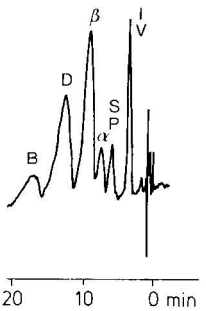

Fig. 1. Chromatogram of some *Strychnos* alkaloids in solvent system I (see text). I = Icajine; V = vomicine; S = strychnine; P = pseudostrychnine; α = α-colubrine; β = β-colubrine; D = diaboline; B = brucine.

ml/min at a pressure of 205 kg/cm^2 for solvent system I (diethyl ether containing 1% of diethylamine) and at a flow-rate of 1.15 ml/min at 200 kg/cm^2 for solvent system II [diethyl ether–methanol (1:1)].

DISCUSSION

The separation of 12 *Strychnos* alkaloids related to strychnine by means of HPLC is shown in Table I and Fig. 1. When the results are compared with the separations obtained by Bisset and co-workers with TLC[1] and GLC[2], the following differences are observed: diaboline is retained more in HPLC than in TLC, perhaps because of the higher amount of diethylamine used in TLC; and α-colubrine and β-colubrine, which could not be separated completely with GLC or TLC, are well separated by HPLC. Although fairly good separation of α- and β-colubrines could also be obtained in a neutral solvent system (system II), tailing made this system less useful because of the acidic properties of the silica gel[4].

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Journal of Chromatography, 109 (1975) 443

CHROM. 8255

Note

A simple method for the evaluation of the translocation behaviour of agrochemicals in soil

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(Received February 11th, 1975)

A detailed knowledge of the translocation behaviour of agrochemicals in soil, especially herbicides and fertilizers, is important for their successful use in agriculture. Suitable analytical methods are column chromatography, with analytical examination of the effluent, and thin-layer chromatography (TLC) using radioactively labelled compounds, or biological methods, *e.g.*, germination of seeds for herbicide examination. In this paper, a simple method is described, based on the translocation in a soil TLC layer followed by simple transfer to a silica gel plate and spraying with an appropriate colour reagent.

EXPERIMENTAL AND RESULTS

Normal plates (20 × 20 cm) were layered with soil of different types, using 100 g of soil and 50 ml of water for three plates and distribution by hand with a glass rod. The plates were air dried at room temperature and the compounds applied. After development with water, the plates were removed from the chamber and immediately covered with a normal silica gel plate (0.25 or 0.50 mm) for 30 min and compressed. The plates were air dried, sprayed with an appropriate reagent and the results interpreted by the usual methods. If the solubility in water of the compound to be transferred is very low, the plate carrying the soil should first be dried and the silica gel plate be impregnated with a solvent in which the compound has a good solubility.

When 25 μg of a compound were applied to the plate carrying the soil, about 30% was transferred to the silica gel plate, as found by using radioactively labelled compounds in our laboratory.

The method was tested with different organophosphorus insecticides, *e.g.*, trichlorphone and dimethoate, which were detected with the common P-reagent[1]. Compounds that are difficult to label with radioisotopes can be studied by this method with advantage, and also mixtures of labelled and unlabelled compounds.

REFERENCE

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Author Index

Errata

J. Chromatogr., 104 (1975) 201–204

Page 201, the title, "Thin-layer chromatography of 3,4-methylenedioxyamphetamine, 3,4-methylenedioxymetamphetamine and other phenethylamine derivatives" should read "Thin-layer chromatography of 3,4-methylenedioxyamphetamine, 3-methoxy-4,5-methylenedioxyamphetamine and other phenethylamine derivatives".

6th and 7th lines of the text, "3,4-methylenedioxymetamphetamine (MMDA)" should read "3-methoxy-4,5-methylenedioxyamphetamine (MMDA)".

J. Chromatogr., 105 (1975) 279–296

Page 282, 2nd line, "η" should read "$\Delta A(c)$".

Page 289, 2nd line, "measured values of $\Delta A_R(0)$ and $\Delta A_T(0)$" should read "measured values of $A_R(0)$ and $A_T(0)$".

Page 291, 6th line, "Fig. 10 indicates" should read "Fig. 10 displays the same parameter in case logarithmic conversion is used, Fig. 11 indicates".

9th line, "Fig. 11" should read "Fig. 12".

11th line, "Fig. 12" should read "Figs. 13–15" and "Fig. 13" should read "Figs. 16 and 17".

12th line, "Fig. 14" should read "Fig. 18".

Page 293, Fig. 17, the scale on the vertical axis should be multiplied by 10^{-1}.

Page 294, Fig. 18, the scale on the vertical axis should be multiplied by 10^{-2}.

1st and 2nd lines, "comparable to" should read "comparable and even superior to".

Page 295, 17th line, "(see Fig. 10)" should read "(see Fig. 12)".

J. Chromatogr., 106 (1975) 1–16

Page 5, eqns. 18 and 19 and the text between, "$\boldsymbol{E}$" should read "E" and "$\boldsymbol{J}$" should read "J".

Page 6, last line, "we obtain $E_j = c\omega[(\mu_j - \mu_0)\varrho_j]$" should read "we obtain $E_j = c\omega/[(\mu_j - \mu_0)\varrho_j]$".

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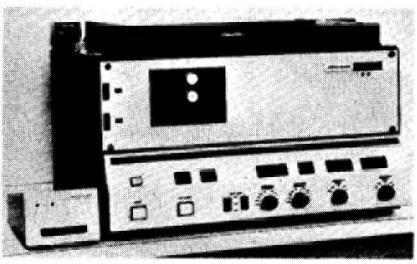
chromatography news section

APPARATUS



N-626

GAS CHROMATOGRAPH

Perkin-Elmer has introduced the Model 910 gas chromatograph which offers temperatures and times set directly by means of a method card as well as digital switches. The system includes a card reader. Once conditions have been optimized, the operator can prepare a card by punching out the appropriate holes. In cases where repetition of the analysis is necessary the card is inserted into the reader. Conditions are set within 2 sec.

After sample injection, the septum can be swung away, and the port closed by a smooth metal surface, thus preventing problems caused by the septum, such as bleed, leakage, and base-line upsets.

Among other points, (i) a control can be set to limit the maximum oven temperature to the value that a particular column can tolerate; (ii) if the FID flame is not lit, a warning light appears on the front panel of the chromatograph; (iii) temperatures and times can be controlled by a programmed computer.

N-598

SEPRACHROM™

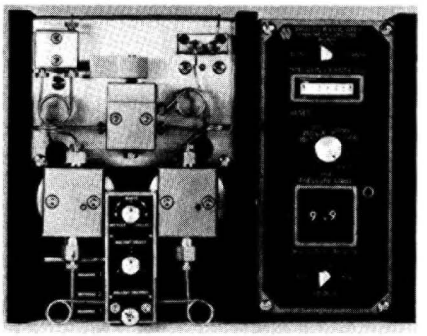
Technical Bulletin 32 from Gelman describes the screening of radiochemicals for impurities by Seprachrom, an ITLC-sytem based on a disposable microchromatography chamber containing ITCL™ (instant thin-layer chromatography) media. The manual contains over 50 references with product instructions for Seprachrom screening of 24 common radiopharmaceuticals such as Technetium-99m.

N-627

PERKIN-ELMER BULLETIN

"Instrument News" Vol. 24, No. 2E from Perkin-Elmer has among others, sections dealing with the Model 601 liquid chromatograph, the Model 1 computing integrator for automation of single chromatographs, the PEP-2 interactive programming data system, and the Model 131T Microstill.

For further information concerning any of the news items, apply to the publisher, using the reply cards provided, quoting the reference number printed at the beginning of the item.



N-624

LIQUID CHROMATOGRAPHY PUMP

Waters Associates has introduced a pump for high-pressure liquid chromatography, the Model 6000A solvent delivery system. This pump includes features such as: choice of three solvent intakes, for step gradients or solvent scouting; three selections for eluent disposition—waste, collect and recycle; and simplified connections for gradient work.

The Model 6000A retains all the features of the Model 6000 *viz.* efficient pumping system, constant volume delivery (solvent compressability compensation), 0.1–9.9 ml/min flow-rates, and 0–6000 p.s.i. pressure capability.

N-625

HEAD SPACE ANALYSIS TECHNIQUE

An accessory for the gas chromatographic determination of volatile substances contained in solutions or solids, by means of the head space analysis technique has been developed by Carlo Erba Strumentazione.

The accessory includes a module for the handling of the samples contained in sealed vials; a module for syringe transfer of head space gas from the vials to the gas chromatograph inlet; and a control module to set the number of analyses, the time between one analysis and the following one, and the temperature of the thermostatic bath.

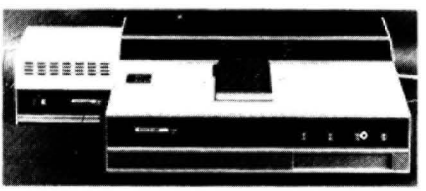
Up to 40 samples contained in 10-ml or 5-ml vials can be automatically processed.

N-628

SPECTROPHOTOMETRIC DETECTOR FOR LIQUID CHROMATOGRAPHY

A new brochure on the Model LC-55 variable-wavelength detector for liquid chromatography is available from Perkin-Elmer. The Model LC-55 detector has an operating wavelength range 190–800 nm and is linear to three absorbance units. This spectrophotometric detector shows no sensitivity to flow-rate or refractive index changes of the mobile phase. It has a high pressure flow cell of 8 μl internal volume which can withstand pressures up to 2500 p.s.i. (170 atm).

The spectral region between 190 and 230 nm has not been used traditionally to characterize organic compounds. However, because of a high



energy beam condensing system the Model LC-55 can be used routinely in this region. This allows analyses which formerly would have required a refractive index detection system and its associated drawbacks.

N-630

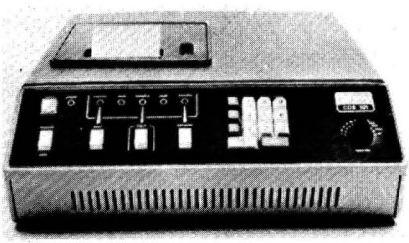
VARIAN MAT 112

Varian MAT has published a 28-page brochure describing the Varian MAT 112 double focusing mass spectrometer. Designed for organic chemical analysis, the MAT 112 has high sensitivity, high resolving power, linear mass scale, and efficient vacuum system. Contents of the brochure include applications with spectra, description of the analyzer, ion generation, vacuum system, GC coupling, inlet system, spectra recording, mass determination, GC recording, automatic data processing accessory, and specifications.

N-631

CHROMATOGRAPHY DATA SYSTEM

The CDS 101 data system from Varian automatically and accurately quantitates data from both gas and liquid chromatographs. Once the sample is injected, the system produces a report on the chromatogram including peak number, separation code, retention time, peak area, and normalized area composition. All the critical peak measuring parameters have been set at optimum values to quantitate most chromatograms. In addition, after each peak during a run, the system automatically adapts its peak measuring parameters to keep them consistent with increasing peak width. After completion of a run, the operator without reanalyzing the sample can edit and re-compute the output report. Solvent peaks or other portions of the chromatogram can be deleted, and a number of peaks can be grouped.



Throughout the analysis, the CDS 101 automatically checks itself and the chromatographic system for errors, such as insufficient or excessive signal from the instrument. If an error is detected, a message is printed denoting the error; if a significant error persists, the system goes on standby to prevent loss of sample.

The CDS 101 interfaces with all chromatography system components including gas and liquid chromatographs, recorders, automatic samplers, and temperature programmers. One CDS 101 can be used alternately with four different chromatographs and their recorders. Four switches, six indicator lights, and a small numeric keyboard, provide control.

N-664

CHROMATOGRAPHY DATA SYSTEM LITERATURE

A 16-page brochure on the Perkin-Elmer chromatography data system, PEP-2, is now available. The illustrated brochure describes the PEP-2 data system as a means of obtaining final-typed analytical reports from raw chromatographic data. The system can simultaneously accept data from 16 separate instruments including gas chromatographs, liquid chromatographs or amino acid analyzers. The PEP-2 can be desgined with from 8–32K of memory and any one of nine analytical programs chosen to optimize the system for both analytical volume and the type of application.

N-642

BASELINE CORRECTION DEVICE

A baseline correction device is currently available from Kontes. The unit accepts output signals from a variety of analytical instruments and permits the user to eliminate baseline drift and suppress background noise without a negative impact on significant peak height. The manufacturer states that resulting corrected baselines can then be integrated on standard apparatus or fed directly to EDP hardware. Designed principally for densitometric scanning where considerable background noise is routine, this device offers the user control of the baseline in other systems including LC and GC applications.

N-634

HIGH PRESSURE SAMPLING VALVE FOR LC

A high pressure sample injection valve together with appropriate accessories is the first product of the Rheodyne Company. Known as the Model 70-10 sample injection valve, the unit offers high-resolution injections with volumetric precision and will withstand up to 7000 p.s.i. operating pressure. The valve injects sample volumes of 10 μl or larger by means of a removable external sample loop. Chief advantages of this type of valve are superior volumetric reproducibility and overall chromatogram consistency.

N-632

NUCLEAR INSTRUMENTATION

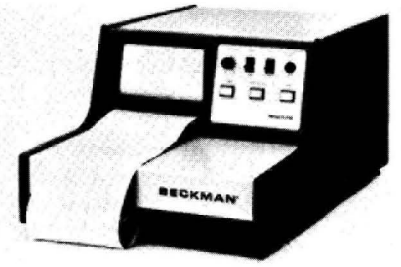
The 6-page publication "Data Systems for Nuclear Instrumentation" from Beckman describes the Model MB data system, a data reduction system designed for clinical radioimmunoassays (RIA). The system, which can be integrated into Beckman gamma-counting instrumentation, accommodates virtually any RIA test and delivers answers quickly and accurately.

N-633

SCANNING DENSITOMETER

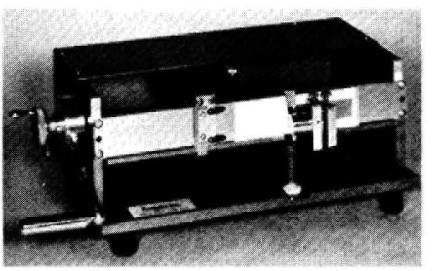
Beckman Instruments has introduced a scanning densitometer for electrophoresis systems that combines automatic gain and zero adjust capabilities with other operating advances. The Microzone® Model R-112 scanning densitometer also offers optional filter selection, automatic overrange detection and multiple electrophoretic medium capability. A dual-scanning control permits the operator to select scanning speeds of 0.63 cm or 0.35 cm/sec. Selectable slit sizes are 0.3 mm x 2 mm and 0.4 mm x 5 mm.

In operation, the Model R-112 automatically scans each sample for maximum density and sets the baseline to zero before sample tracing occurs.



The automatic zero adjust eliminates manual compensation for sample variations and minimizes instrument set-up time and preparatory scanning. The automatic gain control sets the peak reading on the chart to correspond with the front-panel peak height setting. Gain is automatically adjusted during each scan to bring the tracing peak to the desired level.

The scanning densitometer is also equipped with an 8-position filter wheel and four precision interference filters (520 nm, 550 nm, 570 nm and 600 nm) which cover most common dyes. Four blank positions can be fitted with filters from 400 nm–700 nm for special applications.



N-637

TLC ZONAL SCRAPER

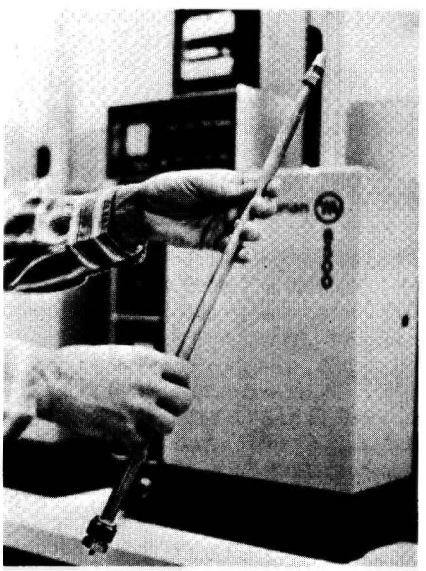
A manual TLC scraper which has the reproducible accuracy of fully-automatic models is announced by Analabs. This readily adjustable manual scraper can handle up to thirty samples in less than two minutes. A TLC plate may be

CHEMICALS

N-629

PREPARATIVE COLUMNS FOR LIQUID CHROMATOGRAPHY

MicroPak® preparative columns for liquid chromatography have been introduced by Varian. Packed with very small particles, the columns have a large surface area exposed to the solute, and high efficiency can be achieved as there is rapid solute mass transfer. The high resolution makes it easier to develop new separations and with the short columns and high flow-rates, preparative separations can be completed in less time.



Two basic types of MicroPak columns are available for preparative chromatography–columns for adsorption chromatography packed with porous silica or alumina, and columns for liquid–liquid partition packed with bonded stationary phases.

N-655

ICN BULLETIN

"Life Sciences" Vol. 1 Issue 3, from ICN Pharmaceuticals has among others, sections on internal standards for amino acid analysis, accurate RNA analysis, and affinity gel chromatography.

N-603

CONTROLLED-PORE GLASS BIBLIOGRAPHY

A bibliography of controlled-pore glass from Electro-Nucleonics is divided into two sections, on permeation and affinity chromatography. It also contains references on topics ranging from theory and technique to applications involving nucleic acids, proteins, enzymes, viruses, polysaccharides and synthetic polymers.

N-661

CHROMATOGRAPHY LIPIDS

"Chromatography Lipids" (Vol. VIII No. 6) from Supelco has sections dealing with OV-275, the highly-polar cyanosilicone; the importance of internal standards in quantitative analyses; determination of impurities in various C_1–C_8 organic compounds with Carbopack. Also included is a separate section describing accessories available from Supelco.

N-662

GAS–CHROM NEWSLETTER

"Gas–Chrom Newsletter" (Vol. 16 No. 1) from Applied Science Labs., describes HI-EFF micropart columns which are pretested microparticle LC columns each containing about 1.5g of silica gel, particle size 5 μm in diameter; column length is 15 cm with an O.D. of 1/4 in. and I.D., 5 mm. The newsletter also has sections on C-13 acids, pure glyceryl ethers, cholesteryl esters, and a discussion on liquid phase polarity.

PROCEDURES

N-613

RADIOIMMUNOASSAY BIBLIOGRAPHY

The second edition of the bibliography of periodical literature on radioimmunoassay is available from Beckman. The 66-page "Bibliography of Radioimmunoassay Periodical Literature" contains over 1700 references indexed by subject matter. A separate author index is also included. The references offer complete details –title, author, publication, date and country of origin. Subject indices identify application and techniques for literature searching.

N-617

TLC BIBLIOGRAPHY

Camag's November 1974 (No. 34) issue of "Camag Bibliography Service" covers new books, review articles, fundamentals, theory and general, instrume nts, methods and techniques relevant to TCL. A cumulative TLC bibliography (Vol. III) is also now available covering the "Camag Bibliography Service" Nos. 21–30 (1969–1973) and containing nearly 5000 references.

N-657

BECKMAN BROCHURE

Beckman Instruments describes its "New Dimension Series" of audio-visual technical programs in a 40-page booklet. The series consists of prepared tape–slide courses in amino-acid and peptide analysis, ultra-centrifugation, electrophoresis, analysis of enzymes, gas chromatography, nuclear instrumental techniques, pH measurement, infrared and UV-visible spectroscopy. Each program consists of a printed script, a set of prepared 35-mm color slides and prerecorded cassette tapes which are keyed to the slides.

Beckman's "New Dimension Series" complements the Technical Education Center's seminars, workshops and training courses in analytical, clinical and research instrumentation methods.

N-656

TRACE PHENOL ANALYSIS

Trace analysis of phenolic compounds by gas chromatography is described in Bulletin 742A from Supelco. Separation not only of *o*-, *m*-, *p*-cresol and ethylphenol isomers, but also a number of dimethylphenol and trimethylphenol isomers is achieved. It is possible to separate most of them without derivitization, but formation of the methyl ethers permits better separation of some isomers. Literature is available.

Part 1 of this bulletin (entitled "GC separation of tar acids- phenolic compounds") presents general background information on the column technology related to these compounds; part 2 describes a number of column materials which can be used for the separation of tar acids; part 3 deals with the separation of the tar acids as derivatives.

N-660

BECKMAN PUBLICATION

Beckman Instruments has announced a new company publication in English entitled "Beckman Information" available free of charge to those engaged in research work in the fields of analytical chemistry, biochemistry, biomedicine, biophysics, medical electronics, physiology as well as to editors of scientific and technical periodicals. Scheduled to appear twice a year, the publication is designed to improve customer–manufacturer communication and also to promote an exchange of information between those involved in all branches of science.

"Beckman Information 1.74" has among others, sections entitled, "A method for the analysis of neutral mono- and disaccharides by column chromatography with the multichrom B", "Gas chromatography determination of fish oil fatty acids" and "Automated Edman degradation of proteins and peptides using the volatile N,N-dimethylbenzylamine buffer". A final section deals with the latest products available from Beckman.

NEW BOOKS

Applications of the newer techniques of analysis (Progress in analytical chemistry, Vol. 6), edited by I.L. Simmons and G.W. Ewing, Plenum Press, New York, London, 1973, viii + 383 pp., price US$ 22.50.

Advances in Chromatography, Vol. 11, edited by J.C. Giddings and R.A. Keller, Marcel Dekker, New York, 1974, xii + 196 pp., price US$ 19.75.

Handbook of chemistry and physics, edited by R.C. Weast, CRC Press, Cleveland, Ohio, 55th ed., 1974, 2305 pp., price US$ 28.95.

Handbook of spectroscopy, Vol. 1, edited by J.W. Robinson, CRC Press, Cleveland, Ohio, 1974, 913 pp., price US$ 51.95.

Handbook of spectroscopy, Vol. 2, edited by J.W. Robinson, CRC Press, Cleveland, Ohio, 1974, 600 pp., price US$ 41.95.

Methodology for analytical toxicology, (update and revision of The manual of analytical toxicology), Edited by I. Sunshine, CRC Press, Cleveland, Ohio, 1975, *ca.* 450 pp., price US$ 30.50.

Practical electrophoresis, by G.J. Moody and J.D.R. Thomas, Merrow, Watford, 1975, vii + 104 pp., price £2.50 (US$ 8.30 overseas).

Gaschromatography der Pflanzenschutzmittel. Tabellarische Literaturreferate IV, Mitteilungen aus der Biologischen Bundesaustalt für Land- und Forstwirtschaft, Heft 161, by W. Ebing, Kommissionsverlag Paul Parey, Berlin, Hamburg, 1974, 101 pp., price DM 10.00.

Gas chromatography of coating materials, by J.K. Haken, Marcel Dekker, New York, 1974, xv + 334 pp., price US$ 29.75.

Recent analytical developments in the petroleum industry, edited by D.R. Hodges, Applied Science Publ., Barking, 1974, ix + 337 pp., 109 figs., 56 tables, price £10.00.

Chromatographic methods, by R. Stock and C.B.F. Rice, Chapman & Hall, London, 3rd ed., 1974, viii + 383 pp., price £2.90 (paperback), £5.25 (hardbound).

1972 Evaluation of some pesticide residues in food (WHO pesticide residue series, No. 2), World Health Organization, Geneva, 1973, 587 pp., price Sw.Fr.25.00.

Pesticides: nomenclature, specifications, analysis, use and residues in foods (Progress in standardization, No. 1; reprinted from *Bull. WHO,* 49, (1973) 169–204), by D.A. Lowe and A.R. Stiles, World Health Organization, Geneva, 1974, 40 pp., price Sw.Fr.6.00.

Ion exchange and solvent extraction–A series of advances, Vol. 6, edited by J.A. Marinsky and Y. Marcus, Marcel Dekker, New York, 1974, xii + 301 pp., price US$ 27.50.

Chemical problems connected with the stability of explosives 3 (Proceedings of the 3rd symposium, Ystad, May 28–30, 1973), edited by J. Hansson, Sektionen för Detonik och Förbränning, Sundbyberg, 1974, vi + 319 pp., price Sw.Kr. 100.00.

Spectroscopic methods of identification of microquantities of organic materials (Applied spectroscopy reviews, by E.G. Brame, Jr., Vol. 8, Part A), by G.M. Ayling, Marcel Dekker, 1974, vii + 163 pp.

MEETINGS

FINNIGAN COURSES

Finnigan Corporation is sponsoring two elementary courses in "Fundamentals of interpreting the mass spectra of organic molecules." The underlying principles relating mass spectra to molecular structure will be discussed, with emphasis on actual practice in the interpretation of unknown mass spectra by the student. The courses will be presented from August 18–22, 1975 at The Hilton Inn in Seattle, Washington, and from October 20–24, 1975 at the Holiday Inn in Atlanta. Registration, textbook and lunches are included in the $275.00 fee. Further information may be obtained from Mr. E.J. Bonelli, Finnigan Corp., Sunnyvale, Calif. 94086, U.S.A.

THIRD INTERNATIONAL CAMAG SYMPOSIUM ON THIN-LAYER CHROMATOGRAPHY

The Third International Camag Symposium on Thin-Layer Chromatography will be held September 10–12, 1975, in London, Great Britain. Papers presented at the Symposium will focus on three themes:
- Detection and Theoretical and Practical Aspects of Thin-Layer Chromatography;
- Quantitative Aspects of Thin-Layer Chromatography;
- Physico-Chemical Techniques Associated with Thin-Layer Chromatography.

Plenary lectures will be presented by the following:
A.A. Boulton, Saskatoon, Canada; T.H. Brodasky, Kalamazoo, U.S.A.; A. Christalli, Rome, Italy; H. Dolezalova, Storrs, U.S.A.; P.E. Flinn, St. Louis, U.S.A.; F. Geiss, Ispra, Italy; R.R. Goodall, Macclesfield, Great Britain; O. Hutzinger, Amsterdam, The Netherlands; H. Jork, Saarbrücken, G.F.R.; R. Kaiser, Bad Durkheim, G.F.R.; K. Krummen, Basel, Switzerland; V. Pollak, Rio de Janeiro, Brazil; K. Randerath, Houston, U.S.A.; G. Rouser, Duarte, U.S.A.; N. Seiler, Frankfurt, G.F.R.; G. Szekely, Basel, Switzerland; L. Treiber, Cleveland, U.S.A.

In addition, an opportunity for participants to present research communications will be provided, and arrangements have been made to publish the complete proceedings of the Symposium. The Symposium and Conference fee for the three days is £75.00 which includes a copy of the proceedings and morning coffee, lunch and afternoon tea.

Further information and application forms may be obtained from Camag or through the Conference Organizers, Laboratory News Europe, 78 Wigmore Street, London W.1.

Participants wishing to present research communications should contact the Symposium chairman, Dr. A.A. Boulton, Psychiatric Research Unit, University Hospital, Saskatoon, Saskatchewan, Canada.

3rd INTERNATIONAL SYMPOSIUM ON ANALYTICAL PYROLYSIS

The 3rd International Symposium on Analytical Pyrolysis will be held at the Hotel CASA 400 in Amsterdam, 7–9 September, 1976. Topics will include the principles and applications of pyrolysis in combination with chromatographic, spectrometric and computer techniques. The main language of the Symposium will be English but assistance will be given to non-English speaking contributors if required. An exhibition of currently available equipment and laboratory prototypes will be on view in the lecture hall complex.

Further information on the meeting can be obtained from Miss Ria Priester, FOM-Institute for Atomic and Molecular Physics, Kruislaan 407, Amsterdam 1006, The Netherlands.

PUBLICATION SCHEDULE FOR 1975

Journal of Chromatography (incorporating *Chromatographic Reviews*)

MONTH	D 1974	J	F	M	A	M	J	J	A	S	O	N	D
JOURNAL	101/1 101/2 102	103/1 103/2 104/1	104/2 105/1 105/2	106/1 106/2	107/1 107/2	108/1 108/2	109/1 109/2	110/1 110/2	111/1	111/2		112	114/1 114/2
REVIEWS*				113/1					113/2		113/3		

* Volume 113 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 Paper Chromatography, Thin-Layer Chromatography, Column Chromatography, Gas Chromatography and Electrophoretic Techniques. (f) Chromatographic Data.

Submission of Papers. Three copies of manuscripts in English, French or German should be sent to: Editorial office of the Journal of Chromatography, P.O. Box 681, Amsterdam, The Netherlands. For *Review articles*, an outline of the proposed article should first be forwarded to the Editorial office for preliminary discussion prior to preparation.

Manuscripts. The manuscript should be typed with double spacing on pages of uniform size and should be accompanied by a separate title page. The name and the complete address of the author to whom proofs are to be sent should be given on this page. Authors of papers in French or German are requested to supply an English translation of the title. A short running title of not more than 50 letters (including spaces between the words) is also required for Full-length papers and Review articles. All illustrations, photographs, tables, etc., should be on separate sheets.

Heading. The title of the paper should be concise and informative. The title should be followed by the authors' full names, academic or professional affiliations, and addresses.

Summary. Full-length papers and Review articles should have a summary of 50–100 words. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes will be published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Particular attention should be paid to the size of the lettering to ensure that it does not become unreadable after reduction. Sharp, glossy photographs are required to obtain good halftones. Each illustration should have a legend, all the *legends* being typed together on a *separate sheet*. Coloured illustrations are reproduced at the author's expense.

References. References should be numbered in the order in which they are cited in the text and listed in numerical sequence on a separate sheet at the end of the article. The numbers should appear in the text at the appropriate places using superscript numerals. In the reference list, periodicals[1], books[2], and multi-author books[3] should be cited in accordance with the following examples:

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*.

Proofs. Two sets of proofs will be sent to the author to be carefully checked for printer's errors. Corrections must be restricted to instances in which the proof is at variance with the manuscript. "Extra corrections" will be inserted at the author's expense.

Reprints. Fifty reprints of Full-length papers, Short communications and Notes will be supplied free of charge. Additional reprints can be ordered by the authors. An order form containing price quotations will be sent to the authors together with the proofs of their article.

News. News releases of new products and developments, and information leaflets of meetings should be addressed to: The Editor of the News Section, Journal of Chromatography, Elsevier Scientific Publishing Company, P.O. Box 330, Amsterdam, The Netherlands.

Subscription orders. Subscription orders should be sent to: Elsevier Scientific Publishing Company, P.O. Box 211, Amsterdam, The Netherlands.

Publication. The *Journal of Chromatography* (including *Chromatographic Reviews*) appears fortnightly and has 14 volumes in 1975. The subscription price for 1975 [Vols. 101–114 and Supplementary Vol. 4 (Bibliography of Electrophoresis 1968–1972)] is Dfl. 1365.00 plus Dfl. 120.00 (postage) (total US$ 631.91). Subscribers in the U.S.A., Canada and Japan receive their copies by air mail. Additional charges for air mail to other countries are available on request. Back volumes of the *Journal of Chromatography* (Vols. 1 through 100) are available at Dfl. 100.00 (plus postage).

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