ISSN 0021-9673

VOL. 136 NO. 2 JUNE 11, 1977

JOURNAL OF CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
AMSTERDAM

PUBLICATION SCHEDULE FOR 1977

Journal of Chromatography (incorporating *Biomedical Applications* and *Chromatographic Reviews*)
In the course of 1977, also the cumulative indexes for Vols. 121–130 and 131–140 will appear.

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

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

Submission of Papers. Papers in English, French and German may be submitted, if possible in three copies. Manuscripts should be submitted to:
The Editor of *Journal of Chromatography*, P.O. Box 681, Amsterdam, The Netherlands
or to:
The Editor of *Journal of Chromatography, Biomedical Applications*, P.O. Box 681, Amsterdam, The Netherlands.
Reviews are invited or proposed by letter to the Editors and will appear in *Chromatographic Reviews* or *Biomedical Applications*. An outline of the proposed review should first be forwarded to the Editors for preliminary discussion prior to preparation.

Subscription Orders. Subscription orders should be sent to: Elsevier Scientific Publishing Company, P.O. Box 211, Amsterdam, The Netherlands. The *Journal of Chromatography, Biomedical Applications* can be subscribed to separately.

Publication. The *Journal of Chromatography* (including *Biomedical Applications* and *Chromatographic Reviews*) has 15 volumes in 1977. The subscription price for 1977 (Vols. 130–144) is Dfl. 1650.00 plus Dfl. 210.00 (postage) (total *ca.* US$ 744.00). The subscription price for the *Biomedical Applications* section only (Vol. 143) is Dfl. 110.00 plus Dfl. 14.00 (postage) (total *ca.* US$ 49.60). Journals are automatically sent by air mail to the U.S.A. and Canada at no extra costs, and to Japan, Australia and New Zealand with a small additional postal charge. Back volumes of the *Journal of Chromatography* (Vols. 1 through 129) are available at Dfl. 100.00 (plus postage). Claims for issues not received should be made within three months of publication of the issue. If not, they cannot be honoured free of charge.

For further information, see page 3 of cover.

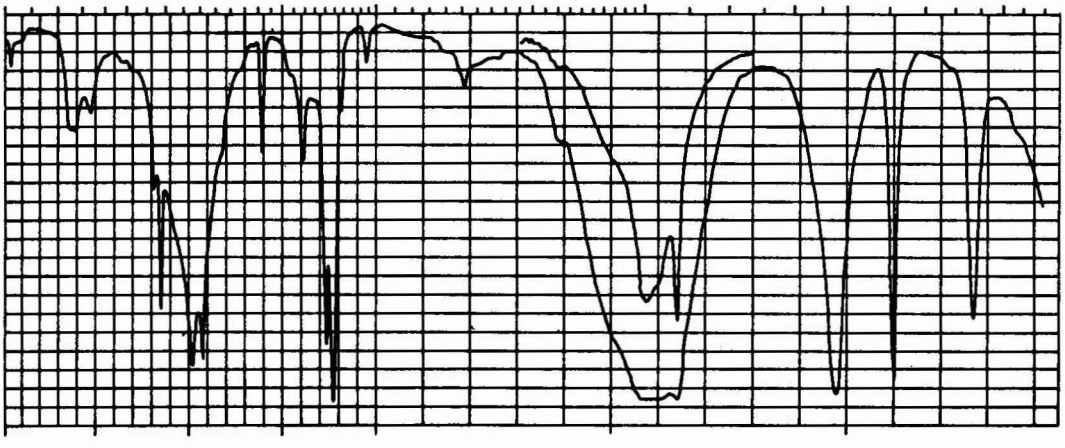
Printed in The Netherlands

CONTENTS

(Continued overleaf)

Contents (continued)

RS solvents for UV and IR spectrophotometry



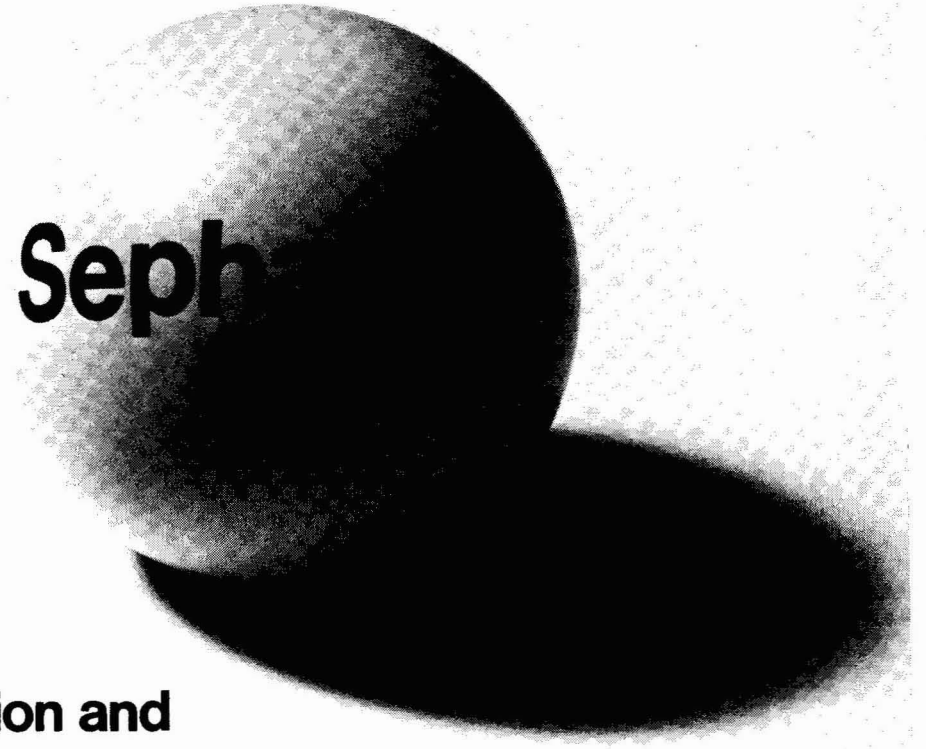
Acetone UV and IR
Acetonitrile UV and IR
Benzene UV and IR
Carbonium sulfide UV and IR
Carbonium tetrachloride UV and IR
Chloroform UV and IR
Cyclohexane UV and IR
N-N-Dimethylformamide UV and IR
Dichloroethane IR
Dimethylsulfoxide UV
Dioxane UV
Ethyl acetate IR
Ethyl alcohol UV 95° and abs.
Ethyl ether UV
n-Heptane UV
n-Hexane UV
Isoctane UV and IR
Isopropyl alcohol UV
Methylene chloride UV and IR
Methyl Alcohol UV
n-Pentane UV
Potassium bromide IR
Tetrachloroethylene IR
Tetrahydrofuran UV and IR
Toluene IR
Trichloroetilene IR

MONTEDISON GROUP
CARLO ERBA

CHEMICALS DIVISION
P.O. Box 3996/20159 Milano/Via Imbonati 24 (Italy)
Telex Erba Mi 36314/Tel. 6995

Ref.no. 224

And now.....



Gel filtration and Affinity chromatography even in 6M GuHCl, organic solvents, 8M urea, 3M KSCN at pH3, pH12, 80°C

CROSS-LINKED SEPHAROSE is a new beaded agarose chromatographic medium with exceptional stability and extremely low non-specific adsorption. It can be used with confidence in the most extreme dissociating media including strong solutions of urea, guanidine hydrochloride and chaotropic salts. Sepharose CL can also be used in organic solvents.

You can sterilize Sepharose CL repeatedly by autoclaving at 110°C - 120°C. Cross-linked Sepharose is available in three types: Sepharose® CL-2B, CL-4B and CL-6B.

Ask for the free booklet with data and details of new techniques, from your supplier of Sephadex®, Sepharose® and other separation media.

Pharmacia Fine Chemicals
Box 175
S-751 04 Uppsala 1
Sweden

ref. nr. 354

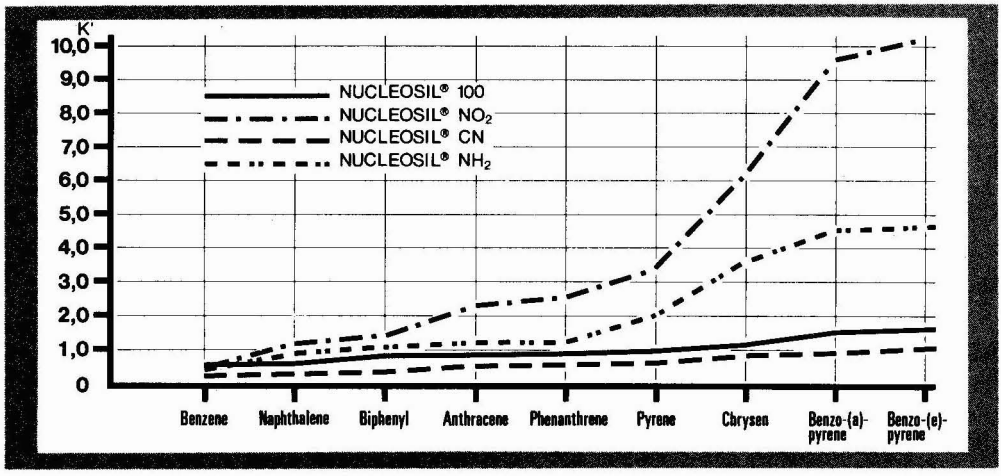
Pharmacia
Fine Chemicals

MN - "THE CHOICE"

HPLC Packings + Prepacked Columns

Your Question:	Our Answer:
Are spherical and totally porous packings available?	YES! NUCLEOSIL®
Are special narrow particle size ranges available?	YES! 5 ± 1.5 µm; 7.5 ± 1.5 µm; 10 ± 1.5 µm
Are different types available?	YES! NUCLEOSIL® for Adsorption and Partition NUCLEOSIL® for Reverse Phase, C_8 and C_{18} groups chemically bonded to silica gel NUCLEOSIL® CN, NO_2, NH_2 or $N(CH_3)_2$ groups chemically bonded to silica gel NUCLEOSIL® for Ion Exchange SA type: strongly acidic, SO_3H groups SB type: strongly basic, $N^+(CH_3)_2Cl^-$ groups
Are irregular gel packings available?	YES! HPLC-Sorb SIL 60 and SIL 60-D
Are prepacked columns available?	YES! 25 (twenty-five) different types of 20, 25 and 30 cm length for all HPLC apparatus
Are "Know-how" instructions of packing methods available?	YES! Have a look in our catalog
Any additional questions?	Request a free copy of our new 36 pages HPLC catalog with 323 literature references

Influence of the functional groups upon the capacity ratio (k') of aromatics. Mobile phase: n-heptane, non dried (100 ppm water).



MACHEREY-NAGEL + CO MN

P.O.Box 307 · D-5160 Düren, Germany · Phone (02421) 61071 · Telex 0833893 mana d

ref. no. 331

Journal of Chromatography, 136 (1977) 189–198

CHROM. 9928

PREPARATIVE COUNTERCURRENT CHROMATOGRAPHY WITH A SLOWLY ROTATING HELICAL TUBE

Y. ITO and R. L. BOWMAN

National Heart, Lung, and Blood Institute, Laboratory of Technical Development, Bethesda, Md. 20014 (U.S.A.)

(Received January 7th, 1977)

SUMMARY

The capability of a simple countercurrent chromatographic scheme for obtaining high-resolution preparative-scale separations was demonstrated with the separations of a series of dinitrophenyl (DNP) amino acids and peptides. Basic studies on stationary phase retention and partition efficiency with a low-viscosity chloroform–acetic acid–0.1 *N* hydrochloric acid (2:2:1) phase system together with the results previously obtained with a viscous *n*-butanol system suggest a general applicability of low-interfacial tension phase systems by the present method.

INTRODUCTION

A simple preparative countercurrent chromatographic scheme has been devised to extend the usefulness of previously described schemes[1,2]. We use a coiled tube rotating slowly in the gravitational field while the mobile phase countercurrents through the stationary phase trapped in each turn of the coil. The principle of the method and the preliminary test results using a *n*-butanol–acetic acid–water (4:1:5) phase system have been reported earlier[3]. Further investigations have demonstrated a broad applicability of low-interfacial tension two-phase solvent systems having various phase properties.

For the present studies, a two-phase system composed of chloroform–acetic acid–0.1 *N* hydrochloric acid (2:2:1) has been selected because of its contrasting phase properties to the *n*-butanol system previously examined. Basic studies on retention of the stationary phase and efficiency of separation are performed according to the procedure previously used for the *n*-butanol phase system in order to compare results obtained with these two solvent systems. Under the optimum operational conditions obtained for these phase systems, preparative-scale separations of a series of dinitrophenyl (DNP) amino acids and peptides are performed using a sample volume of 10 ml each.

EXPERIMENTAL

Apparatus

The test system used in the experiment is illustrated in Fig. 1. A separation column which consists of nine units of coiled PTFE tubes connected in series is mounted around the hollow rotary shaft equipped with a rotating seal at each end. The rotary shaft is driven by a motor (Electro-craft Co.) through a pair of toothed pulleys coupled with a toothed belt. The frame holding all these elements is made adjustable at a desired angle by tightening the center screw against the standing support. A Chromatronix metering pump is used to pump the solvent into the column through a bubble trap, a sample port and then the first rotating seal (right) and the eluate collected through the second rotating seal (left) is monitored with an LKB Uvicord III at 280 nm. Each rotating seal is fabricated with Kel-F block and a PTFE o-ring to prevent corrosion.

Each column unit is prepared by winding PTFE tubing (Zeus Industrial Products, Rariton, N.J., U.S.A.) 5 m long and 2.6 mm I.D. onto a lucite pipe of 50 cm long and 1.25 cm O.D. to make approximately 100 turns with a capacity of 25 ml.

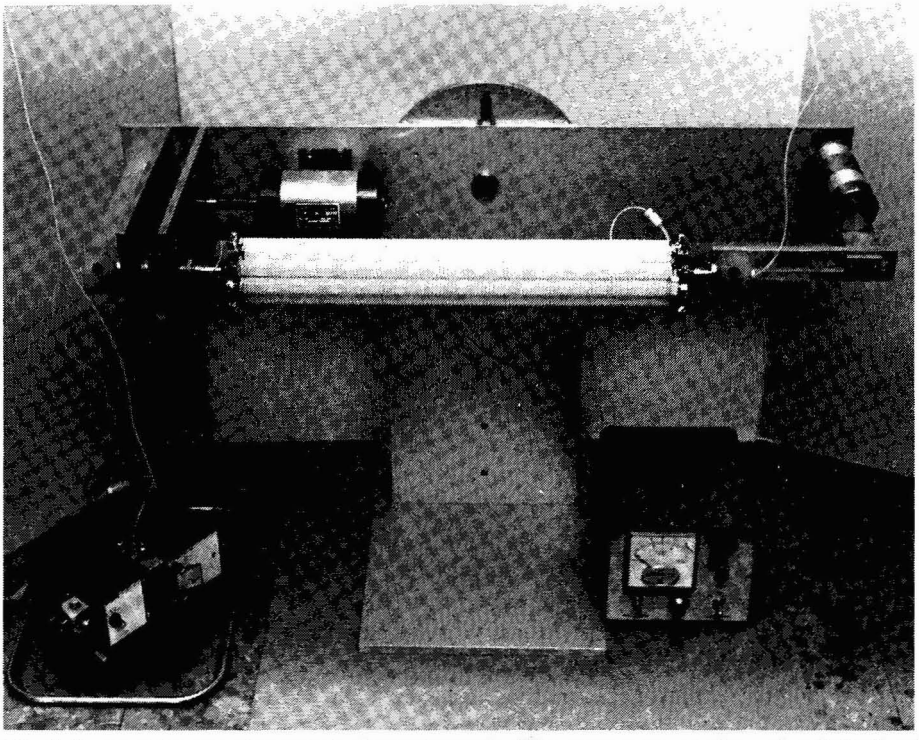


Fig. 1. Preparative countercurrent chromatograph. The apparatus consists of a helical column mounted around the rotary shaft equipped with a rotating seal at each end and driven by a motor through a pair of pulleys coupled with a toothed belt. The solvent is introduced into the column through the first rotating seal (right) and the eluate collected through the second rotating seal (left) is monitored with an LKB Uvicord III at 280 nm. The frame holding the rotary shaft is made adjustable to any desired angle.

The large-capacity preparative column as shown in Fig. 1 is made by connecting nine column units in series.

Two-phase system preparation

A two-phase solvent system composed of chloroform–acetic acid–0.1 *N* hydrochloric acid (2:2:1) is used. The phase mixture is equilibrated at room temperature and separated before use. A two-phase system composed of *n*-butanol–acetic acid–water at 4:1:5 volume ratio is similarly prepared for preparative separation of peptides.

Phase retention studies

Using a single-column unit, retention of the stationary phase is measured under various conditions with respect to column angle, rotational speed, and flow-rate. Both upper aqueous and lower organic phases are tested as a stationary phase. The phase distribution and behavior in the rotating coiled tube is conveniently studied if the stationary phase is colored with a dye which partitions almost entirely to the stationary phase. We used Sudan black B to color the lower phase and basic fuchsin for the upper phase. The column is first filled with the mobile phase and about 5 ml of the lightly colored stationary phase is introduced into the column which is held vertical so that the stationary phase is completely separated from the mobile phase. The length of the column occupied by the colored stationary phase is defined as A. When the column is set to the desired angle, rotated and eluted with the mobile phase at the desired rates, a dynamic equilibrium is reached and the length of the column now containing the colored stationary phase is defined as B. The retention percentage of the stationary phase (R) is given by $R = A/B \times 100$.

Partition efficiency studies

Two DNP amino acids (Sigma), DNP-L-glutamic acid (1.9) and DNP-L-alanine (0.56), are selected on the basis of their suitable partition coefficients indicated in the parentheses since these two mixtures give similar elution curves regardless of the choice of mobile phase. The sample mixture is dissolved into the upper aqueous phase at a concentration of 0.5 g% for each component and stored in dark at 4° before use. In each separation the column is filled with the stationary phase and 0.2 ml of sample solution is injected through the sample port followed by elution with the mobile phase under a given set of conditions of column angle, rotational speed, and flow-rate. Since DNP amino acids are decomposed by exposure to the light, the apparatus is covered with a black sheet during separation. The eluate is continuously monitored at 280 nm to record the elution curve. The partition efficiency in each separation can be evaluated from the relative height of the trough between the two peaks and/or by calculating the number of theoretical plates (T.P.) for each component if the two peaks are well resolved.

RESULTS AND DISCUSSION

Phase retention studies

Retention of the stationary phase in the rotating coiled tube examined under various operative conditions is illustrated in Fig. 2. In each diagram the percentage

of stationary phase volume occupying the column space is plotted against the applied column angle, where 0° indicates the horizontal column position, + 90° and − 90° being the vertical column position with the inlet end upward and downward, respectively. Several curves drawn in each diagram show the effects of flow-rate. Ideal phase retention close to 50% is observed in a wide range of the column angle between 0° ± 60° in most cases.

Each curve exhibits a characteristic shape similar to those observed with the *n*-butanol phase system. An abrupt decrease or increase of phase retention around the horizontal column position is produced by the change of the flow pattern from the

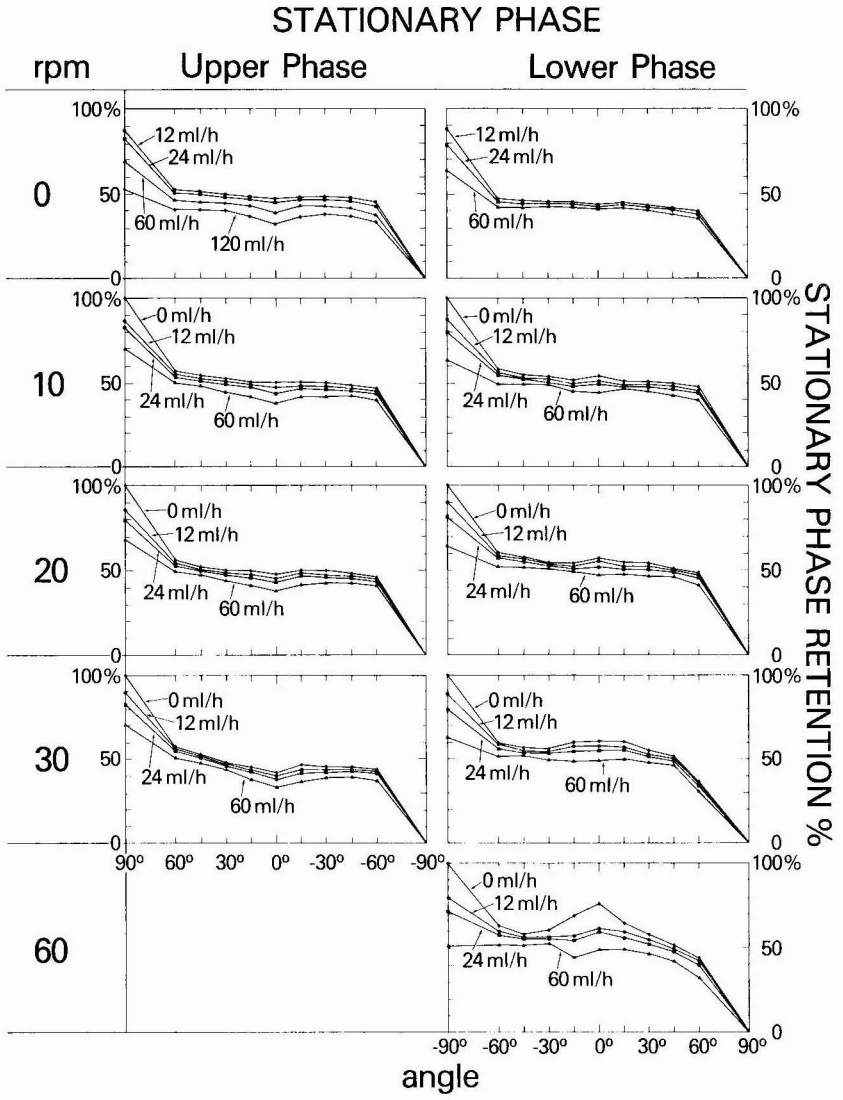


Fig. 2. Retention of the stationary phase in relation to the column angle, rotational speed and flow-rate. The column angle is expressed as 0° being horizontal.

laminary to the droplet. The size of the droplets decreases with the rotational speed and becomes much smaller than the internal diameter of the column at higher rotational speeds. These droplets, however, can be trapped in the coiled tube by gravity to maintain a satisfactory phase retention in the present phase system.

Comparative studies revealed that in both chloroform–acetic acid–0.1 *N* hydrochloric acid (2:2:1) and *n*-butanol–acetic acid–water (4:1:5) phase systems the aqueous phase, having less affinity to the tube wall, tends to form droplets and, if

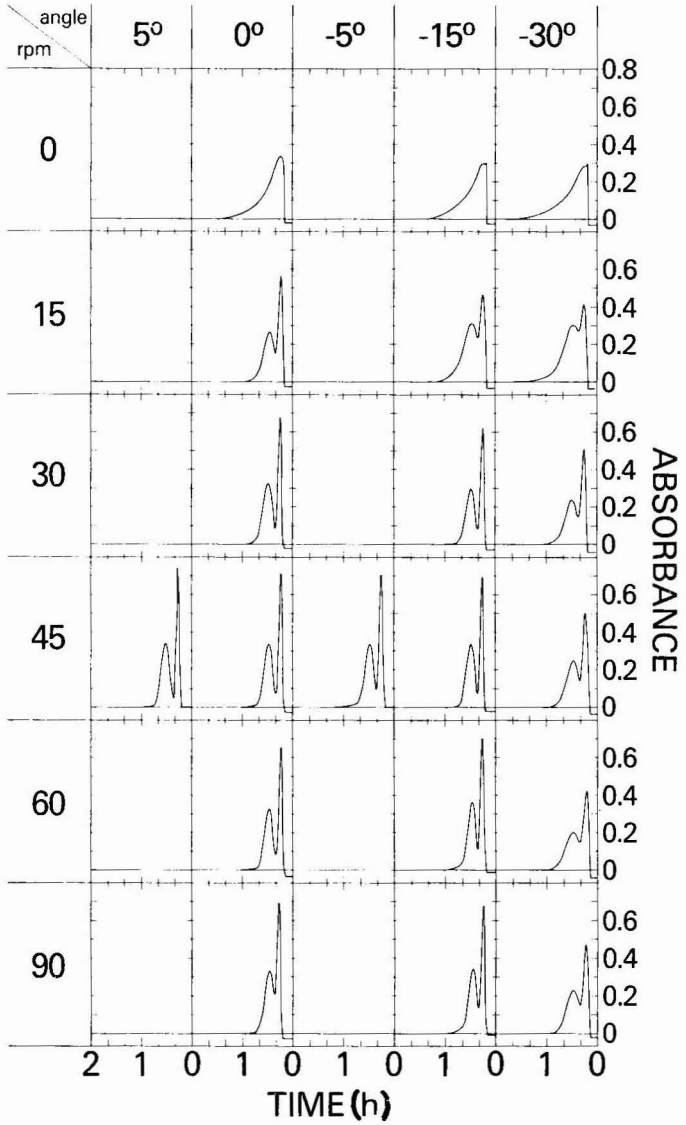


Fig. 3. Effects of column angle and rotational speed on the separation of DNP amino acids. The first peak is DNP-L-glutamic acid; the second, DNP-L-alanine; mobile phase, upper phase; flow-rate, 60 ml/h.

used as the mobile phase, it allows higher rotational speeds or flow-rates for satisfactory phase retention than if used as the stationary phase. The present phase system, which has lower viscosity and a greater density difference between upper and lower phases, permits operations at higher rotational speeds and flow-rates.

Several other two-phase solvent systems have also been tested for phase retention. Satisfactory retention is found with extremely low interfacial tension phase systems such as ethylene glycol ethers–various salt solutions used for separation of proteins[4,5]. However, high interfacial tension phase systems including hexane–water and ethyl acetate–water produce a plug flow in the presently used column and it is necessary to increase the diameter of the column for successful operation.

Partition efficiency studies

Effects of the column angle and rotational speed on separation of two DNP amino acid samples are shown in Fig. 3, where the upper aqueous phase is used as the mobile phase at a flow-rate of 60 ml/h. The partition efficiency is conveniently estimated from the relative height of the trough between the two peaks. The best separation is obtained at 45 rpm with the column set close to the horizontal position. The efficiency rises sharply with the rotational speed from 0–30 rpm.

Using the horizontal column position, further experiments are performed to study the effects of flow-rate and rotational speed on partition efficiency. Fig. 4 shows the results obtained by using the upper aqueous phase as the mobile phase. The best resolution is seen at 60 rpm with a slow flow-rate of 12 ml/h, while the highest partition efficiency in terms of T.P./time is achieved at 45 rpm with a higher flow-rate of 60 ml/h. Fig. 5 shows similarly the results obtained by using the lower organic phase as the mobile phase. Here, the best resolution is seen at 20 rpm with a flow-rate of 24 ml/h where the T.P./time figure shows little improvement with a higher flow-rate of 60 ml/h.

Comparing the above results with those previously obtained with the *n*-butanol phase system, it may be concluded that the aqueous phase, if used as the mobile phase, allows a higher rotational speed and flow-rate to yield a higher partition efficiency regardless of the viscosity of the organic phase. The higher partition efficiency achieved by the present phase system may be attributed to the lower phase viscosity and the greater density difference between the upper and the lower phases.

Preparative countercurrent chromatograms

Preparative-scale separation is carried out with the column consisting of nine column units connected in series which has approximately 1000 turns and 240 ml in capacity. In each separation, the sample mixture is dissolved in the stationary phase and a sample volume of 10 ml is applied.

Fig. 6 shows three chromatograms of DNP amino acids on the present phase system composed of chloroform–acetic acid–0.1 *N* hydrochloric acid (2:2:1). The top and middle chromatograms are obtained by using the upper aqueous phase as the mobile phase under optimum operative conditions of 45 rpm at flow-rates of 60 ml/h and 24 ml/h, respectively. The bottom chromatogram is obtained by using the lower organic phase as mobile phase at 20 rpm and a flow-rate of 24 ml/h.

Fig. 7 shows preparative chromatograms of peptides on the phase system com-

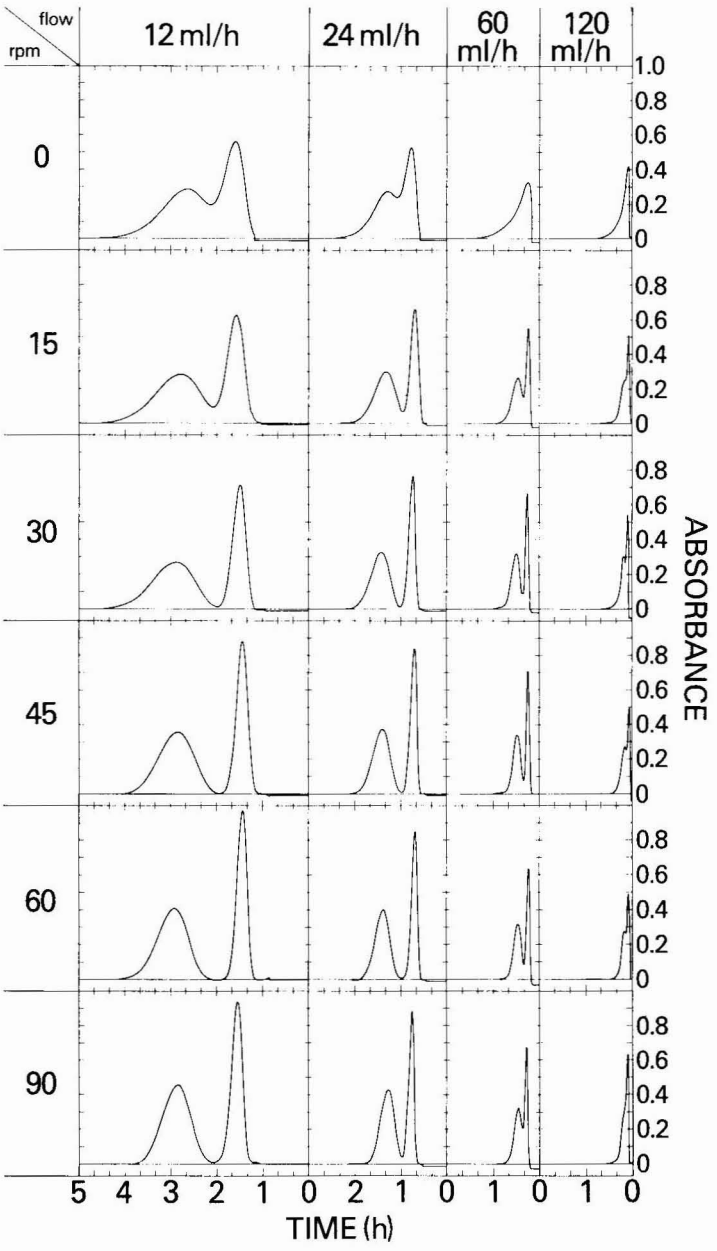


Fig. 4. Effects of flow-rate and rotational speed on the separation of DNP amino acids. The first peak is DNP-L-glutamic acid; the second, DNP-L-alanine; mobile phase, upper phase; column angle, 0°.

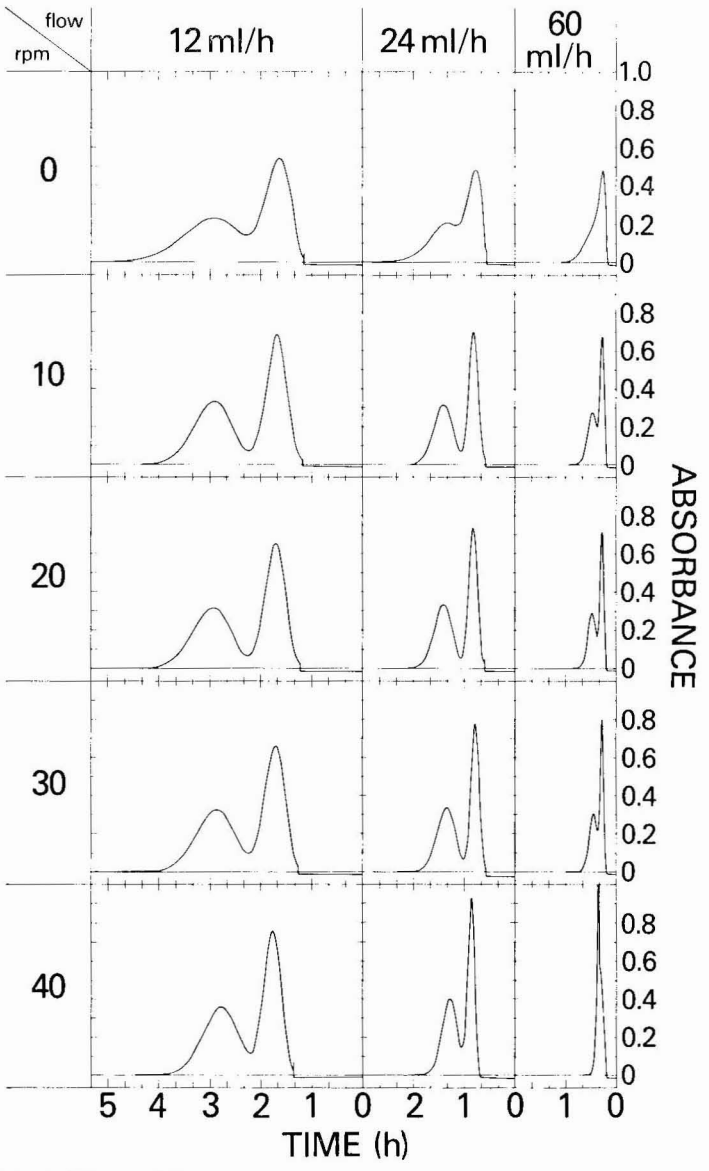


Fig. 5. Effects of flow-rate and rotational speed on the separation of DNP amino acids. The first peak is DNP-L-alanine; the second, DNP-L-glutamic acid; mobile phase, lower phase; column angle, 0°.

posed of *n*-butanol–acetic acid–water (4:1:5) with the same preparative column. The top chromatogram is obtained by using the lower aqueous phase as a mobile phase at 15 rpm and 12 ml/h of flow-rate while the bottom is obtained by using the upper organic phase as a mobile phase at 10 rpm and 12 ml/h.

These preparative separations were produced on the two typical phase systems

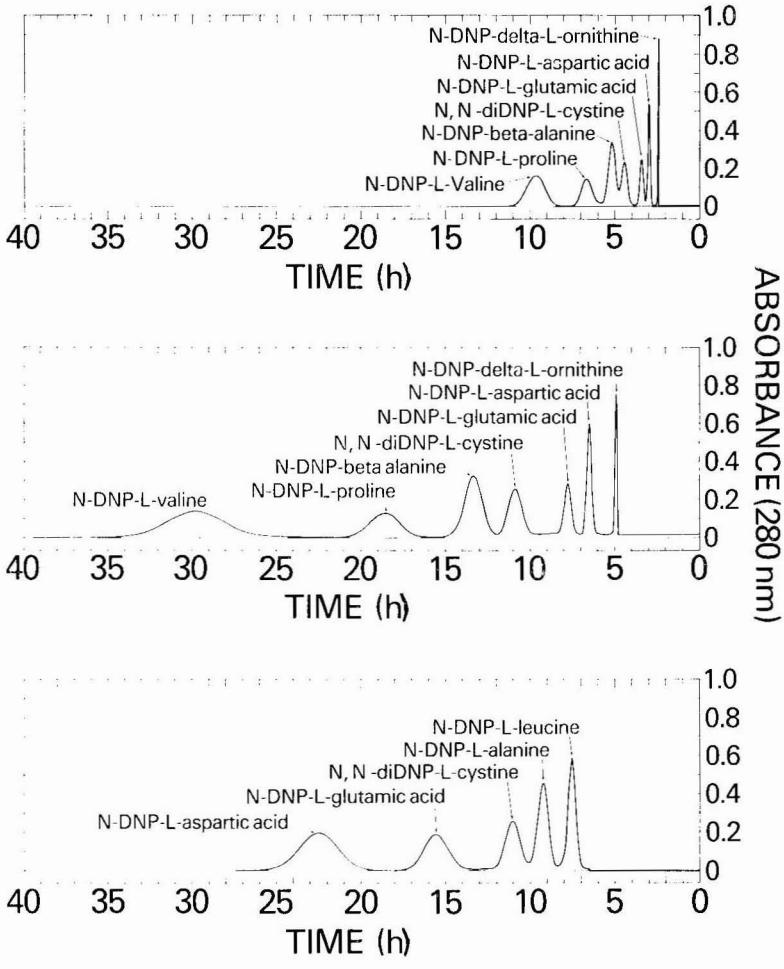


Fig. 6. Preparative countercurrent chromatograms of DNP amino acids on chloroform–acetic acid–0.1 *N* hydrochloric acid (2:2:1). Top: mobile phase, upper phase; flow-rate, 60 ml/h; rotation, 45 rpm; column angle, 0°. Middle: mobile phase, upper phase; flow-rate, 24 ml/h; rotation, 45 rpm; column angle, 0°. Bottom: mobile phase, lower phase; flow-rate, 24 ml/h; rotation, 20 rpm; column angle, 0°.

which differ greatly in viscosity and phase density difference. We think that other low interfacial tension phase systems used for extraction or partition chromatography may be applied successfully by choosing the above operational conditions with minor modifications. The present countercurrent chromatographic scheme allows 10-ml sample charge to yield a high partition efficiency ranging from one thousand to several hundred theoretical plates where either aqueous or organic phase can be used as the mobile phase.

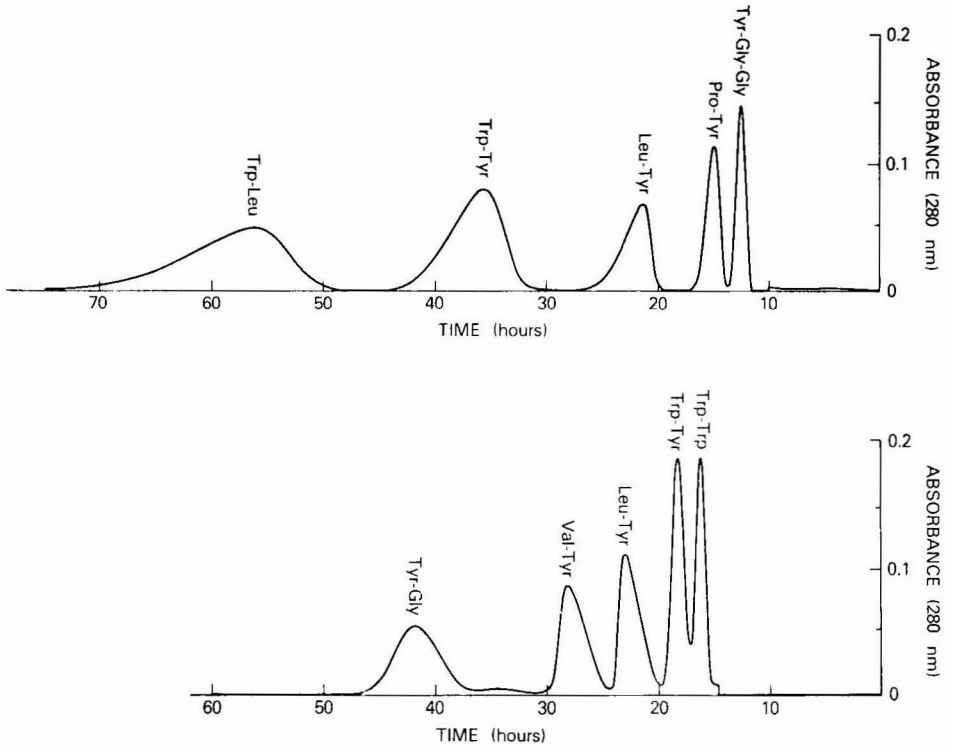


Fig. 7. Preparative countercurrent chromatograms of peptides on *n*-butanol–acetic acid–water (4:1:5). Top: mobile phase, lower phase; flow-rate, 12 ml/h; rotation, 15 rpm; column angle, 0°. Bottom: mobile phase, upper phase; flow-rate, 12 ml/h; rotation, 10 rpm; column angle, 0°.

REFERENCES

1 Y. Ito and R. L. Bowman, *Anal. Chem.*, 43 (1971) 69A.
2 Y. Ito, R. E. Hurst, R. L. Bowman and E. K. Achtor, *Sep. Purif. Methods*, 3 (1974) 133.
3 Y. Ito and R. L. Bowman, *Anal. Biochem.*, in press.
4 A. J. P. Martin and R. R. Porter, *Biochem. J.*, 49 (1951) 215.
5 R. R. Porter, *Biochem. J.*, 53 (1953) 320.

Journal of Chromatography, 136 (1977) 199–212

CHROM. 9956

OPTIMISATION OF THE PERFORMANCE OF A THREE-ELECTRODE THERMIONIC DETECTOR FOR THE DETECTION OF PHOSPHORUS, SULPHUR AND NITROGEN COMPOUNDS BY GAS CHROMATOGRAPHY

R. A. HOODLESS, M. SARGENT and R. D. TREBLE

Department of Industry, Laboratory of the Government Chemist, Cornwall House, Stamford Street, London SE1 9NQ (Great Britain)

(Received December 10th, 1976)

SUMMARY

The main operating parameters and the performance of a commercial thermionic detector have been investigated. The variation of response to phosphorus-, sulphur- and nitrogen-containing compounds is recorded as a function of the position of the electrode assembly for a wide variety of flame conditions. The maximum sensitivities obtainable with the detector are compared with those of the flame ionisation detector (FID). It is shown that the detector may be operated in various modes to improve the simplicity of operation for routine use, to obtain selective responses individually for each type of compound or to give equal response to P-, S- and N-containing compounds while discriminating against hydrocarbons. The recommended conditions for obtaining the various operating modes are summarized and suggestions are made as to the best way of reliably setting-up the detector and using it for routine analysis. Although the detector's sensitivity is by far the greatest for P-compounds, its utility as a selective detector for either S- or N-compounds is also apparent.

INTRODUCTION

The alkali flame ionisation detector (AFID) or thermionic detector (TD) has appeared in several forms since it was first described by Karmen and Giuffrida[1] in 1964 and has been the subject of numerous published studies of varying complexity[2–8]. Nevertheless, there still remains some doubt as to the type of response obtained from each detector design, the way in which performance can vary with operating conditions and whether or not particular designs give a sufficiently stable and reproducible response for routine gas chromatography.

An investigation has been made into the performance of a commercial, three-electrode detector[9] in which the alkali metal salt is mounted in a negatively charged cylinder held just above the jet, while the jet and a central collector electrode are earthed. The effect of various operating parameters on the response of the detector to compounds containing P, N or S atoms is reported. It is shown that even with a

single detector design several different modes of operation can be identified and that it may be necessary to sacrifice some of the inherent sensitivity or selectivity of the detector in return for simple and reliable routine use.

EXPERIMENTAL

Apparatus

The work was carried out using a Pye 104 gas chromatograph fitted with dual FID–thermionic detectors mounted in a temperature stabilised oven. The TD is a standard Pye design supplied as a "nitrogen-selective" detector by using a larger jet aperture and rubidium chloride as the alkali metal salt[10]. The collector electrode and the rubidium chloride crystal and its holder are arranged as a central probe assembly, the performance of the detector being varied by changing the height of this probe relative to the jet. To facilitate the large number of measurements required, a micrometer attachment was fitted to the probe so that its height could be rapidly set to any required value. For routine work, washers of known thickness were used to ensure reproducible settings of the height. The standard gas controls were used for the FID but it was found essential to use precision regulators and gauges for the TD. The equipment used (summarized in Table I) allowed the air and hydrogen flow-rates to be maintained within limits of 0.1 and 0.02 ml/min, respectively. The auxiliary nitrogen flow was supplied to the detector by a connection to the column outlet, allowing the total nitrogen flow through the detector to be increased while maintaining the carrier gas flow constant at 25 ml/min. All measurements were made using 1.5 m × 4 mm I.D. glass columns packed with 5% OV-17 on Gas-Chrom Q.

The rubidium chloride crystal and its holder (a perforated, cylindrical electrode concentric with the detector body and the central collector electrode) were maintained at −170 V. Signals on the collector electrode were measured with a Pye wide-range amplifier giving a maximum sensitivity of 1×10^{-12} A f.s.d. when used in conjunction with a 10-mV chart recorder. The detector was modified to provide rigid clamping of

TABLE I

GAS CONTROLS USED WITH THERMIONIC DETECTOR

Gas	*Type of regulation*	*Range of flow-rate (ml/min)*	*Regulator*	*Gauge*
Nitrogen to column	Constant flow controller	0–60	Brookes, type 8744A	None
Auxiliary nitrogen	Fixed orifice and constant pressure regulator	0–60	Manostat, type 10A	Budenburg, 3-in. Simplex gauge, 0–30 p.s.i.
Air	Fixed orifice and constant pressure regulator	0–550	Manostat, type 10A with multi-turn control spindle	Budenburg, 4-in. bronze tube gauge 3–15 p.s.i.
Hydrogen	Fixed orifice and constant pressure regulator	0–60	Brookes, type 8601	Budenburg, 10-in. standard test gauge, 0–20 p.s.i.

the connecting cable at the point of entry; with the original design it was found that any movement of the cable caused slight movement of the collector electrode and considerably increased the noise level of the detector. This modification resulted in excellent noise levels without the need to resort to additional damping of the recorder signal.

Performance studies

The total number of variable parameters with this type of detector is large. It includes the nitrogen, air and hydrogen flows, the height of the collector electrode above the flame, the position and shape of the alkali crystal relative to the collector and the flame, the polarizing voltage applied to the crystal, the temperature of the detector oven, and the type of alkali salt used. In order to reduce the complexity of the task several of the parameters were fixed, the selection being based on experience with the use of the detector as well as on practical limitations. Thus only RbCl crystals were used, although CsBr is also available from Pye and other salts can be pressed into suitable holders without difficulty. The detector oven was maintained at 350° (the maximum usable without overheating the coaxial lead to the collector); lower temperatures simply reduce the background current obtained with a given set of conditions. The polarising voltage was kept constant as it produces only small changes in response provided the field strength is sufficient to ensure proper collection of ions.

The geometry of the detector (*i.e.*, the position of the electrodes and crystal relative to the flame) is of considerable importance. However, the design of the Pye detector makes it difficult to observe the effects of each parameter unambiguously, the effects of the detector geometry being closely linked to the flame conditions since changes of gas flow vary not only the flame composition and temperature but also its size and shape. The height of the central collector electrode is a critical parameter, as at least part of the selectivity of the thermionic detector is thought to arise from differences in the lifetimes of the ions formed by different heteroatoms. That is, these ions persist to different heights in and above the flame so that a change of collector electrode height can vary the relative responses of different heteroatoms. With the Pye design, variation of the collector height by movement of the central probe simultaneously changes the position of the RbCl crystal. It is possible to avoid this by repositioning the crystal in the holder but this is laborious and difficult to carry out accurately. Movement of the crystal has two possible effects; the most important is the change in rate of vaporisation of RbCl (*i.e.*, a change in background current) as the temperature of the crystal surface varies. A second effect may occur with the Pye design because a negative potential is applied to the crystal to remove unwanted ions (*e.g.*, hydrocarbon ions formed by a normal FID mechanism) before they reach the collector electrode. Thus, movement of the crystal assembly may alter the response of the detector to "unwanted" compounds such as hydrocarbon solvents, even though there should be no direct effect due to the change in background current.

Experience with the detector suggests that an increase of background current increases the sensitivity of the detector to those heteroatoms responding through the thermionic mechanism, but has little effect on their relative response. In normal use, the maximum usable value of the background current is governed by the increases of signal noise and of background drift as the crystal burns away more rapidly. For most of the experiments described here it was decided to locate the crystal in its

holder so that a usable background current was obtained over a reasonable range of probe heights, allowing the effect of changes in collector height to be observed. The best location was found to be where the tip of the jet is level with the lower rim of the crystal when the central probe is at its lowest position. This setting gives the minimum separation of the flame and the collector electrode, the end of the collector then being 8.5 mm above the tip of the jet. Thus, this figure should be added to the probe heights given in the results in order to obtain the true height of the collector.

As mentioned above, changes in the gas flow-rates vary the dimensions of the flame. Thus the effect of a change in flame conditions will also depend on the position of the probe assembly. It was therefore decided to base the main study of the detector's parameters on plots of the effect of probe height on background signal and response under different flame conditions. The sets of flame conditions were chosen so as to illustrate the effects of air flow-rate, of the total hydrogen plus nitrogen flow-rate through the jet, and of the hydrogen to nitrogen ratio. In order to carry out these measurements it was necessary not only to decide on the best compromise for the position of the RbCl crystal in its holder (see above), but also the way in which it should be "conditioned" to the flame shape. As RbCl is vaporised during operation, a new crystal tends to lose RbCl until its inner surface has assumed the shape of the flame, when it settles down to a steady background current with low loss of RbCl. Any change in the flame conditions or probe height which produces a large increase in background signal will therefore irreversibly alter the shape of the crystal. Conditioning of the crystal was standardised by using the following procedure:

(1) Set the crystal in the probe assembly so that its metal holder protrudes 11.5 mm and check the parallel alignment of crystal, collector and probe assembly.

(2) Light the flame with gas flow-rates of air 100, nitrogen 30, and hydrogen 30 ml/min. Push the probe assembly hard down into the detector body taking care not to extinguish the flame.

(3) Increase the air flow-rate until a maximum background current is obtained.

(4) Leave the crystal to burn in this way for about 5 min, then reduce the air flow-rate to obtain a background current of about 1×10^{-11} A.

(5) Leave running for about 1 h or until steady conditions are obtained.

(6) Reset the air flow-rate to 100 ml/min and adjust the probe height for maximum background current. If the crystal is correctly conditioned, a background current of 5×10^{-12} to 5×10^{-11} A should be obtained.

(7) Set optimum conditions for the performance required.

Using this technique it was found that a sufficiently large background signal for testing the response was obtained with a wide range of operating conditions but the loss of RbCl only occasionally became high enough to alter the shape of the crystal significantly. Where that did occur the set of measurements was completed after allowing the system to stabilise, but a new crystal was conditioned as above before proceeding further. This would not be necessary for routine use of the detector, as a used crystal (including one giving erratic signals due to a worn RbCl surface) can be reconditioned several times using the same technique. Usually it is then necessary to increase the nitrogen and/or hydrogen flow-rates in order to burn away the crystal significantly. When this procedure is used the setting of the probe height for a particular mode of operation will usually change slightly.

Most of the measurements were carried out using 1-μl injections of hexane solutions of triethyl phosphite, dibutyl sulphide, *o*-toluidine and tetradecane. These compounds were chosen to allow easy interpretation of the results, each containing only one atom of one of the elements of interest. They are also well separated on the OV-17 column at 120°–140° and simultaneous injections are possible.

Concentrations of 1 to 1 $\times$ 10^4 μg/ml were used as appropriate. Most of the results were recorded as peak areas in units of C/mole to provide correction for the differing retention times and molecular weights.

RESULTS AND DISCUSSION

A large part of the experimental work was based on plots of signal *versus* probe height such as the example shown in Fig. 1. The shape of the curves varies with the conditions used, a major factor being the range of probe heights over which a measurable response can be obtained. However, those shown are fairly typical with a background current which is relatively constant until it rapidly drops to zero when the probe is raised too far. The increased nitrogen response at higher probe settings was observed for a wide range of gas flow-rates but the relative sizes of the P, S and N signals vary considerably, allowing the selectivity of the detector to be altered.

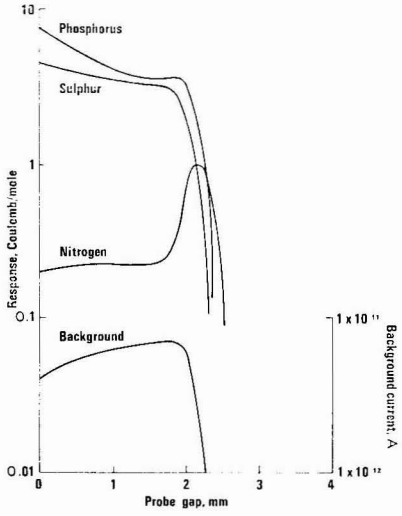


Fig. 1. Variation of detector response with probe height. Flow-rates: air, 100 ml/min; nitrogen, 30 ml/min; hydrogen, 30 ml/min.

The results obtained from these plots of signal *versus* probe height are summarized in Fig. 2 (effect of air flow-rate), Fig. 3 (effect of total hydrogen and nitrogen flow-rate) and Fig. 4 (effect of nitrogen–hydrogen flow-rate ratio). Increased air flow-rate was found to increase the maximum attainable response while reducing the range of probe heights for which a response can be obtained. This effect continues up to the maximum of 550 ml/min air obtainable from the gas control unit, but the effect of probe movement was too critical above about 300 ml/min to obtain reproducible plots. Increasing the total (hydrogen + nitrogen) flow-rate had the converse

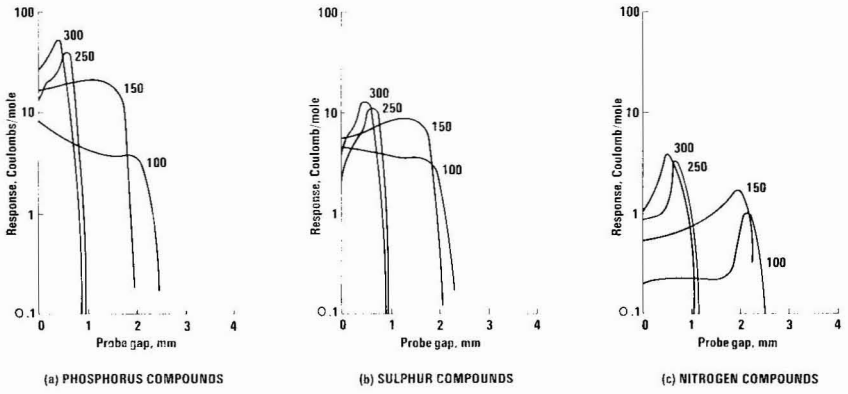


Fig. 2. Detector response at different air flow-rates. Air flow-rates shown in ml/min. Hydrogen flow rate, 30 ml/min; nitrogen flow-rate, 30 ml/min.

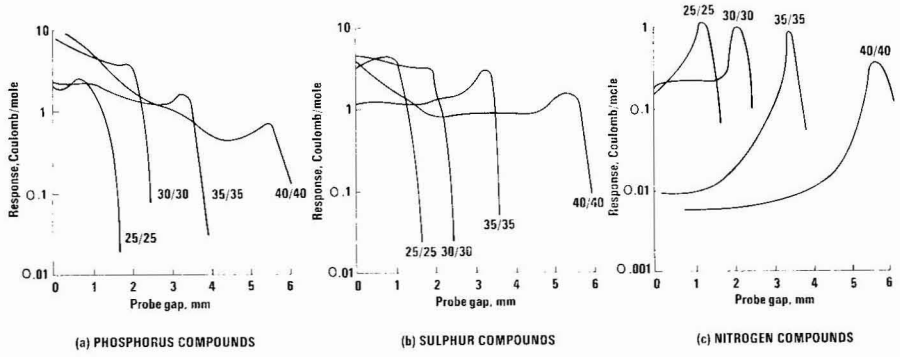


Fig. 3. Detector response at different total (hydrogen + nitrogen) flow-rates. Nitrogen/hydrogen flow-rates shown in ml/min. Air flow-rate, 100 ml/min.

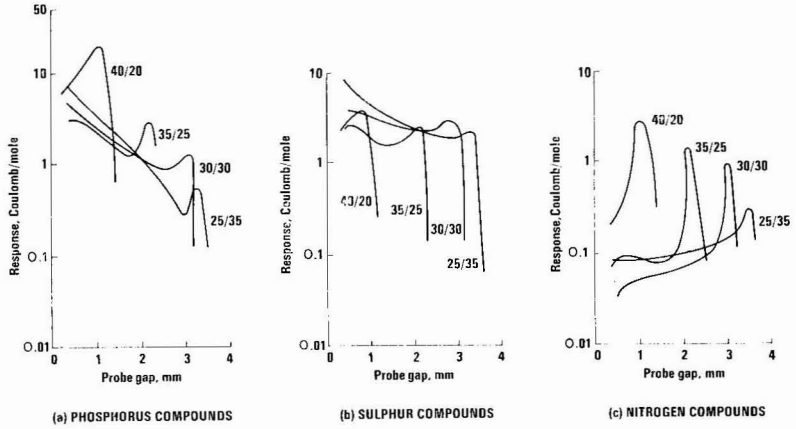


Fig. 4. Detector response at different nitrogen/hydrogen ratios. Nitrogen/hydrogen flow-rates shown in ml/min. Air flow-rate, 100 ml/min.

effect on usable probe range, *i.e.*, the range increases with flow-rate, while the sensitivity decreases somewhat (particularly for phosphorus). A total of 60 ml/min seems to offer a useful probe range for many purposes (with air at 100 ml/min) and was used to test the effect of nitrogen/hydrogen ratio. As shown in Fig. 4, the range of usable probe heights increases as the mixture is varied from nitrogen rich to hydrogen rich. At the same time, phosphorus and nitrogen compounds show a considerable drop in sensitivity, whereas that for sulphur compounds remains fairly constant. Thus use of a hydrogen rich flame allows operation of the detector in its most sulphur specific mode.

While recording the data for these plots, it was realised that they showed a steady trend: the smaller the probe gap corresponding to the maximum of a particular plot, the greater the sensitivity at that maximum. This effect appears to be independent of the combination of the other variables leading to a maximum at any given probe height. The consistency of this trend can be seen in Fig. 5 in which all the points plotted correspond to maxima of the plots in Figs. 2–4. Thus if the sole objective when setting up the detector is maximum sensitivity, it should be used with the probe in its lowest position and the other variables adjusted to give optimum performance at this point.

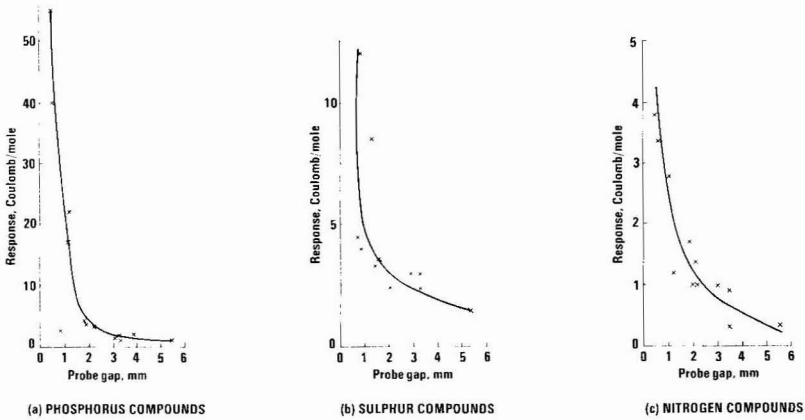


Fig. 5. Dependence of maximum attainable detector response on probe height. Each point on the curves represents one of the maxima obtained on the curves in Figs. 2–4.

The variation of detector response to hydrocarbon compounds was not included in the above measurements even though it is of considerable importance from the point of view of detector specificity. This was unavoidable because of the complex variation of the hydrocarbon peak shape with both quantity injected and small variations of the probe height of the detector. Some idea of the situation can be obtained from Fig. 6 which shows chromatograms for tetradecane injected in quantities of 0.5–50 μg with three slightly different detector settings. It can be seen that the response may vary from a normal "positive" peak, through various stages of "reversal" (in which the peak is split into two by a central trough), until it becomes completely "negative". Furthermore this change can occur either with injected quantity at one detector setting (Fig. 6b) or at one concentration as a result of small

changes of probe height (*e.g.*, the 5- or 10-μg injections of Fig. 6). As a general rule, the hydrocarbon response (positive or negative) will be larger at higher probe settings but there is considerable variation, depending on the flame conditions. Fig. 6 also illustrates that, because of the effect of concentration, the solvent peak does not automatically indicate the type of hydrocarbon response to be expected from samples. However, with practice it can often give useful information on the mode in which the detector is operating (see below).

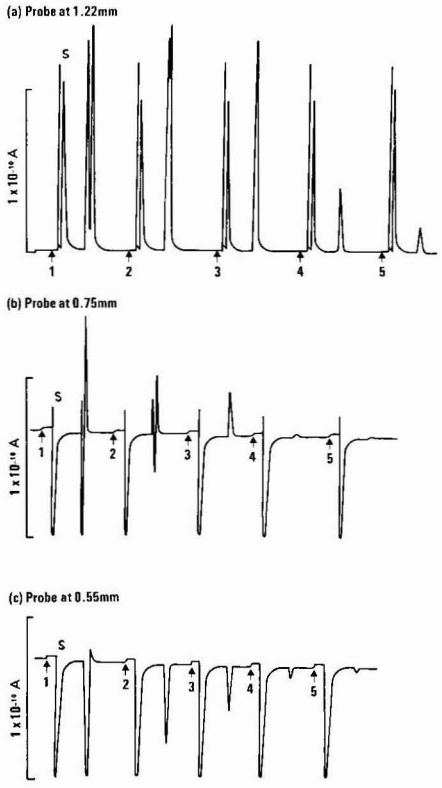


Fig. 6. Variation of hydrocarbon peak shape with concentration. Flow-rates: air, 100 ml/min; nitrogen, 30 ml/min; hydrogen, 30 ml/min. S = solvent (hexane) peak. Injected quantities of *n*-dodecane: 1 = 50 μg, 2 = 10 μg, 3 = 5 μg, 4 = 1 μg, 5 = 0.5 μg.

The effects of concentration on peak shape can also be seen to a lesser extent with compounds other than hydrocarbons once their linear response range is exceeded. This is particularly true of sulphur compounds and can result in chromatograms which can be highly misleading to the unwary. The effect is illustrated in Fig 7 which shows a set of injections of dibutyl sulphide at levels from 0.1 to 10 μg under detector conditions giving a narrow range of linear response for sulphur (see below). The retention time of the dibutyl sulphide is marked C and the solution also contains three trace sulphur-containing impurities (marked A, B and D) which were present in the sample. The first injection (0.1 μg) is approaching the limit of linear calibration for the main peak C under the conditions used and B and D are just apparent. This

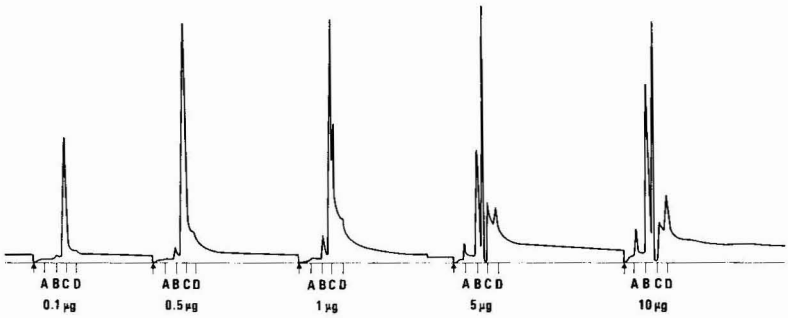


Fig. 7. Variation of sulphur peak shape with concentration. Flow-rates: air, 100 ml/min; nitrogen, 25 ml/min; hydrogen, 35 ml/min.

is the type of chromatogram the user would expect to obtain from this sample. At 0.5 μg, C has increased in height but the response is obviously non linear. B and D have increased as expected and A is now just apparent. By 1 μg A, B and D are still showing a normal, linear response but C is well into the "reversed" region and no longer increases in height. It could easily be mistaken for two partially resolved peaks. At 5 μg the "reversal" of C is comp ete; the centre now reaches zero and the right hand side has almost been lost. It has the appearance of another impurity giving a severely tailing peak. The left hand side is easily mistaken for a normal, well-resolved

TABLE II

COMPARISON OF FID AND THERMIONIC DETECTOR SENSITIVITIES

Responses are given in C/mole (signal measured as peak area) and in A/ng (signal measured as peak height and without any correction for the differing retention times of the test compounds); the latter are presented in parentheses.

Parameter	*Thermionic detector set to maximum sensitivity* for:*		*FID*
	Phosphorus and nitrogen	*Sulphur*	
Air flow-rate (ml/min)	550	450	400
Nitrogen flow-rate (ml/min)	68.0	68.0	25
Hydrogen flow-rate (ml/min)	34.0	34.5	25
Probe gap	zero	zero	
Background current (A)	1.2×10^{-9}	9.0×10^{-10}	8.0×10^{-12}
Background noise level (A)	1.0×10^{-12}	2.0×10^{-13}	2.0×10^{-14}
Triethyl phosphite response	3150 (3.1×10^{-9})	400 (4.0×10^{-10})	0.8 (1.0×10^{-12})
Dibutyl sulphide response	3.0 (2.5×10^{-12})	15.0 (1.2×10^{-11})	1.50 (1.3×10^{-12})
o-Toluidine response	13.5 (9.2×10^{-12})	2.2 (1.5×10^{-12})	1.0 (7.5×10^{-13})
Tetradecane response	0.5 (7.5×10^{-14})	−0.2 (-3×10^{-14})	2.4 (7.0×10^{-13})

* This does not necessarily correspond to a setting recommended for optimum detector performance (see text).

TABLE III

USEFUL OPERATING MODES OF THE THERMIONIC DETECTOR

Mode	*Probe position**	*Flame condition (flow-rate in ml/min)***			*Advantages*	*Disadvantages*	*Comment*
		Air	*Nitrogen*	*Hydrogen*			
1	Zero	550	68–70	34–35	Maximum sensitivity to P- and N-compounds with extremely sensitive, selective response to P-compounds in particular	Rapid consumption of crystal. Relatively poor stability and high noise level.	Should only be used for routine work when maximum sensitivity is essential.
2	Zero	450	68–70	34–35	Maximum sensitivity to S-compounds	As 1	As 1
3	Low	300	30	30	High sensitivity with high discrimination against hydrocarbons (which give a negative response)	Crystal consumption still high. Probe setting critical because of restricted usable range and rapid variation with probe height of the relative response to P, N or S.	Better than 1 and 2 but may cause problems when used routinely. Note that maximum sensitivity is not obtained simultaneously at one probe setting for all three types of compound.
4	Medium	250	30	30	Allows simultaneous determination of P-, N- and S-compounds with similar sensitivity while discriminating against hydrocarbons.	Sensitivity is similar to FID, but well below that attainable for each type of compound individually. Hydrocarbon response is positive, so that better selectivity against hydrocarbons can be obtained for each type of compound individually.	Satisfactory for routine use provided care is taken to standardise setting-up and operating techniques. Very useful for many trace analysis problems such as pesticide or drug residues.

5	High	100	30	30	As 4	As 4, except sensitivity is somewhat poorer.	As 4, except that operation in this mode is more convenient and stable. Particularly useful for long term operation with minimum attention.
6	High	100	25	35	Gives a selective response to S compounds against P and N compounds as well as hydrocarbons	Linearity range for S is restricted. Sensitivity well below maximum attainable for S.	Easy to use routinely. The linearity range for S can be increased by increasing the background (*e.g.*, increase hydrogen flow-rate) at the expense of less selectivity against P. This also considerably improves selectivity against N.
7	Medium to high	300	30	30	Gives a selective response to N compounds against P and S compounds as well as hydrocarbons	Better selectivity against hydrocarbons obtainable under other conditions. Much better sensitivity can be obtained if a larger P response can be tolerated.	Better for regular use than 3 because of the lower background current but the restricted range of probe movement means that practice is needed to set up this mode reproducibly.

* Relative to the range available under the flame conditions in use.

** These are intended only as a guide; the hydrogen flow-rate in particular should be optimised (see text).

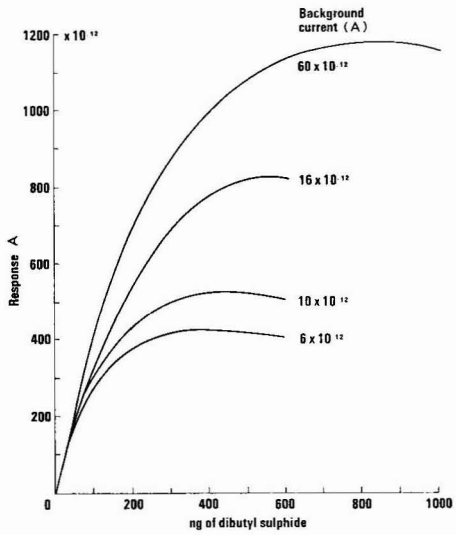


Fig. 8. Calibration curves for dibutyl sulphide at various background currents. Flow-rates: air, 100 ml/min; hydrogen, 28.5 ml/min. The nitrogen flow-rate was varied between 35 and 55 ml/min to obtain the range of background currents shown. The response was measured as the peak height.

peak. The three impurities continue to show a linear response. Finally at 10 μg there is little further change in C but B is now beyond the linear part of the calibration; at higher concentrations it, too, would begin to reverse.

Although similar effects are sometimes seen for P- and N-containing compounds, these give a much wider linear calibration range and problems would not usually arise under the conditions used to determine them. Even with sulphur compounds it is possible to extend the range of linearity (and increase the sensitivity) by working with a larger background current. This is apparent from Fig. 8 which shows a set of calibration curves for dibutyl sulphide obtained with detector background currents between 6×10^{-12} A (approximately the value in the chromatograms in Fig. 7) and 6×10^{-11} A.

In addition to its ability to give a selective response, the thermionic detector is widely recommended for its ability to show an enhanced sensitivity to certain compounds compared with the normal FID. The results shown in Table II represent the maximum sensitivity we were able to obtain with the detector but they could probably be improved by altering the shape of the crystal (by varying the conditioning procedure) to increase the background current. It can be seen that by far the greatest enhancement (several thousand fold) occurs with the phosphorus compounds for which the detector was originally recommended. For sulphur and nitrogen compounds the enhancement is much smaller (around ten fold) and from the point of view of detection limits is off-set by the higher signal noise levels. Thus for these compounds the detector is mainly of value for its selectivity. Although the sulphur response has been known for some time[11,12], it is only recently that it has been recognised[13,14] as of similar analytical utility to the widely used nitrogen response. Thus the data reported here for sulphur is of considerable practical importance. It should be emphasized, however, that the conditions used to obtain the maximum

sensitivities for all of the compounds in Table II are not normally suitable for routine operation of the detector for long periods. The high background current obtained (about 1×10^{-9} A) results from rapid vaporization of the RbCl crystal. Thus detector performance shows a steady drift, the crystal needs frequent replacement and it is necessary to remove RbCl from the collector electrode and the jet at regular intervals.

A more useful approach to routine use of the detector is to identify some of the possible modes in which it may be operated and then to select the most appropriate for any particular application, remembering that it is not usually possible to obtain maximum sensitivity, maximum selectivity and maximum convenience of use simultaneously even for just one compound. Table III represents an attempt to list some of the possibilities based on the results given above and our own experience with the detector. They were mostly designed to deal with particular problems and many other useful sets of operating conditions could probably be devised. The recommendations

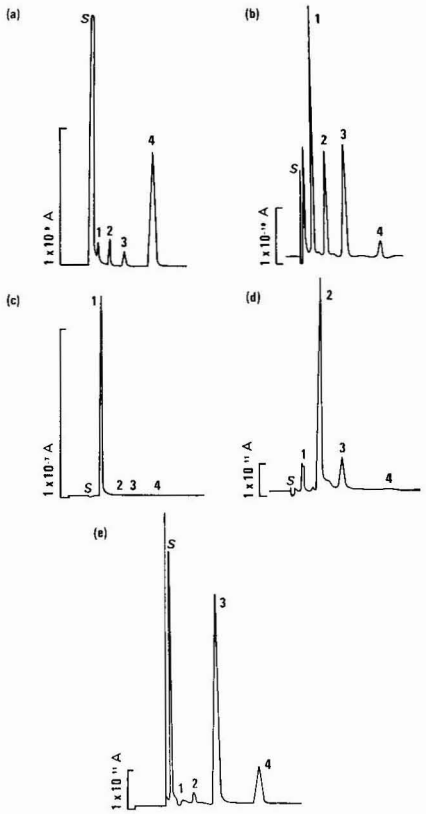


Fig. 9. Comparison of the response of an FID and the thermionic detector operated in different modes of selectivity. Flow-rates (ml/min) of air, nitrogen and hydrogen in Figs. a–e are given in this order: (a) FID; 465, 25.0, 26.4. (b) TD in general mode; 250, 30.0, 29.2; probe gap, 1.37 mm. (c) TD in phosphorus-selective mode; 550, 68.0, 34.0; probe gap, zero. (d) TD in sulphur-selective mode; 100, 25.0, 34.6; probe gap, 3.60 mm. (e) TD in nitrogen-selective mode; 300, 30.0, 29.8; probe gap, 1.22 mm. Peak identification: S = solvent, 1 = triethyl phosphite (100 ng), 2 = dibutyl sulphide (100 ng), 3 = *o*-toluidine (100 ng), 4 = tetradecane (1000 ng).

in Table III can only be used as a guide since even with one detector the results obtained will vary with factors such as the way the crystal is inserted in its holder, its previous operating history and the accuracy with which the probe height is reset. A useful approach is to decide on the performance required, set the air, nitrogen and hydrogen flow-rates to the recommended values and position the probe in approximately the right area (*i.e.*, low, medium, high, etc.) using the variation of background current as a guide. Then using a suitable test mixture (*e.g.*, of P-, S- and N-containing compounds as in this paper) adjust the probe height until the detector is operating in the required mode. Finally, optimise the performance by very small adjustments to the hydrogen flow-rate. In most cases it is far easier to optimise the detector and understand its operation if these tests are made with a suitable mixture rather than by injecting just standard solutions of the sample compound. When the detector is in regular use for long periods, it will be found most convenient if satisfactory performance can be achieved using low background currents (*i.e.*, low air flow-rates or a high probe) to minimise loss of RbCl. In addition, use of low air flow-rates extends the range of probe heights over which the detector operates and greatly facilitates accurate positioning and resetting of the probe.

The variety of operating conditions which can be achieved is illustrated by Fig. 9 which compares the chromatogram obtained using an FID for a standard mixture with some of the thermionic detector modes listed in Table III. For routine application of the detector to pesticide residue analysis we have found the general mode to be particularly useful. It offers similar sensitivity to the FID for P-, S- or N-containing pesticides with considerable discrimination against the solvent and sample co-extractives. In fact several different versions of this mode can be used depending on the requirements for sensitivity and long term stability and the concentrations of the coextractives in the injected sample.

ACKNOWLEDGEMENT

The authors thank the Government Chemist for permission to publish this paper.

REFERENCES

1 A. Karmen and L. Giuffrida, *Nature (London)*, 201 (1964) 679.
2 V. V. Brazhnikov, M. V. Gur'ev and K. I. Sakodynsky, *Chromatogr. Rev.*, 12 (1970) 1.
3 W. A. Aue, *Advan. Chem. Ser.*, 104 (1971) 39.
4 D. J. David, *Gas Chromatographic Detectors*, Wiley-Interscience, New York, 1974, Ch. 5.
5 J. Ševčík, *Detectors in Gas Chromatography*, Elsevier, Amsterdam, 1976, Ch. 6.
6 B. Kolb and J. Bischoff, *J. Chromatogr. Sci.*, 12 (1974) 625.
7 W. P. Cochrane and R. Greenhalgh, *Chromatographia*, 9 (1976) 255.
8 G. R. Verga and F. Poy, *J. Chromatogr.*, 116 (1976) 17.
9 F. P. Speakman, *Column*, 3 (1968) 2.
10 D. F. K. Swan, *Column*, 14 (1972) 9.
11 M. Dressler and J. Janák, *Collect. Czech. Chem. Commun.*, 33 (1968) 3970.
12 M. Dressler and J. Janák, *J. Chromatogr. Sci.*, 7 (1969) 451.
13 N. Mellor, *J. Chromatogr.*, 123 (1976) 396.
14 R. A. Hoodless, M. Sargent and R. D. Treble, *Analyst (London)*, 101 (1976) 757.

Journal of Chromatography, 136 (1977) 213–220

CHROM. 9972

IMPROVED MINIATURE FLOW FLUOROMETER FOR LIQUID CHROMATOGRAPHY

L. H. THACKER

Oak Ridge National Laboratory, Oak Ridge, Tenn. 37830 (U.S.A.)*

(Received December 15th, 1976)

SUMMARY

A simple, reliable, and inexpensive miniature flow fluorometer has been developed for use as a detector in liquid chromatography. The new instrument has been designed after approximately 15 years cumulative experience with a previously reported model. All of the desirable features of the earlier instrument are retained, and a number of simplifications and improvements have been added. These include three light-source alternatives for meeting a wide range of excitation requirements. For many applications a 1–10 ng limit of detection has been attained[2].

INTRODUCTION

The improved miniature flow fluorometer has evolved from an earlier version[1] after extensive use as a detector for liquid chromatographs in various programs at the Oak Ridge National Laboratory[3–9]. In many of those applications, the instrument was used to detect fluorescent Ce(III) generated by eluted components which reduce reagent Ce(IV) added to the column eluate. In other applications, the natural fluorescence of the eluted components was detected directly[8].

EQUIPMENT

The fluorometer (Fig. 1) consists of the fluorometer body and a box for electronic components and circuits; the electronic system includes an internal high-voltage supply which replaces the separate supply previously required. A schematic diagram of the instrument system is shown in Fig 2.

The fluorometer body (Figs. 1 and 3) is a machined aluminum block of simple design. The earlier design was assembled from two halves to permit milling rectangular slit optical apertures within the body; the improved version is a monolithic block with circular drill holes for all apertures. The use of a 1/2-in.-diameter side-viewing photomultiplier that fits inside the fluorometer body also yields additional savings

* Operated for the Energy Research and Development Administration under contract with the Union Carbide Corporation.

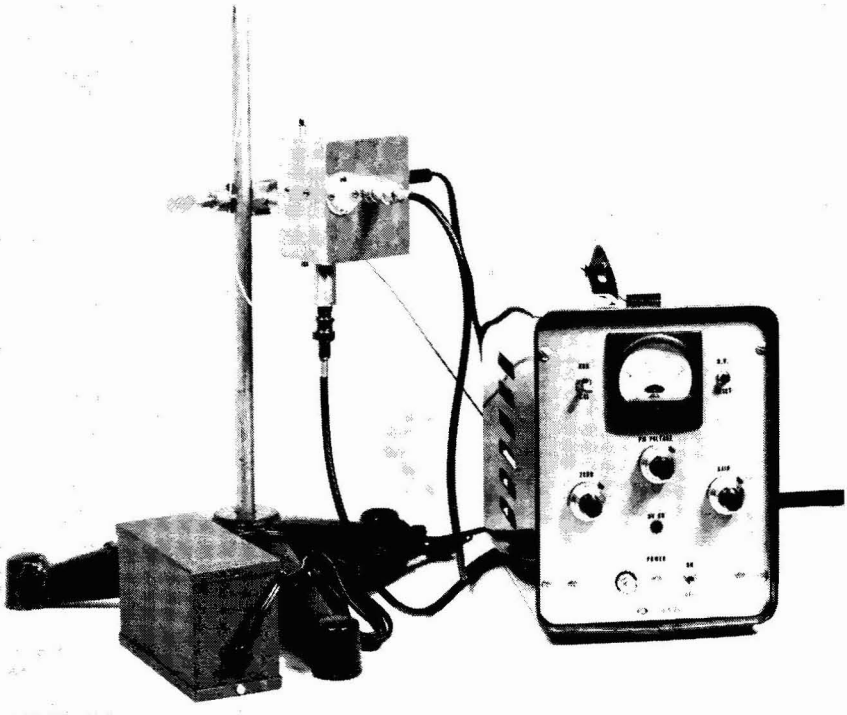


Fig. 1. The improved miniature flow fluorometer.

in fabrication costs. The $3 \times 2\frac{5}{8} \times 2$-in. block contains the desired light-source or adapter, an appropriate excitation filter, a quartz-tube flow cell, an appropriate blocking filter, the photomultiplier, and a photoconductor which compensates for changes in lamp intensity. The mounting accommodations for all of these components are machined into the aluminum block along with the required optical apertures; therefore, the problem of establishing or maintaining proper alignment of components does not exist. The fluorescence from the sample is observed at a right angle to the excitation radiation.

The low-pressure mercury lamp option (Ultra-Violet Products; Model 11SC-2) dissipates approximately 4.6 W, with an approximate 4% conversion efficiency for emission of the mercury spectral lines at 253.7, 312.5, 365, 404.7, 435.8, 546.1, and 577 nm. Of the total irradiance, approximately 90% is in the 253.7-nm line, with the remainder divided approximately equally among the next five longer wavelengths. An Ultra-Violet Products SCT-1 power supply drives the mercury lamp.

The halogen cycle incandescent lamp option uses the new miniature General Electric type 3027 bulb which dissipates 12 W and is mounted in an adapter with external cooling fins. Although the bulb is not made of quartz, it transmits 13% at 2800 Å, 40% at 3000 Å, 36% at 3200 Å, and 71% at 3400 Å[10]; thus, it is very useful with "cut-off" and "cut-on" interference filters for many types of fluorescence studies.

The quartz fiber optic option (Fig. 3) makes possible the use of almost any choice of external light source or monochromator. This option is used only when the

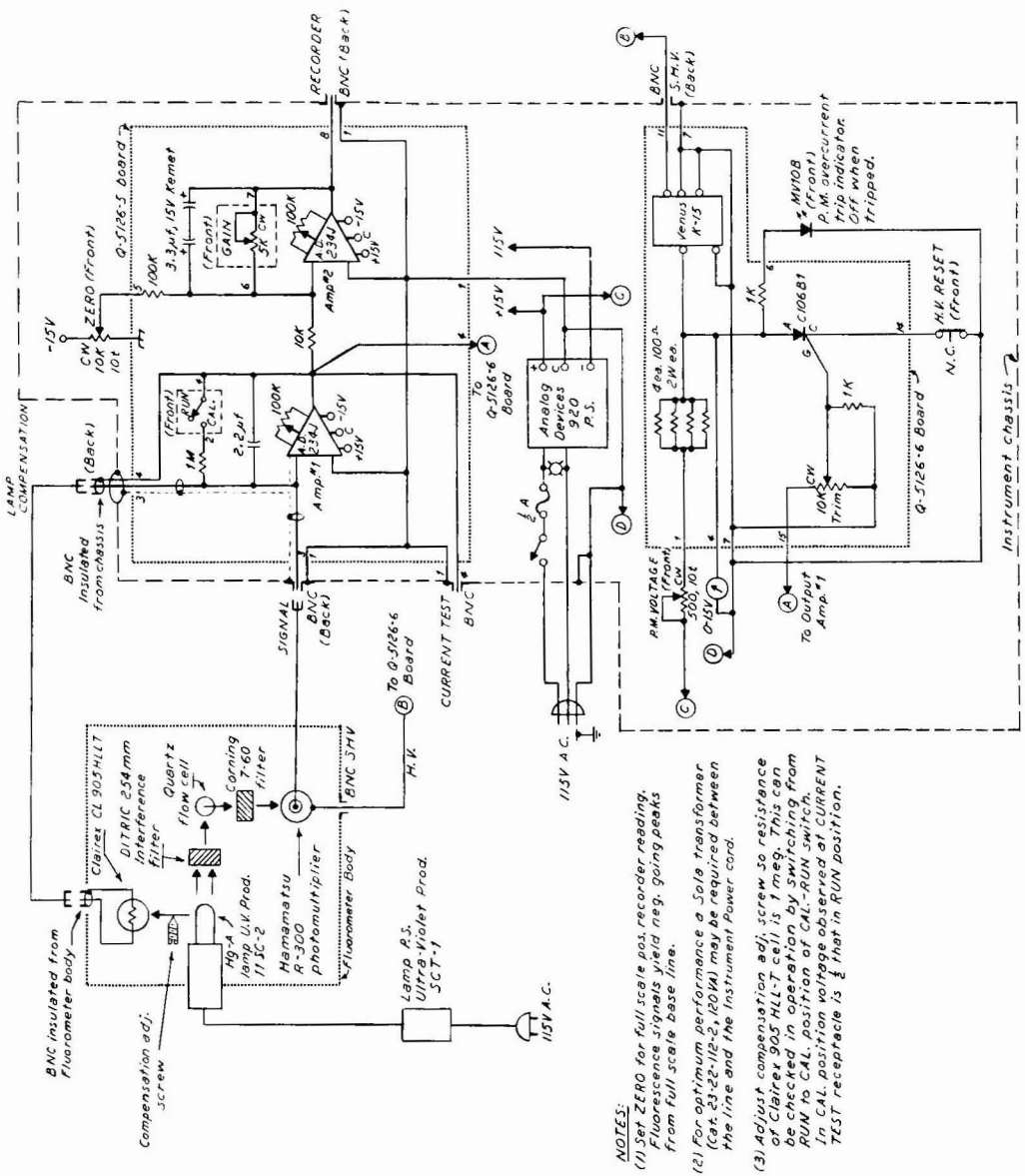


Fig. 2. Fluorometer schematic diagram.

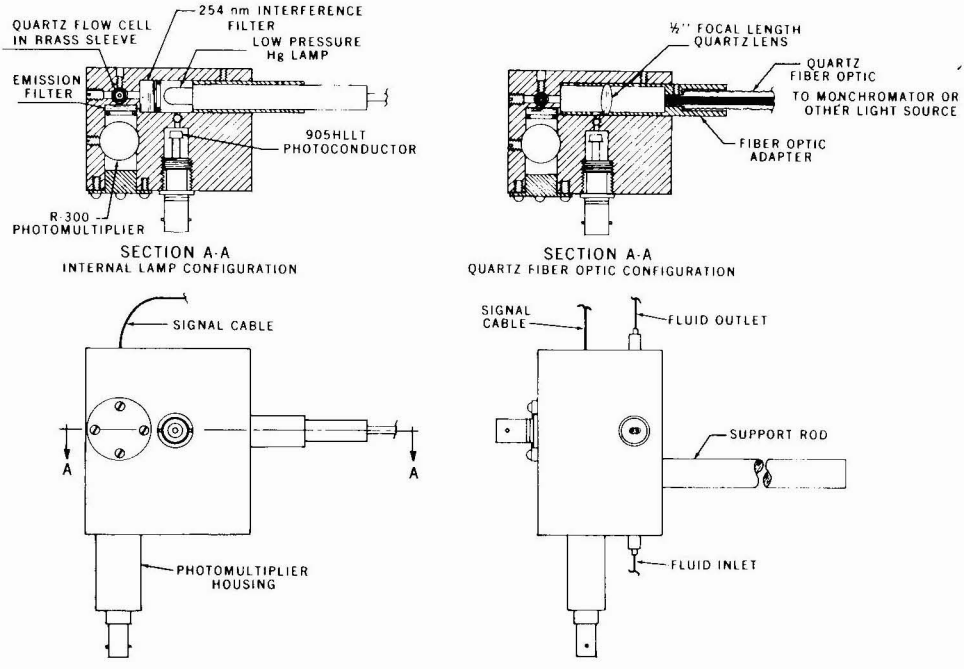


Fig. 3. Fluorometer body.

much more convenient internal lamps cannot supply the required excitation power at the desired wavelength.

The excitation filter is chosen to select the desired spectral band for exciting the sample. Because the excitation maximum for Ce(III) ions is at 260 nm, the instrument is operated with a 254-nm interference filter and the low-pressure mercury lamp for that application. The choice of a particular interference filter should be based on high transmittance in the pass band and high blocking (10^{-4}–10^{-6}) at longer wavelengths, rather than on a narrow pass band. Appropriate filters are available from Ditric Optics.

Several flow-cell designs have been tested. The highest sensitivity and signal-to-noise ratio have been obtained with a cell consisting of a 2.5 cm × 4 mm O.D. by 0.5 mm wall commercial quartz tubing with ends smoothly pulled down to 1.6 mm O.D. Each end of the flow cell is connected to 1.6 mm O.D. PTFE tubing. The quartz-to-PTFE connection is made by etching the end of the PTFE tube with commercial etchant, abutting the PTFE tube to the quartz tube, covering the joint w th a short length of No. 16 PTFE, and sealing it by shrinking a sleeve of heat-shrinkable plastic over it. The tubulated flow cell is mounted in a brass tube with appropriately cross drilled excitation and emission apertures. These flow-cell assembles are tested for leaks at 30 p.s.i.g. before they are installed in the instrument. At a small sacrifice in sensitivity and signal-to-noise ratio, a flow cell having a volume of only 15.7 μl (less than one-tenth the volume of the optimum cell) has been used. It is fabricated by slightly expanding the ends of 1.6 mm O.D. PTFE tubes; these ends were then pushed inside a length of 3 mm O.D. by 0.5 mm wall quartz tube to leave a clear gap of

about 5 mm. The quartz-to-PTFE seal is again made with heat-shrinkable plastic tube.

The emission filter that defines the band of wavelengths passed to the photomultiplier is selected to block the excitation wavelengths while passing as much of the emitted light as possible. Since the fluorescence emission spectrum of Ce(III) corresponds almost precisely with the pass band of the Corning 7-60 filter, that filter material is used in many applications.

The photomultiplier is a Hamamatsu R-300 1/2-in.-diameter side-viewing tube with an S-5 photoresponse characteristic. This response spectrum imposes an ultimate 185–650-nm limit on the detectable fluorescence emission spectrum. The voltage divider is contained in the 5/8 in. O.D. photomultiplier housing tube; a coaxial high-voltage cable is connected at one end of the tube, and a miniature coaxially shielded signal cable emerges from the opposite end.

A Clairex CL 905HLL-T photoconductor senses the emission of the light source and adjusts the gain of the electronic circuit to compensate for variations in lamp intensity. An aperture-adjusting screw determines the amount of light falling on the photoconductor; the aperture screw is set initially so that the photoconductor resistance is 1 MΩ. Subsequent checks and adjustments can be made during normal operation through use of the Run-Calibrate switch on the electronic chassis, as described below.

The principal components in the electronic chassis are a ±15-V d.c. power supply (Analog Devices 920), two high-quality chopper-stabilized operational amplifiers (Analog Devices 234-J), and a miniature d.c.-to-d.c. converter (Venus Scientific K-15) which generates the high voltage for the photomultiplier from the 15-V power supply. The amplifier electronics and a high-voltage power supply that has an over-current safety system are implemented on individual printed circuit boards (Fig. 2) which plug into sockets on the chassis.

Amplifier 1 is operated as a current-to-voltage converter; the signal current from the photomultiplier is taken directly to the summing junction of the amplifier, and the photoconductor in the fluorometer head is connected as the feedback resistor. Thus, when the intensity of the mercury lamp decreases, the signal current also decreases proportionately; since the resistance of the photoconductor increases proportionately, the output of amplifier 1 remains essentially unchanged. The resistance of the compensating photoconductor can be checked while the system is operating by switching the toggle switch on the front panel from the Run to the Calibrate position. In the Calibrate position, the photoconductor is paralleled by a 1-MΩ resistor; since the desired resistance of the photoconductor is 1 MΩ, the voltage observed at the Current Test receptacle with the switch in the Calibrate position should be exactly one-half the value observed when the switch is in the Run position If this is not the case, the photoconductor resistance can be adjusted by the aperture screw at the top of the fluorometer body.

Amplifier 2 provides zero and gain adjustments for interfacing with a variety of standard recorders. Because workers in our programs want to record peaks in the same format (and often on the same chart) as transmittance and absorbance peaks from other instruments, we use zero adjustment to place the base line at positive full scale on the recorder; fluorescence peaks are recorded as down-scale (negative) deflections of the recorder pen. In situations not demanding ultimate sensitivity, amplifier 2 could be an inexpensive, integrated circuit amplifier.

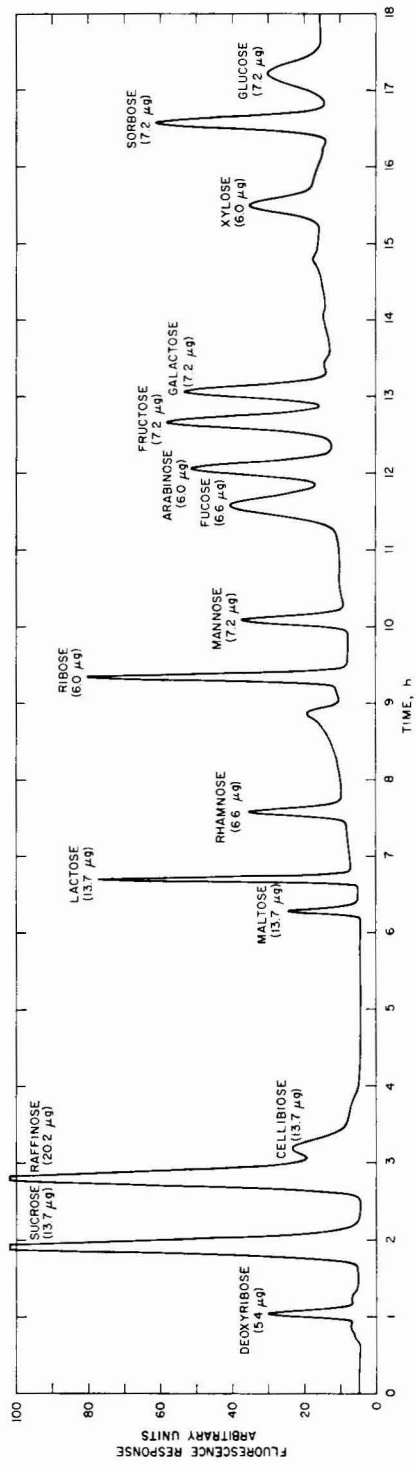


Fig. 4. Fluorescence response in anion-exchange separation of 16 carbohydrates.

The photomultiplier voltage is adjustable from about 300 V to 1500 V from a front-panel dial. A silicon controlled rectifier monitors the photomultiplier current and, if the current exceeds that selected by an adjustable trip point, shorts the d.c.-to-d.c. converter input to ground, thus reducing the output voltage and protecting the photomultiplier tube.

Unless line voltages are clean and stable, it has been necessary to provide power through a Sola transformer (Catalog No. 23-22-112-2; 120 V-A) for operation at high sensitivities. Since the capacity of the Sola transformer is adequate, power for the mercury-lamp supply is also taken from that source.

INSTRUMENT APPLICATIONS

The miniature flow fluorometer has been used in a large number and variety of applications. A cross section of these applications is summarized and referenced below.

In a liquid-chromatographic analysis of neutral carbohydrates in serum glycoproteins, Mrochek *et al.*[3] used the instrument in a cerate oxidimetric system[4]. Fig. 4 shows results they obtained in separating and detecting 16 carbohydrates. A highly linear response to eluted amounts of fucose, ranging from 0.23 to 2.3 μg (Fig. 5), is shown, and a sensitivity to 1 nmole of fucose is demonstrated.

Katz *et al.*[5] compared the sensitivity of fluorescence detection with UV photometry for 12 substituted aromatic acids using the fluorometer in a cerate oxidimetric

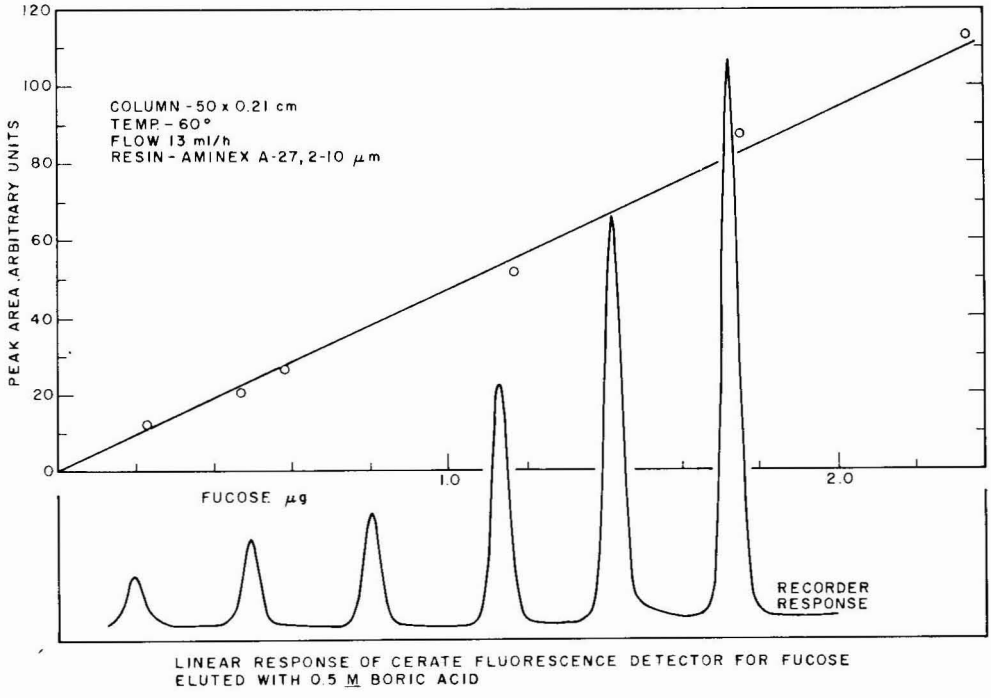


Fig. 5. Linear response of cerate fluorescence detector for fucose eluted with 0.5 *M* boric acid.

system. For 11 of the compounds, they found the ratio of fluorescence response to UV response to range from 2 to greater than 50; for one aromatic acid (2-hydroxybenzoic), the ratio was only 0.8.

Katz *et al.*[6] used dual monitoring by UV absorption and fluorescence produced by cerate oxidation to obtain sensitive and wide-ranging detection capabilities in a comparative serum and urine analysis by anion-exchange chromatography.

In another report, Katz *et al.*[7] found that the cerate oxidative detector system, using the reported fluorometer, provides better sensitivity and more effective peak resolution than other monitors for eluted carbohydrates, but the system is less specific than most of the earlier detectors.

Mrochek *et al.*[8] used the detector in a study of acetaminophen metabolism in man. Fluorescence and UV absorbance chromatograms as a function of time after ingestion are presented in their report.

In a personal communication[9], Katz reported that the fluorometer was used extensively for about a year in studies of coal-derived liquids on both a preparative and an analytical scale in series with a UV detector. The solvents used included heptane, hexane, isooctane, and various alcohols. In this application, a wide-band emission filter was installed, and the instrument was used to monitor the natural fluorescence of polycyclic aromatic hydrocarbons. Although the fluorometer was operated at reduced sensitivity, the response was generally as good or better than the UV detector. One particular advantage reported was that the fluorometer was less sensitive to flow disturbances.

ACKNOWLEDGEMENT

Research sponsored by the National Institute of General Medical Sciences, National Institutes of Health.

REFERENCES

1 L. H. Thacker, *J. Chromatogr.*, 73 (1972) 117.
2 S. Katz, personal communication, August 12, 1975.
3 J. E. Mrochek, S. R. Dinsmore and T. Phillip-Waalkes, *Clin. Chem.*, 21 (1975) 1314.
4 S. Katz and W. W. Pitt, Jr., *Anal. Lett.*, 5 (1972) 177.
5 S. Katz, W. W. Pitt, Jr. and G. Jones, Jr., *Clin. Chem.*, 19 (1973) 817.
6 S. Katz, W. W. Pitt, Jr. and J. E. Mrochek, *J. Chromatogr.*, 104 (1975) 303.
7 S. Katz, W. W. Pitt, Jr., J. E. Mrochek and S. Dinsmore, *J. Chromatogr.*, 101 (1974) 193.
8 J. E. Mrochek, S. Katz, W. H. Christie and S. R. Dinsmore, *Clin. Chem.*, 20 (1974) 1086.
9 S. Katz, personal communication, August 19, 1975.
10 W. L. Scully, General Electric Co., personal communication, November 15, 1973.

Journal of Chromatography, 136 (1977) 221–231

CHROM. 9883

HIGH-SPEED LIQUID CHROMATOGRAPHY ON CADMIUM-MODIFIED SILICA GEL

CORAZON R. VOGT*, TIMOTHY R. RYAN** and JANET S. BAXTER

Environmental Trace Substances Research Center, University of Missouri, Columbia, Mo. 65201 (U.S.A.)

(Received December 15th, 1976)

SUMMARY

A novel procedure for the preparation of low-surface-area porous silica adsorbents, modified by cadmium salts, is described. The salt is introduced concurrently with a reduction of the surface area of an inexpensive silica gel by hydrothermal treatment. The resulting materials contain mg/g amounts of the metal. Liquid chromatography on the cadmium-modified adsorbent is shown. Charge-transfer chromatography is favored when the salt is introduced at high hydrothermal temperature which yields ultra low surface area and dense cadmium population.

INTRODUCTION

Silica gels are the most widely used adsorbents for column liquid chromatography. This preference prevails because these materials have large surface areas, diverse surface activities, easily controllable porosity and finally because they are available in narrow-size-range spherical particles. The preparations and characteristics of silicas for highly efficient liquid chromatography have been described by Kirkland[1] and Unger *et al.*[2]. While water[3,4] and polar organic compounds[1] are generally used as modifiers on active silica surface, Bebris *et al.*[5] used layers of carbon and weakly polar polymers.

Charge-transfer chromatography of organic compounds on surfaces containing impregnated metal salts has been reviewed[6,7]. The reviews include its use in gas–liquid chromatography (GLC), gas–solid chromatography (GSC) and liquid chromatography (LC).

Silica surfaces are made more selective to olefins by coating the adsorbent with silver ions. In particular, this technique has been used for column, paper, and thin-layer chromatography (TLC)[8]. Impregnation of TLC silica adsorbent with silver oxide[9], cadmium sulfate[10], cadmium acetate[11], and zinc salts[12] yields efficient isomeric separations of aromatic amines.

* To whom correspondence should be addressed.

** Present address: Department of Chemistry, Iowa State University, Ames, Iowa 50010, U.S.A.

Chromatography on complex forming materials has been extended to high-speed liquid chromatography. Mikeš *et al.*[13] obtained good separations of various olefins with Corasil II coated with either rhodium(II) acetate or silver nitrate–ethylene glycol. Heath *et al.*[14] used silver nitrate coated silica adsorbent to obtain preparative separations of unsaturated acetates, aldehydes and hydrocarbons by high-pressure LC. Columns packed with metal ion-impregnated silica gel usually suffer fast deterioration due to the loss of the salt with the mobile phase. Improved stability and chromatographic reproducibility of nitrogen-containing compounds on silica-based adsorbents impregnated with silver nitrate has been reported by Vivilecchia, Thiebaud and Frei[15]; with cadmium iodide by Kunzru and Frei[16]. Aigner, Spitzy and Frei[17] reported the bonding of silver to silica gel. In each case, the metal ions were introduced, initially or totally, to the silica surface by way of NaOH treatment. Chromatography on the resulting supports demonstrated largely a donor–acceptor mechanism with some contributions from adsorption.

Treatment of silica gels with water at high temperatures results in a reduction of surface area and better chromatographic properties[18]. Aue *et al.*[19] and Kapila *et al.*[20] converted technical silica gels by a similar "hydrothermal treatment" in liquid water to well-performing supports for GLC. In our own systematic study of the latter process, some evidence was obtained indicating that the activity of the silica gel changed with the hydrothermal temperature, presumably caused by the re-deposition of the dissolved silica on to the particles in the cooling solution. In this work, we report the hydrothermal treatment of silica gel in the presence of cadmium chloride and the performance of the resulting materials in modern LC.

EXPERIMENTAL

A borosilicate glass insert was used with the 500-ml cylinder of the Model 4652 Parr bomb (Parr, Moline, Ill., U.S.A.). The unit included the electric heater and the automatic temperature controller.

Preparation of the supports

A saturated solution of cadmium chloride was poured into the glass insert. Silica gel Davison 62, ground to 325–400 mesh, was added to form a slurry. The insert was placed in the bomb assembly and the heat turned on. It generally took 1–1.5 h to reach the desired temperature (250° or 350°). After 16 h of hydrothermal exposure, the bomb was taken out of the heating assembly and allowed to cool. In approximately $2\frac{1}{2}$ h the temperature decreased to less than 100° and the bomb was opened. The treated silica gel was then transferred to a sintered glass funnel and washed with at least 500 ml of distilled water, followed by washings with methanol and hexane. The packing was dried at 45° under vacuum and re-sieved to 325–400 mesh.

At a hydrothermal temperature (HT) of 350°, the silica gel particles became considerably finer, and re-sieving yielded only a small fraction of 325–400 mesh. Therefore, in succeeding hydrothermal treatment at this temperature, silica gels of larger mesh size (270–325) were used to improve the yield of the 325–400 fraction.

The sieved portion was slurry-packed using a balanced-density mixture of tetrabromoethane and carbon tetrachloride. A Haskel air-driven pump was used at

about 5000 p.s.i. on the chloroform packing solvent. All columns were 11 cm × 4.5 mm I.D., precision bore, stainless steel and fitted with 5-μm stainless-steel snubbers at the inlet and outlet.

Portions of 325–400 fractions were digested with perchloric acid and cadmium was determined by atomic absorption (AA).

Chromatography

A Model 202 (Waters Assoc.) liquid chromatograph with UV (254 nm) detector was used. The columns were tested with model isomeric compounds typically used with this type of columns: mixture 1 consisted of *o*-, *m*- and *p*-chloroaniline, containing 0.67 μg/μl of the *ortho* and *meta*, and 0.33 μg/μl of the *para* isomer. Mixture 2 consisted of 2-, 3- and 4-picolines, containing 0.17, 0.33 and 0.50 μg/μl, respectively. Both mixtures were in hexane.

Two binary solvent mixtures were used as mobile phase: acetonitrile–hexane and methanol–hexane. Hexane was also used to demonstrate that the blank supports can effect separations.

Capacity factor

The k' values of the columns were determined by using 1-μl injections of a series of 2-picoline solutions in hexane. At the given composition of the eluting binary solvent, the retention time of benzene was 19–22 sec at a flow-rate of 4 ml/min.

RESULTS AND DISCUSSION

Table I lists the specific surface area and the amount of cadmium present in the adsorbents produced in this study. Hydrothermally treated silica gel in the absence of cadmium was analyzed for the trace metal by flameless AA, others by flame AA. The sensitivity of the former was approximately 100 times better.

Using the more conventional chromatographic term, cadmium is present in 0.3–0.6% load. Incidentally, in a batch equilibration procedure of loading copper(II) complexes on silica gel, Datar and Ramanathan[21] reported a decrease uptake of the copper complexes with increasing surface area, which is opposite to those shown in Table I.

Figs. 1A and 2A show the LC separations obtained with the acetonitrile–hexane system on columns modified by cadmium. Also shown are the chromatographies on the blank columns (≡ hydrothermally treated in the absence of cadmium) obtained under identical solvent composition (Figs. 1B and 2B) and when hexane is vsed alone (Figs. 1C and 2C). The strong influence of cadmium is apparent. While metal-loaded columns give excellent separations of the chloroaniline isomers with acetonitrile–hexane, the same solvent system is inept on the blank columns. Use of pure hexane on cadmium with HT = 250° and 350° (HT-250 and HT-350) results in excessively retained *o*-chloroaniline while the *meta* and the *para* isomers are practically irreversibly adsorbed. On the other hand, the weak adsorption sites produced by hydrothermal treatment without the benefit of cadmium can effect minor separations (especially at HT-250) if hexane is used. Fig. 3 shows the separation of the same mixture when acetonitrile is replaced by methanol. Methanol is a strong solvent. However, it is less likely to compete in complex formation with cadmium than acetonitrile. Therefore, one would assume better sepa-

TABLE I

HYDROTHERMALLY MODIFIED SILICA GEL

Hydrothermal temp. (°C)	*$CdCl_2$ modifier*	*Surface area (m^2/g)**	*Amount of Cd (ppm)*
250	No	14.84	1.6
250	Yes	87.33	3000
300	No		0.32
300	Yes		6440
350	No	1.64	0.23
350	Yes	21.85	6340

* Multipoint BET analysis using krypton (Micromeritics, Norcross, Ga., U.S.A.).

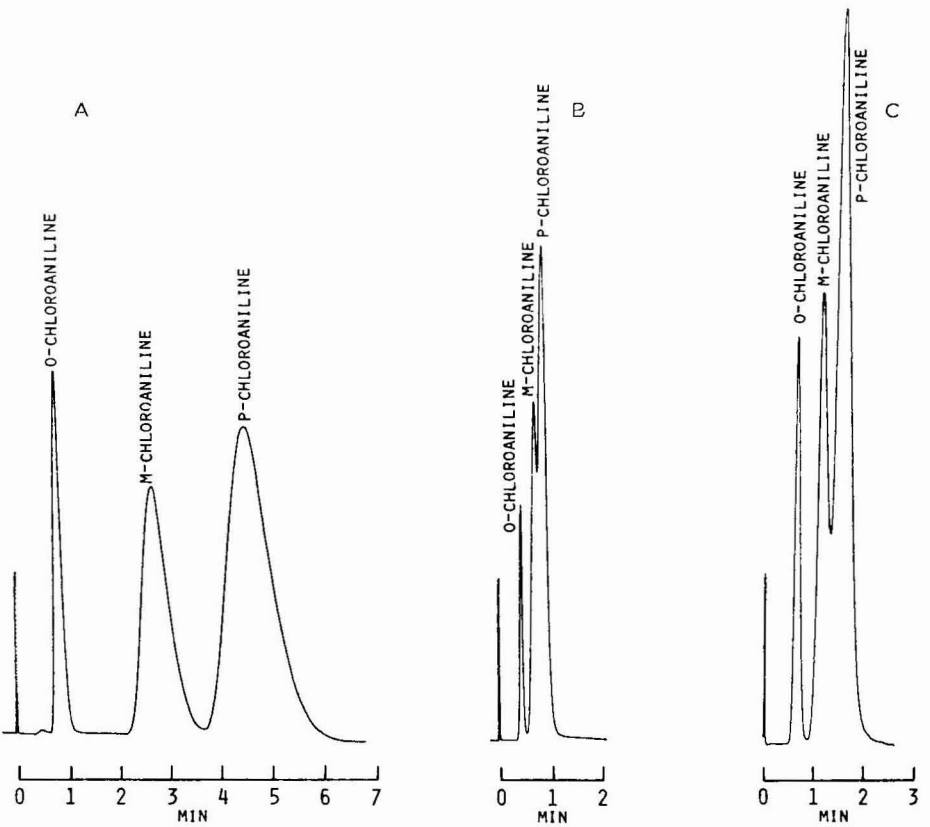


Fig. 1. Separation of chloroaniline isomers on hydrothermally (250°) treated silica gel in the presence and absence of cadmium chloride. (A) HT-250; 0.5% acetonitrile in hexane; attenuation, × 0.05. (B) Blank HT-250; 0.5% acetonitrile in hexane; attenuation, × 0.2. (C) Blank HT-250; hexane; attenuation, × 0.05. Column, 11 cm × 4.5 mm I.D.; eluent (as shown) at a flow-rate of 4 ml/min; detector, UV (254 nm). Injected amount, 2 μg each of *o*- and *m*-, and 1 μg of *p*-chloroaniline.

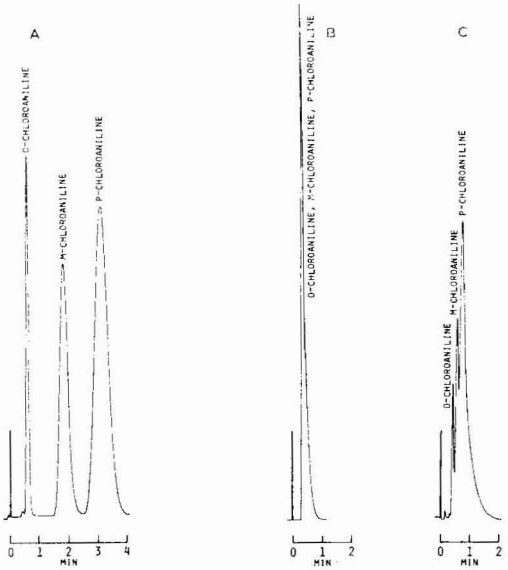


Fig. 2. Conditions are the same as in Fig. 1 except the hydrothermal temperature (350°). (C, attenuation × 0.1.)

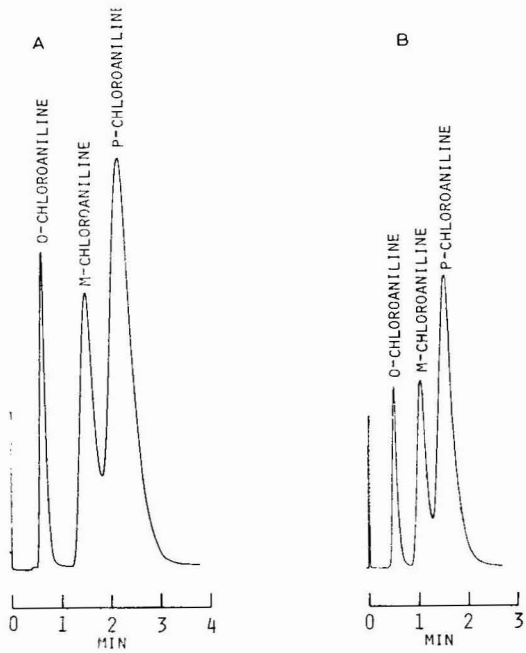


Fig. 3. Separation on cadmium-loaded silica gel. Eluent, 0.1% methanol in hexane (4 ml/min); column, 11 cm × 4.5 mm I.D.; detector, UV (254 nm). (A) HT-250; attenuation, × 0.05. (B) HT-350; attenuation, × 0.2.

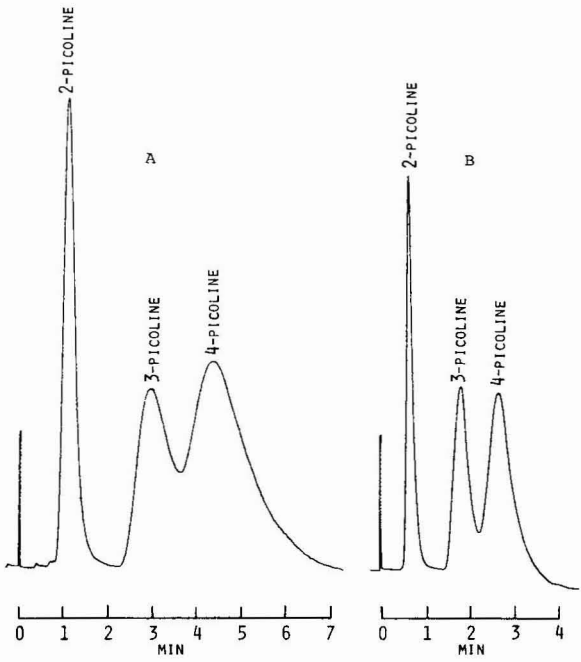


Fig. 4. Separation of picoline isomers on cadmium-loaded silica gel at (A) HT-250, 0.3% methanol in hexane (4 ml/min); attenuation, × 0.02; (B) HT-350, 0.25% methanol in hexane (4 ml/min); attenuation, × 0.05. Injected amounts of 0.5, 1.0 and 1.5 μg of 2-, 3-, and 4-picoline, respectively.

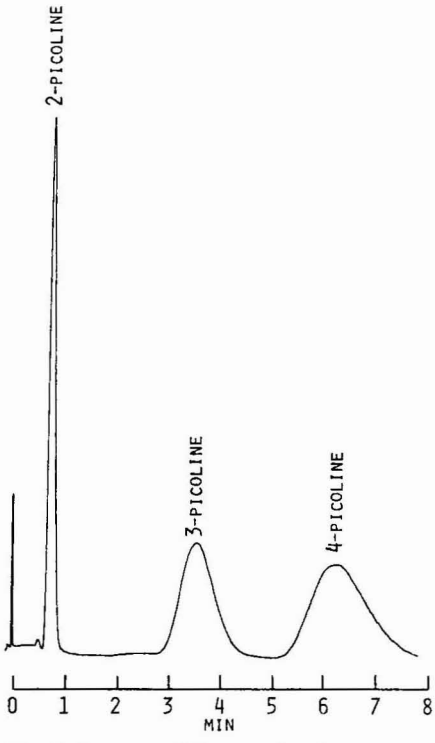


Fig. 5. Same conditions as in Fig. 4B except eluent (2% acetonitrile in hexane).

ration with acetonitrile if a donor–acceptor mechanism predominates. On the chromatograms shown, the cadmium HT-250 (0.03 mg Cd per m^2) is more retentive but less efficient than cadmium HT-350 (0.29 mg Cd per m^2). This seems to suggest the significant contribution of surface adsorption on two different sites in the former. Initial testing on cadmium HT-300 showed close chromatographic resemblance to cadmium HT-350; unfortunately however, the packed bed was disturbed before further evaluations could be made.

Chromatography of the more basic compounds, the isomeric mixture of picolines, is shown on Fig. 4. In this separation, the methanol–hexane composition is approximately twice that for the chloroanilines. Again, the important role played by a competing solvent acetonitrile in the separation is realized by comparing Figs. 4B and 5. Separation is obviously improved in the latter case but efficiency is decreased. Chromatographic efficiency shown on Fig. 5 can be, and was considerably, improved after introducing trace amounts of *n*-propylamine to the column. The separation of α- and β-naphthylamines is shown on Fig. 6A.

The sorption activity appears to have been decreased sufficiently to enable the sorbent to perform satisfactorily without the polar compound. The change of one

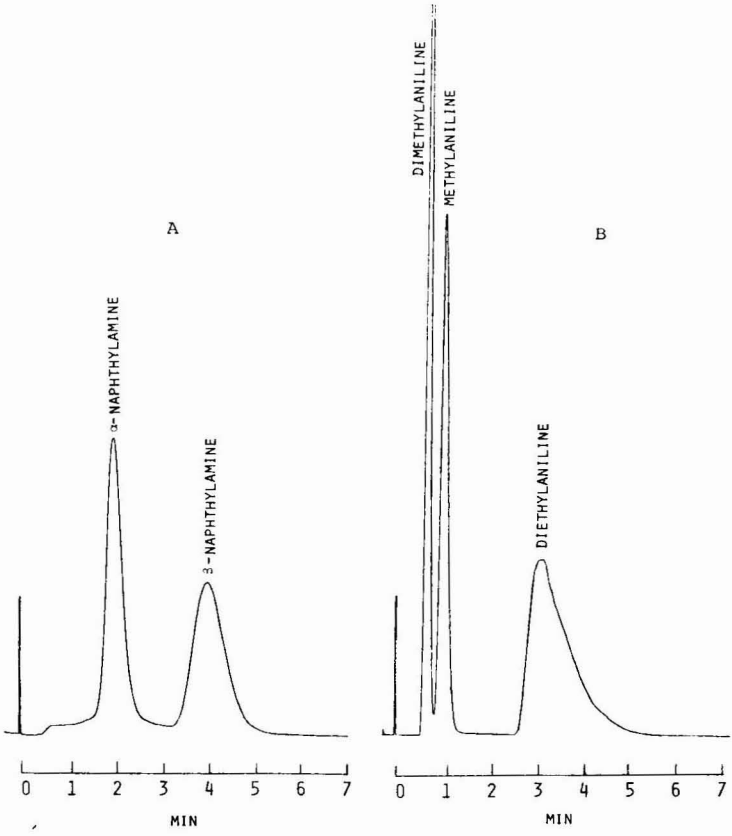


Fig. 6. Separations on 11 cm × 4.5 mm I.D. column packed with cadmium HT-350. (A) 0.5% acetonitrile–hexane; (B) 0.1% acetonitrile–hexane. Flow-rate, 4 ml/min.

mobile phase to another, or return to the initial condition in a gradient elution, requires only a short time for equilibration except for one solvent containing more than trace amount of *n*-propylamine (0.1%) which, however, was still useful.

The chemical form of the cadmium finally introduced into the silica gel by hydrothermal treatment is unknown at this time. On chromatography, the cadmium can act as surface moderator, electron-pair acceptor, or both. In the first case, the cadmium may deactivate the sorption sites effecting to what may be equivalent to a more uniform surface; it may alter the accessible surface area, and lastly, it may effect changes in pore sizes and distributions. In the second case, the cadmium would form a donor–acceptor complex with the nitrogen atom of the solutes to effect separations. If this is the case, the cadmium must be able to function as electron-pair acceptor. It appears that both factors are contributing to the chromatographic properties of cadmium-modified silica gel. It would be desirable to test solutes unlikely to form complexes with cadmium. In this regard however, the UV detector is limiting.

The contribution of the donor–acceptor complexing to the retention is influenced by the accessibility of the electron pair and the pK_a of the solutes. Both these effects are shown in Table II by the retention times on cadmium HT-350 of a number of amines. The toluidines followed the elution order *para* > *meta* > *ortho*. However, the elution compared to chloroanilines is: *p*-toluidine > *p*-chloroaniline ≈ *m*-toluidine > *o*-toluidine ≈ *m*-chloroaniline > *o*-chloroaniline. It is interesting to note that while N,N-dimethylaniline elutes before N-methylaniline, N,N-diethylaniline elutes after N-ethylaniline (Fig. 6B). In a similar separation, the use of 0.05% acetonitrile–hexane (instead of 0.1%) gave 60% resolution of methylaniline from ethylaniline while diethylaniline is excessively retained with a broad tailing peak. Pure acetonitrile

TABLE II

RETENTIONS ON CADMIUM HT-350

Compound	pK_a *(ref. 22)*	t_R *(sec)*	*Mobile phase (4 ml/min)*
o-Chloroaniline	2.62	38	0.05% Acetonitrile–hexane
Dimethylaniline	5.07	72	
Methylaniline	4.85	102	
Ethylaniline	5.11	138	
Diethylaniline	6.56	52	0.2% Acetonitrile–hexane
m-Chloroaniline	3.32	100	
o-Toluidine	4.38	105	
Aniline	4.62	166	
m-Toluidine	4.67	166	
p-Chloroaniline	3.81	176	
p-Toluidine	5.07	262	
Pyridine	5.14	238	2% Acetonitrile–hexane
m-Phenylenediamine	4.88	119	100%Acetonitrile
2-Aminopyridine	6.7	55	10% Methanol–acetonitrile
p-Phenylenediamine	6.08	55	10% Methanol–acetonitrile
Benzylamine	9.34	109	0.001% *n*-Propylamine–methanol
o-Phenylenediamine	4.47	143	

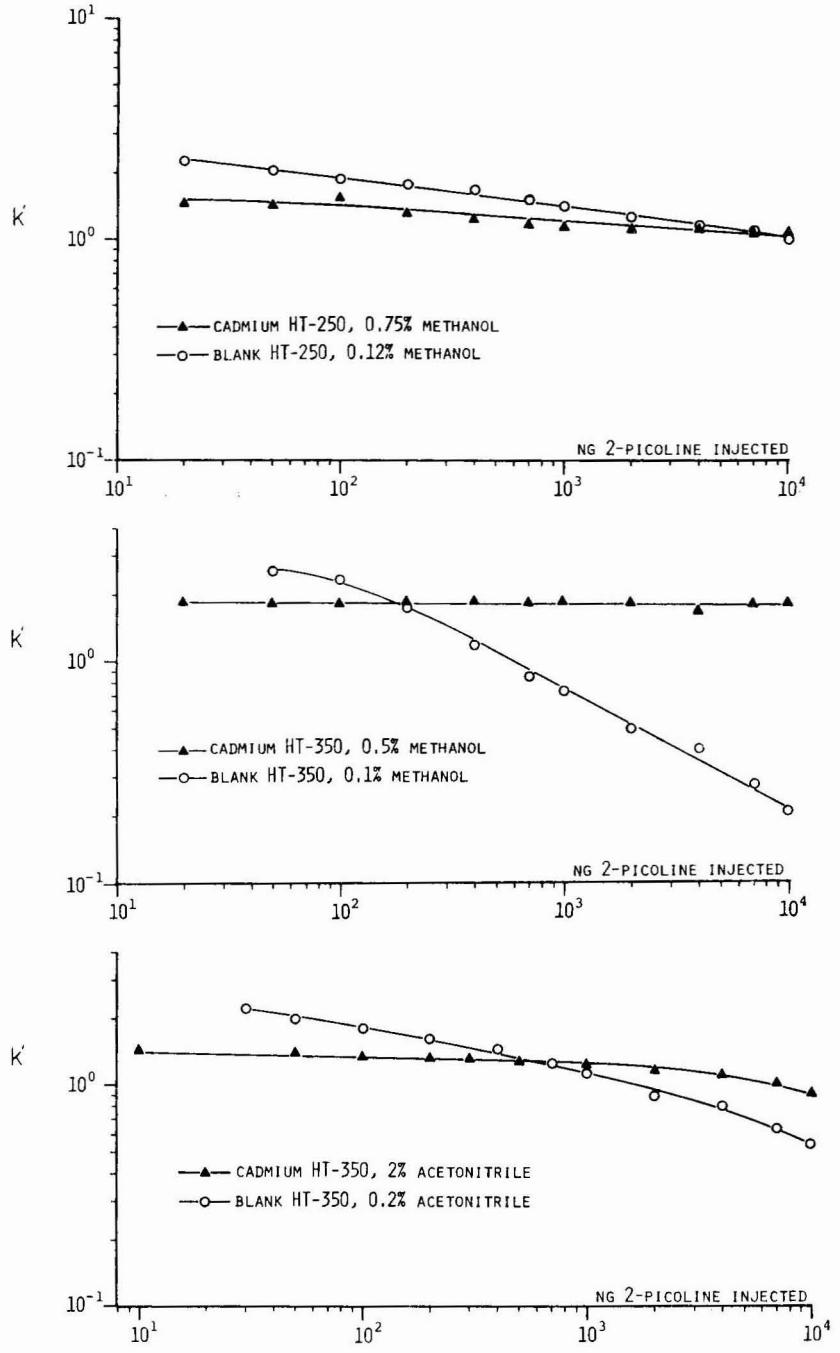


Fig. 7. Sorption isotherms expressed by the capacity factor (k') *versus* amount of 2-picoline injected to 11 cm × 4.5 mm I.D. column packed with silica gel modified with cadmium. Binary solvent as indicated in hexane; flow-rate, 4 ml/min.

will elute *m*-phenylenediamine while the *para* and *ortho* isomers will not. 10% methanol–acetonitrile and 0.001% *n*-propylamine–methanol are required to elute *p*-phenylenediamine and *o*-phenylenediame, respectively.

The column packed with cadmium HT-350 was heavily used with organic solvents for three months without loss of efficiency. At the end of the 3-month testing, the column was subjected to 3 h of continuous flushing with phosphate buffer (pH 5). Re-testing of the column with the chloroaniline mixtures gave tailing peaks not observed before.

In the present study, the capacity factor, k', is expressed as the ratio of the corrected retention time of 2-picoline to the retention time of benzene. Sorption isotherms (expressed as k' *versus* amount injected) of the cadmium-modified silica gel and their corresponding blanks are compared on Fig. 7. The adsorbent loaded with cadmium-HT-250 yields a non-linear isotherm while the HT-350 gives linearity up to at least 10 μg. Nonetheless, the wide linearity range observed with cadmium HT-350 using methanol–hexane is diminished when acetonitrile–hexane is used. It is well known, that strong adsorbing solvents give wide linear ranges. The amount

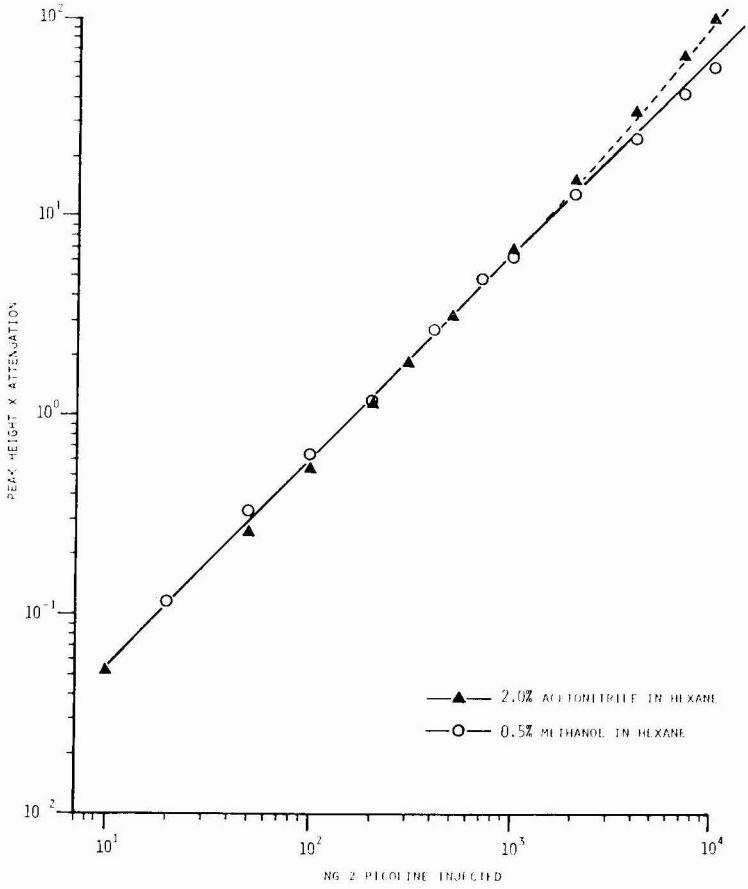


Fig. 8. Response linearity of silica gel loaded with cadmium at 350° hydrothermal temperature.

of 2-picoline injected is plotted *versus* the detector response (peak height × attenuation) on Fig. 8. While solvent strength increases linear range capacity, the separation usually degrades. Therefore, sorbent deactivation is generally used to improve capacity. The metal deactivation described in this work is concurrent with the alteration (decrease) of surface area and porosity in the presence of a saturated solution of the salt. The consequence of an unsaturated cadmium chloride solution on the amount deposited at different HT temperatures was not investigated. The effect of cooling rate on the size and shape of the particles has likewise not been investigated. Yet, it is easy to imagine that a variety of cadmium loadings with different surface areas can be made by this procedure.

CONCLUSIONS

Low-surface-area LC adsorbents can be derived from low-priced silica gel by hydrothermal treatment. The LC activity and selectivity is modified by the presence of a cadmium salt introduced during the treatment, and can be manipulated, to a degree, to yield separations of model amines.

ACKNOWLEDGEMENTS

We are grateful to Mr. E. Hinderberger and Dr. F. Lichte for the atomic absorption analysis. Thanks are due to Dr. W. A. Aue for helpful suggestions. This study was supported by the National Science Foundation, Grant No. MPS74 18084

REFERENCES

1 J. J. Kirkland, *J. Chromatogr.*, 83 (1973) 149.
2 K. Unger, J. Schick-Kalb and K-F. Krebs, *J. Chromatogr.*, 83 (1973) 5.
3 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, Ch. 4.
4 H. Engelhardt, J. Asshauer, U. Neue and N. Weigand, *Anal. Chem.*, 46 (1974) 336.
5 N. K. Bebris, R. G. Vorobieva, A. V. Kiselev, Y. S. Nikitin, L. V. Tarasova, I. I. Frolov and Y. I. Yashin, *J. Chromatogr.*, 117 (1976) 257.
6 O. K. Guha and J. Janák, *J. Chromatogr.*, 68 (1972) 325.
7 R. J. Laub and R. L. Pecsok, *J. Chromatogr.*, 113 (1975) 47.
8 A. S. Gupta and S. Dev, *J. Chromatogr.*, 12 (1963) 189 and refs. cited therein.
9 S. Tabak and M. R. M. Verzola, *J. Chromatogr.*, 51 (1970) 334.
10 K. Yasuda, *J. Chromatogr.*, 60 (1971) 144.
11 K. Yasuda, *J. Chromatogr.*, 72 (1972) 413.
12 K. Yasuda, *J. Chromatogr.*, 74 (1972) 142.
13 F. Mikeš, V. Schurig and E. Gil-Av, *J. Chromatogr.*, 83 (1973) 91.
14 R. R. Heath, J. H. Tumlinson, R. E. Doolittle and A. T. Proveaux, *J. Chromatogr. Sci.*, 13 (1975) 380.
15 R. Vivilecchia, M. Thiebaud and R. W. Frei, *J. Chromatogr. Sci.*, 10 (1972) 411.
16 D. Kunzru and R. W. Frei, *J. Chromatogr. Sci.*, 12 (1974) 191.
17 R. Aigner, H. Spitzy and R. W. Frei, *Anal. Chem.*, 48 (1976) 2.
18 A. V. Kiselev and Y. I. Yashin, *Gas Adsorption Chromatography*, Plenum Press, New York, 1969.
19 W. A. Aue, S. Kapila and K. O. Gerhardt, *J. Chromatogr.*, 78 (1973) 228.
20 S. Kapila, W. A. Aue and J. M. Augl, *J. Chromatogr.*, 87 (1973) 35.
21 A. G. Datar and P. S. Ramanathan, *J. Chromatogr.*, 114 (1975) 29.
22 H. A. Sober, *Handbook of Biochemistry*, CRC Press, Cleveland, Ohio, 1970, pp. J-203–220.

Journal of Chromatography, 136 (1977) 233–243

CHROM. 9950

HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF GLYCOSPHINGOLIPIDS (WITH SPECIAL REFERENCE TO GANGLIOSIDES)

U. R. TJADEN*

Laboratory for Analytical Chemistry, University of Amsterdam, Amsterdam (The Netherlands)

and

J. H. KROL, R. P. VAN HOEVEN**, E. P. M. OOMEN-MEULEMANS and P. EMMELOT

Division of Cell Biology and Department of Biochemistry, Antoni van Leeuwenhoek-huis, The Netherlands Cancer Institute, Amsterdam (The Netherlands)

(Received December 9th, 1976)

SUMMARY

The analysis of mixtures of gangliosides from adult human or bovine brain, supplemented with Tay-Sachs ganglioside, and haematoside from dog erythrocytes by high-pressure liquid chromatography using a moving-wire detector system is described.

The complete separation of six gangliosides within 40 min has been achieved, using silica as the stationary phase and acidified chloroform–methanol–water mixtures as the eluent on a 25-cm column. Neutral glycosphingolipids, *viz.*, the major components from normal human erythrocytes, can be completely separated on the same column, using non-aqueous and non-acidic eluents.

It is shown that the methods described are useful for both analytical and (micro)-preparative purposes.

INTRODUCTION

In this paper, for gangliosides the standard Svennerholm nomenclature[1] is used, while for neutral glycosphingolipids the symbols used by Dawson[2] have been adopted. For structures and synonyms, see ref. 3.

Since the pioneering work of Wagner *et al.*[4] and Jatzkewitz and Mehl[5], the thin-layer chromatography (TLC) of gangliosides and neutral glycosphingolipids has become the method of choice for the analysis of complex mixtures of these lipids from natural sources[6–10]. However, in spite of the simplicity of TLC, this technique has several severe drawbacks. The chloroform–methanol–water mixtures most commonly used as the mobile phase in the TLC analysis of gangliosides create gradients of individual solvent components in both the thin layer and the surrounding gaseous

* Present address: Department of Analytical Chemistry and Pharmaceutical Analysis, Gorlaeus Laboratories, State University, P.O. Box 75, Leiden, The Netherlands.

** To whom correspondence should be addressed.

phase, as demonstrated by the use of the Sandwich and Vario-KS chambers[11] (*cf.*, ref. 12). Thus, the system in which TLC is carried out in conventional paper-lined tanks is in fact ill-defined and may respond unpredictably to accidental variations in handling and in the surroundings.

Further, quantitative analysis by means of resorcinol staining[13] of the individual ganglioside classes after TLC separation is laborious and relatively insensitive. Such staining, being destructive, prevents the isolation of the separated components by extraction from the plates for further analysis. Although the recent non-destructive use of the universal primuline spray[14] is an important improvement in analysis, there still remains a need for a satisfactory quantitative and micro-preparative assay.

Glycosphingolipids, and gangliosides in particular, have so far withstood high-pressure liquid chromatographic (HPLC) analysis. This difficulty has partially been caused by the fact that a simple conversion of the TLC system to a column system appears to fail (see below) and by the absence of a significant ultraviolet absorption of the solutes, which hampers detection using the commonly available ultraviolet detectors.

As far as we know, only one report has been published[15] in which a column liquid chromatographic separation of glycosphingolipid fractions from normal, Gaucher's and Fabry's plasma and normal erythrocytes is described. However, in that study the glycosphingolipids were converted into their benzoyl derivatives prior to chromatography, in order to permit ultraviolet detection.

Classical column liquid chromatography on silica, followed by off-line detection by means of TLC, has been described for the preparative separation of gangliosides[1,16,17], but the procedure is very time consuming and considerable overlapping between peaks occurs.

In this paper, the complete separation of gangliosides and of neutral glycosphingolipids by means of HPLC within 40 min is described. By using a universal detector, derivatization is unnecessary. Finally, the importance of this technique in future research on these complex lipid classes is outlined.

EXPERIMENTAL

Glycosphingolipids

Gangliosides from adult bovine brain were purchased from Koch-Light (Colnbrook, Great Britain), Sigma (St. Louis, Mo., U.S.A.) and Supelco (Bellefonte, Pa., U.S.A.). These preparations contained essentially all of the principal gangliosides (GM1, GD1a, GD1b and GT1) in different proportions. Preparations, enriched in mono-, di- and trisialogangliosides from normal brain and pure GM2 from Tay-Sachs brain, were obtained from Supelco. Highly enriched preparations of GM1, GD1a, GD1b and GT1 were isolated from adult human brain[16]. Haematoside (GM3) was isolated from dog erythrocyte ghosts[18] by the method of Koscielak[19] with the modification of Hakomori and Strycharz[20]. The crude preparation exhibited only one resorcinol-positive spot with higher mobility than authentic GM2 on TLC. It was purified by treatment with methanolic sodium hydroxide solution and subsequent HPLC, as described below. The purified haematoside showed a molar ratio of glucose, galactose and sialic acid of 1:1:1.

Galactocerebroside (GL1b), a preparation from bovine brain, was obtained

from Koch-Light and glucocerebroside (GL1a), isolated from Gaucher's spleen, from Supelco. Lactosylceramide (GL2a) was a synthetic preparation (Miles Labs., Slough, Great Britain) consisting of the stearoyl derivative only. Authentic digalactosylglucosylceramide (GL3) originated from *post mortem* kidney of a patient with Fabry's disease[21]. GL3 was also isolated, together with GL2a and ceramide tetrahexoside (globoside, GL4), from human erythrocyte ghosts[18–20]. Ceramide, prepared from bovine brain cerebrosides, was a product of Koch-Light.

Solvents

All solvents were of analytical-reagent grade and were used without prior purification.

Column materials

Silica SI 60 (E. Merck, Darmstadt, G.F.R.) with a particle size range of 63–200 μm was ground in an agate mortar and then fractionated by means of an air classifier (Model 100 MZR; Alpine, Augsburg, G.F.R.). The particle size distributions of the classified fractions were determined with a Coulter counter and the fraction of $9 \pm 1.5\,\mu$m was used for column packing. The columns were packed using a pressurized balanced-slurry technique[22].

Apparatus

The liquid chromatograph was constructed from custom-made and commercial parts and consisted of a thermostated eluent reservoir, a high-pressure pump (DMP 1515, Orlita, Giessen, G.F.R.), a flow-through manometer as damping device, a sampling valve (Rheodyne, Model 7120) and a stainless-steel 316 column. Column tubings of length 10 and 25 cm, I.D. 2.8 mm and O.D. 6.35 mm were constructed from precision-bore stainless-steel tubing. The detector was the improved moving-wire system equipped with a flame-ionization detector (Pye Unicam, LCM2)[23]. The chromatograms were recorded using a linear potentiometric recorder (Philips PM 8220).

The gas chromatograph (Varian Model 2100) was equipped with a flame-ionization detector and an electronic integrator (Infotronics Model CRS-208). A glass column of length 150 cm, I.D. 2 mm and O.D. 6.35 mm was used, packed with Gas-Chrom Q, 100–120 mesh (Applied Science Labs., State College, Pa., U.S.A.), coated with 3% SE-30 by an evaporation technique.

Determination of the chromatographic parameters

The capacity ratio, k_i', of a component i was determined from the retention time, t_{Ri}, and the time of a non-retarded compound, t_{Ro}:

$$k_i' = \frac{t_{Ri} - t_{Ro}}{t_{Ro}} \tag{1}$$

The selectivity coefficient, r_{ji}, of two compounds was calculated from their capacity ratios:

$$r_{ji} = \frac{k_j'}{k_i'} \tag{2}$$

Ceramide was used as a non-retarded compound.

Thin-layer chromatography

All separations were performed on pre-coated silica plates (Merck) in conventional TLC tanks lined with Whatman 3 MM paper.

Gangliosides (1–20 μg) were applied as 1-cm long streaks, about 3 mm wide. The plates were developed by two successive runs over 15 cm using chloroform–methanol–water (containing 200 mg/l of potassium chloride)[10] in the volume ratio 60:35:8.5 as solvent. Between the two runs, the plates were dried for exactly 10 min by means of an infrared lamp. Resorcinol staining, which is specific for sialic acid, was used for detection[24]. Neutral glycosphingolipids were separated by two successive runs in the same solvent and rendered visible by staining with orcinol[25] or naphthoresorcinol[26]. Contaminating phospholipids were detected with Zinzadze reagent[27].

Preliminary monitoring was performed by means of a primuline spray[14] and inspection at 350 nm. This procedure did not interfere with the subsequent application of the aforementioned sprays.

Gas chromatography

The individual glycosphingolipid classes were analysed for their sugar content by gas–liquid chromatography. In principle the method of Sweeley and Walker[28] was used, except for the following modifications.

Methanolysis was performed in 0.5 *N* anhydrous methanolic hydrochloric acid for 5 h at 90°, followed by neutralization with silver carbonate. Re-N-acetylation, which is essential for reproducible (high) yields of amino-sugars and sialic acid to be obtained, was performed by reaction with acetic anhydride (0.1 by volume) for 5 h at room temperature. After evaporation at 50° in a stream of nitrogen, the residue was dissolved in water and shaken with chloroform. The aqueous upper layer, containing the methylglycosides, was freeze-dried and the residue was silylated in pyridine with a mixture of 1% (v/v) trimethylchlorosilane (TMCS) in N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) at 50° for 1 h. Aliquots of the contents of the vials were injected into the chromatograph with an injection port temperature of 200°. Chromatography was carried out with a linear temperature programme of 4°/min from 180 to 250°. The reliability of the method was checked with standard glycosphingolipids.

RESULTS AND DISCUSSION

Separation of gangliosides

In order to develop a selective phase system for gangliosides, a number of experiments were carried out. The choice of adsorption chromatography was based mainly on earlier TLC experiments, which showed that a polar solvent had to be used in order to elute these compounds within a reasonable period. Conversion of the TLC phase system, consisting of silica as stationary phase and a chloroform–methanol–water mixture as the mobile phase, to columns led to disappointing results. Whereas in TLC good results with, *e.g.*, chloroform–methanol–water (60:35:8) were obtained, in column chromatography the use of this phase system led to very broad, asymmetric and consequently severely overlapping peaks.

Glycosphingolipids consist of sugar chains of various lengths and compositions, attached to a ceramide molecule. As ceramides are not retarded in this phase system,

it can be concluded that the ceramide moieties of the glycosphingolipids contribute only slightly to their chromatographic properties. The hydrophilic character of the gangliosides, which is due primarily to the presence of sialic acid residues in the molecule, causes a very strong adsorption on silica. The addition of water to the eluent in order to accelerate the separation strongly promotes the dissociation of the sialic acid residues. The consequence is a very strong interaction of the dissociated acid residue(s) of the molecule with the silica surface, which causes the unsatisfactory peak shapes. This effect can be counteracted by adding a strong acid to the eluent, which converts the gangliosides into neutral molecules, leading to significant improvements in peak shape and capacity ratios. In addition, the presence of a strong acid in the eluent increases the selectivity coefficients, because the contribution of the dominating acid residues to the overall retention decreases and the influence of the structure of the solutes becomes more pronounced.

The optimal composition of the phase system with respect to selectivity and speed was investigated more precisely by determining the effect of the type of acid and the water content on the capacity ratio, selectivity coefficient, peak shape and stability of the gangliosides.

As the use of the moving-wire detector system prohibits the use of organic acids, only volatile mineral acids can be applied. Nitric acid was chosen originally, but further TLC analysis of the eluted fractions was hampered owing to the high salt concentrations formed upon neutralization, leaving gas chromatography as the only supporting technique. It was found that use of hydrochloric acid permits subsequent TLC analysis when the eluted fractions are collected in vials containing silver carbonate. In addition to minimizing the time during which the gangliosides are exposed to lower pH values, only low salt concentrations in the fractions are obtained. In general, the type of acid does not influence the capacity ratios and the selectivity coefficients.

The results with different amounts of acid are presented in Table I. At low acid concentrations, almost no improvement in the peak shape was noticed. The reliability of the data with 0.003 *M* hydrochloric acid is therefore relatively low. On the other hand, the low stability of gangliosides in highly acidic solvents tends to reduce the reliability and reproducibility of the data with 0.05 *M* hydrochloric acid.

TABLE I

EFFECT OF THE ACID CONTENT OF THE MOBILE PHASE ON THE CAPACITY RATIOS AND SELECTIVITY COEFFICIENTS OF SOME GANGLIOSIDES ON SILICA (LICHROSORB SI 60)

Eluent: chloroform–methanol–aqueous HCl (60:35:5).

Component	*Final HCl concentration (M)*									
	0.003		*0.005*		*0.01*		*0.02*		*0.05*	
	k'_i	r_{ji}	k'_i	r_{ji}	k'_i	r_{ji}	k'_i	r_{ji}	k'_i	r_{ji}
GM1	2.32	—	2.14	—	1.71	—	1.58	—	1.45	—
GD1a	3.37	1.45	3.11	1.45	2.48	1.45	2.30	1.46	2.04	1.41
GD1b	4.66	1.38	3.97	1.28	3.16	1.27	2.96	1.29	2.81	1.38
GT1	6.59	1.41	5.93	1.49	4.68	1.48	4.12	1.39	3.62	1.29

It is known that gangliosides lose their sialic acid residues in aqueous solution at acid concentrations of 0.05 *M* and above, leaving lower gangliosides and/or asialo derivatives, especially at higher temperatures[29]. Therefore, a final hydrochloric acid concentration of 0.01 *M* was chosen as a compromise between the demands of reasonable peak shape, time of analysis and decomposition.

In Table II, the influence of the water content of the eluent on the capacity ratios and the selectivity coefficients is summarized. For all eluents tabulated, the final concentration of hydrochloric acid was maintained at 0.01 *M*. An increase in the water content decreases the capacity ratios of the individual gangliosides, and decreases the selectivity coefficient of GD1a and GD1b. Hence the system containing 4% of water and a final acid concentration of 0.01 *M* appears to be optimal.

TABLE II

EFFECT OF THE WATER CONTENT OF THE MOBILE PHASE ON THE CAPACITY RATIOS AND SELECTIVITY COEFFICIENTS OF SOME GANGLIOSIDES ON SILICA (LICHROSORB SI 60)

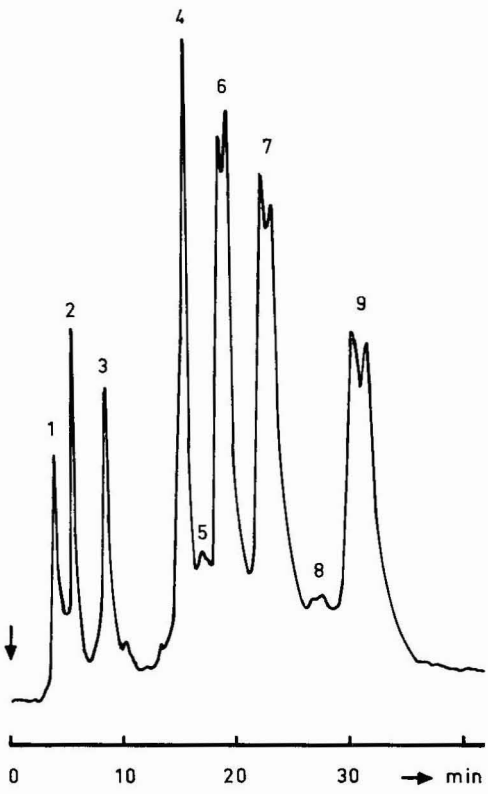
Final HCl concentration: 0.01 *M*. Eluent: chloroform–methanol–aqueous HCl.

Component	*Chloroform–methanol–aqueous HCl*							
	60:35:3		*60:35:4*		*60:35:5*		*60:35:6*	
	k'_i	r_{ji}	k'_i	r_{ji}	k'_i	r_{ji}	k'_i	r_{ji}
GM3	—		0.38	—	—		—	
GM2	—		0.98	2.54	—	—	—	
GM1	2.33	—	2.16	2.21	1.71	—	1.64	
GD1a	3.13	1.34	2.98	1.38	2.48	1.45	2.50	1.52
GD1b	5.02	1.60	4.31	1.45	3.16	1.27	2.93	1.17
GT1	6.67	1.33	6.02	1.40	4.68	1.48	4.52	1.54

Fig. 1 shows a chromatogram of a test mixture of six gangliosides obtained by using this phase system and a 25-cm column. Good separation of the major components was achieved, although the unknown compounds 5 and 8 (apparently impurities) interfered. All of the eluted compounds except 5 and 8 (owing to the considerable overlapping on both sides) were collected and subsequently inspected by means of gas and thin-layer chromatography, as described before. For all collected peaks the expected molar ratios of glucose, galactose, galactosamine (if present) and sialic acid were found. TLC confirmed the identity of the fractions as indicated and showed the high purity of the monosialoganglioside fractions. The purity of the di- and trisialogangliosides was poorer, as judged by visual observation of the primuline-sprayed thin-layer plates. While the liquid chromatogram showed doublets for the higher gangliosides, it appears from gas chromatography that the molar ratios were identical for fractions collected from the front and back of these peaks.

Separation of neutral glycosphingolipids

As neutral glycosphingolipids do not contain sialic acid residues, the hydrophilic character is considerably reduced in comparison with the gangliosides. Chloro-



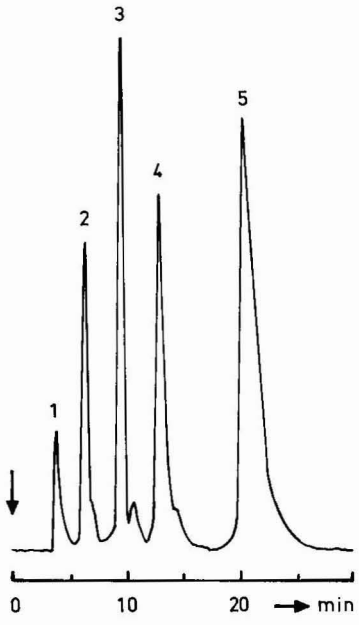


Fig. 1. Separation of a sample mixture of six gangliosides by HPLC. Column: 250 × 2.8 mm I.D. packed with LiChrosorb SI 60 (9 μm). Eluent: chloroform–methanol–aqueous HCl (60:35:4); final HCl concentration, 0.01 *M*. Detection: moving wire with flame-ionization detector. Arrow denotes time of injection. Peaks: 1, 5, 8 = unknown; 2 = GM3; 3 = GM2; 4 = GM1; 6 = GD1a; 7 = GD1b; 9 = GT1.

Fig. 2. Separation of a sample mixture of four neutral glycosphingolipids by HPLC. Conditions as in Fig. 1. Eluent: chloroform–methanol (3:1). Arrow denotes time of injection. Peaks: 1 = unknown; 2 = GL1a; 3 = GL2a; 4 = GL3; 5 = GL4. A thin-layer chromatogram of peaks 2–5 is shown in Fig. 3.

form–methanol (3:1) proved adequate to achieve a good separation of four neutral glycosphingolipids (GL1a, GL2a, GL3 and GL4) (Fig. 2). The pertinent data for the individual compounds in this system are collected in Table III.

All glycolipid fractions were analysed by means of TLC before and after liquid chromatography, as illustrated in Fig. 3. The high purity of the eluted fractions deserves special attention.

It was further shown that the same column can be used for the separation of gangliosides and neutral glycosphingolipids alternately without a severe change in the retention parameters. The silica is reactivated reproducibly after ganglioside analysis by pumping chloroform–methanol (3:1) through the column for 1 h at a flow-rate of 0.5 ml/min.

The chloroform–methanol system thus enables one to separate natural mix-

TABLE III

CAPACITY RATIOS AND SELECTIVITY COEFFICIENTS OF SOME NEUTRAL GLYCOSPHINGOLIPIDS ON SILICA (LICHROSORB SI 60)

Eluent: Chloroform–methanol (3:1).

Component	k_i'	r_{ji}
GL1a	0.63	—
GL2a	1.38	2.19
GL3	2.21	1.60
GL4	4.06	1.84

tures of glycosphingolipids into groups, *viz.*, the neutral glycosphingolipids and the more polar gangliosides, by using a step gradient of chloroform–methanol (3:1) to chloroform–methanol–dilute aqueous acid. The suitability of the method is enhanced by its application to the purification of crude preparations of glycosphingolipids, which contain considerable amounts of contaminating phospholipids and neutral lipids (see below).

Quantitative aspects

In order to improve the applicability of the method described, its use in quantitative analysis was examined. As an example, GL1a as a neutral glycosphingolipid and GM1 as a ganglioside were investigated. The relationships between peak area and amount injected, ranging from 2 to 400 μg for GL1a and from 2 to 200 μg for GM1, were linear, provided that the eluent flow-rate and wire speed were kept constant during the analysis. As the ceramide moieties of the glycosphingolipids differ widely in fatty acid and sphingosine composition within one glycosphingolipid class, the overall carbon content should be known for each compound eluted, prior to final quantification.

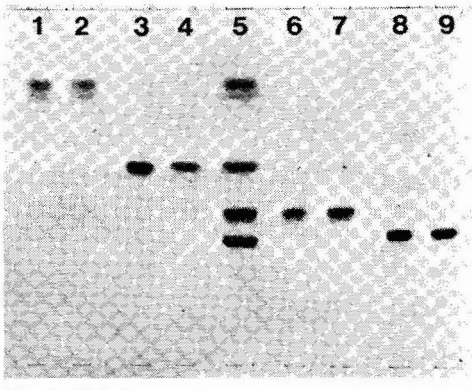


Fig. 3. Thin-layer chromatogram of neutral glycosphingolipids before and after HPLC. Plate: silica pre-coated. Solvent: chloroform–methanol–water (60:35:8.5) containing 200 mg/l of KCl. Development: twice, over 15 cm. Detection: naphthoresorcinol spray. Lane 1, GL1a from Gaucher's spleen; lane 3, synthetic GL2a; lane 5, mixture of GL1a, GL2a, GL3 and GL4 to be separated by HPLC; lane 6, GL3 from Fabry kidney; lane 8, GL4 from human erythrocyte ghosts; lanes 2, 4, 7 and 9, fractions 2, 3, 4 and 5, respectively, from Fig. 2.

Preparative aspects

As the moving-wire detector system renders the derivatization of glycosphingolipids superfluous and its construction is such that only about 1% of the effluent is used for detection, we investigated whether the method can be used on a preparative scale.

Preliminary experiments indicated that the method has to be adapted to the chemical nature of the lipid extract. It is obvious that lipid extracts from the grey matter of brain tissue, in which gangliosides are abundantly present, require less prepurification in order to be suitable for HPLC than extracts from non-neural tissues, in which gangliosides are only minor components[30,32]. In the latter instance considerable amounts of neutral lipids (as cholesterol) and phospholipids are present, of which the latter interfere severely with the separation of gangliosides. We found that treatment of such lipid extracts with methanolic sodium hydroxide solution, by which glycerophospholipids are split and (glyco)sphingolipids left intact, followed by neutralization with methanolic hydrochloric acid gave good results. The fatty acid methyl esters produced are eluted almost unretarded, together with cholesterol, immediately after the solvent front. As only the alkaline-stable sphingomyelin may now interfere, its chromatographic properties were studied in more detail. With chloroform–methanol (3:1) as the eluent it was completely retarded, whereas with chloroform–methanol–aqueous hydrochloric acid as the eluent it was eluted in the midst of the gangliosides. It was found, however, that the capacity ratio for sphingomyelin was affected far more by water content and far less by the acidity of the eluent than were the capacity ratios for gangliosides. Hence the composition of the eluent can be chosen in such a way that sphingomyelin is not co-chromatographed with one of the gangliosides present in the sample.

In micro-preparative applications, we observed lower yields of individual components in the collected fractions than was expected from the quantitative analysis. Small amounts of gangliosides chromatographed on silica with chloroform–methanol–dilute acid as the eluent showed considerable losses of individual gangliosides, apparently due to a loss of sialic acid during evaporation of the solvent at 40° in a stream of nitrogen, as was confirmed by the presence of less hydrophilic compounds in TLC experiments. Even neutralization with silver carbonate did not improve the recoveries. It was observed that decomposition of the gangliosides occurred only during evaporation of the eluent that passed through the column. A mixture of gangliosides, dissolved in freshly prepared (acidic) eluent was stable for at least 12 h at room temperature, while no decomposition was observed in TLC after evaporation of the neutralized solution. Therefore, the interference could be attributed to trace amounts of silica, present in colloidal form in the eluent. It is known that methanol solubilizes silica to some extent[31], especially from very finely ground material. The solubility of silica in ethanol is much lower than in methanol and, for preparative purposes, the replacement of the latter solvent with ethanol leads to a remarkable improvement in the recoveries. Chromatography on silica with chloroform–ethanol–dilute hydrochloric acid (45:40:10) as the eluent was found to be useful in micro-preparative applications. Although the separation of the higher gangliosides, especially GD1a and GD1b, is far less satisfactory than in the methanol system, no loss of gangliosides was observed. It should be borne in mind that in this instance the sample should be introduced in the proper (ethanolic) eluent, as otherwise severe

de-mixing occurs, resulting in ghost peaks near the eluent front containing all components of the sample.

CONCLUSIONS

The proposed method offers good prospects for future research in the fields of, *e.g.*, neurochemistry and membrane biochemistry. By choosing conditions appropriate for the nature of the biological specimen, complete analysis of gangliosides and the lower neutral glycosphingolipids is now possible. Advantages over previously used methods include greater speed and resolution and possibilities of quantitative and preparative applications.

Further research on the glycosphingolipids of plasma membranes from normal liver and hepatomas is at present being carried out[32].

ACKNOWLEDGEMENTS

The authors express their sincere thanks to Professor J. F. K. Huber (University of Vienna) and Dr. J. C. Kraak (University of Amsterdam) for stimulating discussions, to Professor B. Berra (University of Milan) for his generous gifts of pure samples of the gangliosides GM1, GD1a, GD1b and GT1, to Professor J. M. Tager (University of Amsterdam) for a gift of GL3 and to the Centre National de la Recherche Scientifique for a travel grant to one of us (R.P.v.H.) to attend a ganglioside conference at Strasbourg (France) in 1973.

REFERENCES

1 L. Svennerholm, *J. Neurochem.*, 10 (1963) 613.
2 G. Dawson, *J. Lipid Res.*, 13 (1972) 207.
3 S. I. Hakomori, *Biochim. Biophys. Acta*, 417 (1975) 55.
4 H. Wagner, L. Hörhammer and P. Wolff, *Biochem. Z.*, 334 (1961) 175.
5 H. Jatzkewitz and E. Mehl, *Hoppe Seyler's Z. Physiol. Chem.*, 320 (1960) 251.
6 S. R. Korey and J. Gonatas, *Life Sci.*, 2 (1963) 296.
7 E. Svennerholm and L. Svennerholm, *Biochim. Biophys. Acta*, 70 (1963) 432.
8 V. P. Skipski, A. F. Smolowe and M. Barclay, *J. Lipid Res.*, 8 (1967) 295.
9 J. R. Wherrett and J. N. Cumings, *Biochem. J.*, 86 (1963) 378.
10 D. H. van den Eijnden, *Hoppe Seyler's Z. Physiol. Chem.*, 352 (1971) 1601.
11 E. P. M. Oomen-Meulemans and R. P. van Hoeven, unpublished results.
12 R. A. de Zeeuw, *Anal. Chem.*, 40 (1968) 2134.
13 K. Suzuki, *Life Sci.*, 3 (1964) 1227.
14 V. P. Skipski, *Methods Enzymol.*, 35 (1975) 396.
15 J. E. Evans and R. H. McCluer, *Biochim. Biophys. Acta*, 270 (1972) 565.
16 G. Tettamanti, L. Bertona, B. Berra and V. Zambotti, *Ital. J. Biochem.*, 13 (1964) 315.
17 S. I. Hakomori and B. Siddiqui, *Methods Enzymol.*, 32 (1974) 345.
18 J. T. Dodge, C. Mitchell and D. J. Hanahan, *Arch. Biochem. Biophys.*, 100 (1963) 119.
19 J. Koscielak, *Biochim. Biophys. Acta*, 78 (1963) 313.
20 S. I. Hakomori and D. B. Strycharz, *Biochemistry*, 7 (1968) 1279.
21 R. J. Desnick, C. C. Sweeley and W. Kr vit , *Lipid Res.*, 11 (1970) 31.
22 U. R. Tjaden, J. C. Kraak and J. F. K. Huber, *J. Chromatogr.*, 143 (1977) 183.
23 R. P. W. Scott and J. G. Lawrence, *J. Chromatogr. Sci.*, 8 (1970) 65.
24 L. Svennerholm, *Biochim. Biophys. Acta*, 24 (1957) 604.
25 L. Svennerholm, *J. Neurochem.*, 1 (1956) 42.

26 G. Pastuska, *Z. Anal. Chem.*, 179 (1961) 427.
27 J. C. Dittmer and R. L. Lester, *J. Lipid Res.*, 5 (1964) 126.
28 C. C. Sweeley and B. Walker, *Anal. Chem.*, 36 (1964) 1461.
29 R. Ledeen, *J. Amer. Oil Chem. Soc.*, 43 (1966) 57.
30 E. Mårtensson, *Progr. Chem. Fats Other Lipids*, 10 (1969) 365.
31 J. J. Wren, *J. Chromatogr.*, 4 (1960) 173.
32 R. P. van Hoeven, E. P. M. Oomen-Meulemans, J. H. Krol and P. Emmelot, in preparation.

Journal of Chromatography, 136 (1977) 245–252

CHROM. 9893

GAS–LIQUID CHROMATOGRAPHY OF RESIN ACID ESTERS

D. F. ZINKEL and C. C. ENGLER

Forest Products Laboratory, Forest Service U.S. Department of Agriculture**, P.O. Box 5130, Madison, Wisc. 53705 (U.S.A.)*

(Received December 22nd, 1976)

SUMMARY

A number of alkyl esters of resin acids were prepared with dialkyl acetals of dimethylformamide, and their gas chromatographic characteristics evaluated with several packed columns. The cyanosilicone liquid phases allow for simultaneous determination of levopimaric and palustric acids, a determination previously unattainable by gas–liquid chromatography.

INTRODUCTION

Hudy[1] in 1959 applied gas–liquid chromatography (GLC) to the analysis of resin acid methyl esters using Apiezon N and three polyesters. A number of workers have since extended that work to include a variety of other liquid phases. An overview of the field can be found in the reviews of Zinkel *et al.*[2] and Bardyshev and Bulgakov[3].

Although a number of gas chromatographic systems have been studied, satisfactory resolution of certain resin acid methyl esters has not been attained, such as in analyzing natural products containing levopimaric and palustric acids. Two possible approaches for satisfactory resolution are (1) increasing liquid phase polarity, and (2) accentuating the differences of the double-bond character of the resin acid esters by reducing the common leveling effect of the ester carbonyl–liquid phase interaction. The applicability of polar cyanosilicone liquid phases and hindered *tert.*-butyl esters in the GLC of resin acids to effect these approaches is the subject of this paper.

EXPERIMENTAL

Hewlett-Packard (Models 5750 and 5830) and Research Specialties (Model 600) gas chromatographs equipped with flame-ionization detectors (FIDs) were used. Column packings were prepared by the slurry techniques using Chromosorb W AW

* Maintained in cooperation with the University of Wisconsin.

** Mention of trade or proprietary names is for identification only and does not imply endorsement by the Forest Service of the U.S. Department of Agriculture.

support and the liquid phase, followed by fluidized drying. Stainless-steel columns were used during acquisition of retention characteristics but were later replaced by glass columns. Columns were packed by both free-fall and pressure techniques appropriate for the column configuration.

Liquid phases included the methyl silicone SE-30 [on a support precoated with ethylene glycol isophthalate (EGiP)], the trifluoropropyl silicone SP-2401 (Supelco, Bellefonte, Pa., U.S.A.), the phenyl methyl silicone OV-17, Carbowax 20M, the phenyl cyanopropyl methyl silicone OV-225, diethylene glycol succinate (DEGS), and the cyanosilicones Silar 10C (Applied Science Labs., State College, Pa., U.S.A.) and OV-275.

Diazomethane, prepared from N-methyl-N-nitroso-*p*-toluenesulfonamide, was used to prepare the resin acid methyl esters[4]. Other resin acid esters were prepared with the dialkyl acetals of dimethylformamide (DMF). The ethyl, propyl, isopropyl, butyl, and *tert.*-butyl reagents were obtained from Pierce (Rockford, Ill., U.S.A.), the cyclohexyl reagent from Aldrich (Milwaukee, Wis., U.S.A.). To prepare esters with the dialkylacetal reagents, 10 mg of resin acids and 200 mg of the neat reagent were heated in a dry, capped vial at 60° overnight. The excess reagent was removed at reduced pressure with heating. The esters were dissolved in petroleum ether for GLC.

Resin acid methyl esters for determination of the response factor were purified by the methods required in preparing standards for reference spectra[2].

RESULTS AND DISCUSSION

Columns

In a previous study[5], DEGS was found the most generally useful liquid phase for the GLC of resin acid methyl esters (we have found the retention values on EGSS-X are almost identical with those for DEGS; the liquid phases are interchangeable for this purpose with the minor disadvantage of a smaller solute capacity with EGSS-X). However, the very long retention characteristics of other resin acid esters having additional oxygen-containing functionality (CHO, COH, COOMe) require a non-polar packing. SE-30 on a support precoated with a small amount of EGiP has been most useful in this respect[5]. The EGiP precoating considerably reduces support-catalyzed levopimarate decomposition, but does not eliminate it.

In earlier work in this laboratory, Anakrom ABS was found the most inert support available for GLC of resin acid methyl esters because it did not cause isomerization of palustrate and even more susceptible levopimarate. Over the years, however, the degree of Anakrom ABS silanization was increased by the manufacturer; this provided an improved support for many applications but concurrently resulted in an unsatisfactory support for preparation of efficient polyester packings. Fortunately, improvements in the preparation of Chromosorb W AW have provided an acceptable replacement.

In earlier observations at this laboratory on the effect of the column material we found that glass columns were of no advantage over stainless-steel columns containing DEGS packings when using the requisite amount of resin acid esters for an adequate thermal conductivity detector response. We have now verified this observation for an EGSS-X packing even at the much lower sample-size levels used with the FID.

This is not the case, however, with a cyanosilicone liquid phase such as Silar 10C. Extensive decomposition of the readily isomerizable methyl levopimarate can only be avoided by using glass columns. As a general precaution, glass columns are now used in the major portion of our GLC investigations involving resin acid esters.

Resin acid esterification

To facilitate gas chromatography of resin acids, the methyl ester is usually prepared. Although several reagents can be used to accomplish the methylation of the analogous fatty acids[6], only two methods, reaction with diazomethane and pyrolysis of the tetramethyl ammonium salt[7], can be used with the more hindered resin acid carboxyl group.

On comparing the functional groups of the various resin acid methyl esters (18-carboxylates) it seems reasonable that the carbomethoxy function will have interactions of the same magnitude for polar liquid phases. Hindered alkyl esters such as the trimethylsilyl ester, which is readily prepared, should accentuate the differences in unsaturation, thus, enhance the separations. Evidence from the retentions of the trimethylsilyl esters on several liquid phases of low to moderate polarity indicated, indeed, the approach was valid[8]. However, the trimethylsilyl esters are very susceptible to hydrolysis or transesterification during GLC on polar liquid phases (a fact overlooked in several publications on the GLC of trimethylsilyl esters of various acids).

Not until the relatively recent availability of the dialkyl acetal dimethylformamide reagents was there a potential for convenient preparation of a variety of other esters for analytical purposes. For example, saturated fatty acid esters are prepared rapidly and quantitatively by this method[9], although care must be exercised to avoid spurious GLC peaks[10]. We have found the reaction of resin acids with the neat reagent at 60° overnight is essentially quantitative. As an interesting aside, the phenolic hydroxyl as well as the axial carboxyl of podocarpic acid was found to be methylated by the dimethyl acetal reagent. The determination of the specific conditions for quantitative methyl ether formation for podocarpic acid or other phenolics was outside the scope of this investigation.

Methyl, ethyl, propyl, isopropyl, butyl, *tert.*-butyl, and cyclohexyl esters of pimaric acid were prepared using the dialkyl acetal–DMF reagents. Comparative retention data on eight liquid phases with a broad range of polarities are presented in Table I. Inspection of the data shows (1) the expected increase in retention with increasing molecular weight for the *n*-alkyl and cyclohexyl esters and (2) a pattern of decreasing retention with increasing liquid phase polarity for the more hindered isopropyl and *tert.*-butyl esters (the retention times for the isopropyl and *tert.*-butyl esters on the more polar liquid phases are less than that of the corresponding methyl ester). The retention characteristics for esters of the other usual pine resin acids (sandaracopimaric, palustric, levopimaric, isopimaric, abietic, dehydroabietic, and neoabietic acids) paralled the data in Table I for pimaric acid esters.

GLC separation of resin acid esters

Because of the possibility for improved separations coupled with decreased retention time, the *tert.*-butyl esters appeared a promising replacement for the methyl esters in the GLC of resin acids. Relative retention data for these two esters are compared for nine liquid phases (including the new polar phase, OV-275) in Table II.

TABLE I

GLC RETENTION CHARACTERISTICS OF ALKYL ESTERS OF PIMARIC ACID

Conditions			*Retention time of methyl ester (min)*	*Retention time relative to methyl ester*					
Liquid phase	*Column temp. (°C)*	*Nitrogen flow-rate (ml/min)*		*Ethyl*	*n-Propyl*	*n-Butyl*	*Cyclo-hexyl*	*Iso-propyl*	*tert.-Butyl*
9% SE-30–EGiP (1/8 in. × 6 ft.)	200	90	10.4	1.20	1.63	2.18	5.32	1.27	1.35
3% SP-2401 (1/8 in. × 10 ft.)	200	35	8.6	1.15	1.54	2.07	4.72	1.27	1.20
3% OV-17 (1/8 in. × 6 ft.)	210	40	6.1	1.16	1.54	2.08	5.32	1.12	1.14
3% Polyphenyl ether (6-ring)	210	35	9.6	1.13	1.54	2.18	6.56	1.04	1.01
5% OV-225 (1/4 in. × 6 ft.)	210	70	8.9	1.09	1.41	1.77	4.53	1.00	0.97
5% Carbowax 20M (1/8 in. × 6 ft.)	210	20	8.5	1.06	1.35	1.72	4.59	0.94	0.87
10% DEGS (1/8 in. × 8 ft.)	200	50	21.0	0.98	1.23	1.52	4.04	0.86	0.75
10% Silar 10C (1/8 in. × 11 ft.)	200	20	21.8	1.00	1.23	1.49	3.70	0.83	0.73

TABLE II

GLC RETENTION CHARACTERISTICS OF METHYL (Me) AND *tert.*-BUTYL (t-Bu) ESTERS OF RESIN ACIDS AT 200°

Liquid phase	*Ester*	*Resin acid*							
		Pimaric	*Sandaraco-pimaric*	*Levo-pimaric*	*Palustric*	*Iso-pimaric*	*Abietic*	*Dehydro-abietic*	*Neo-abietic*
SE-30–EGiP	Me	1.00	1.06	1.22	1.22	1.20	1.55	1.34	1.84
	t-Bu	1.00	1.05	1.24	1.17	1.09	1.41	1.27	1.82
SP-2401	Me	1.00	1.08	1.21	1.17	1.28	1.60	1.39	1.77
	t-Bu	1.00	1.09	1.21	1.11	1.16	1.41	1.34	1.77
OV-17	Me	1.00	1.07	1.33	1.31	1.28	1.74	1.67	2.17
	t-Bu	1.00	1.08	1.32	1.31	1.17	1.63	1.62	2.20
PPE	Me	1.00	1.09	1.38	1.39	1.39	2.08	2.01	2.57
	t-Bu	1.00	1.10	1.37	1.37	1.23	1.91	1.98	2.55
OV-225	Me	1.00	1.08	1.29	1.27	1.29	1.75	1.69	2.12
	t-Bu	1.00	1.09	1.30	1.22	1.19	1.41	1.65	2.12
Carbowax 20M	Me	1.00	1.10	1.34	1.36	1.39	2.01	2.04	2.40
	t-Bu	1.00	1.09	1.33	1.33	1.22	1.83	1.92	2.38
DEGS	Me	1.00	1.12	1.33	1.34	1.46	2.08	2.25	2.46
	t-Bu	1.00	1.11	1.33	1.31	1.25	1.86	2.15	2.39
Silar 10C	Me	1.00	1.15	1.42	1.36	1.51	2.13	2.48	2.53
	t-Bu	1.00	1.14	1.40	1.31	1.32	1.87	2.30	2.42
OV-275*	Me	1.00	1.15	1.38	1.29	1.54	2.14	2.56	2.54
	t-Bu	1.00	1.15	1.46	1.27	1.35	1.92	2.37	2.49

* Column of 1/8 in. × 10 ft. packed with 20% OV-275 on 80–100 Chromosorb W AW at 200°; nitrogen flow-rate, 17 ml/min. Retention time of methyl ester, 15.0 min.

Although improved separation is seen for *tert.*-butyl levopimarate and palustrate with several of the packings, overlap of other previously resolved components now occurs.

Previous to our work with OV-275, we used the methyl and *tert.*-butyl esters for determining the palustric and the levopimaric acid contents in pine extractives by GLC analysis on Silar 10C but GLC analysis on another (DEGS or EGSS-X) column was necessary to resolve the methyl dehydroabietate–neoabietate or the *tert.*-butyl palustrate–isopimarate overlaps. The separations shown in Table II for the *tert.*-butyl esters of the common resin acids indicate that OV-275 would be the liquid phase of choice. However, we and others[11] have not been able to obtain as efficient columns with OV-275 using Chromosorb W supports as with Silar 10C and other liquid phases. In addition, we have observed that the 15% OV-275 on Chromosorb P AW DMCS column packings reported[12] to be efficient for resolution of fatty acid esters such as methyl oleate–elaidate cause complete decomposition of levopimarate and very extensive decomposition of palustrate. The longer time in preparation of the *tert.*-butyl ester by the *tert.*-butyl acetal of DMF reagent compared with the quick preparation of the methyl ester with diazomethane also can be an important factor in a specific analytical situation. Thus we are now analyzing the resin acids in most pinewood extractives and oleoresins as their methyl esters with a two-column GLC system using 10% EGSS-X for the primary data and 20% OV-275 for the determination of methyl levopimarate and palustrate.

Routine GLC of resin acid esters on polyester liquid phases has been limited to column temperatures of about 200° because of liquid phase instability. The greater stability of the cyanosilicones, however, permits an evaluation of possible improved separation at higher temperatures not only for palustrate–levopimarate, but also for other less common and difficultly resolved pairs such as communate–8,13β-epoxy-14-labden-19-oate (found in *Pinus resinosa* needles[13]) and anticopalate–isopimarate[14]. Little change is seen, however, in separation of the resin acid methyl esters with increasing column temperature (Table III) except for some small improvement in the dehydroabietate–neoabietate pair. Thus, GLC on two, and in some cases even three

TABLE III

EFFECT OF TEMPERATURE ON RETENTION OF RESIN ACID METHYL ESTERS ON CYANOSILICONE LIQUID PHASES

Methyl ester	*10% Silar 10C*			*20% OV-275*		
	200°	*215°*	*230°*	*200°*	*215°*	*230°*
Pimarate	1.00	1.00	1.00	1.00	1.00	1.00
Sandaracopimarate	1.16	1.14	1.12	1.15	1.14	1.13
Levopimarate	1.42	1.38	1.34	1.38	1.34	1.30
Palustrate	1.37	1.32	1.26	1.29	1.24	1.20
Communate	1.40	1.34	1.27	1.38	1.31	1.25
8,13β-Epoxy-14-labdenoate	1.39	1.35	1.31	1.41	1.36	1.32
Anticopalate	1.48	1.42	1.37	1.41	1.34	1.31
Isopimarate	1.53	1.49	1.44	1.54	1.50	1.46
Abietate	2.17	2.05	1.92	2.14	2.02	1.90
Dehydroabietate	2.54	2.35	2.15	2.50	2.27	2.11
Neobaietate	2.57	2.42	2.24	2.54	2.35	2.21

(*e.g.*, OV-275, EGSS-X and SE-30), columns is necessary to obtain complete analyses for the individual resin acids in pine extractives.

Response factors

The relative response, calculated as correction factors[5], of resin acid methyl esters are compared in Table IV with data reported by Pensar and Bruun[15]. The large correction factors, reported by Pensar and Bruun most likely reflect the state of purity of their samples; the resin acids, especially the abietadienoic acids (and methyl esters), are readily oxidized by air.

TABLE IV

FID CORRECTION FACTORS FOR RESIN-ACID METHYL ESTERS

Methyl ester	*Correction factor*	
	Present work	*Calculated from data ref. 15*
Pimarate	1.00	1.00
Sandaracopimarate	1.03	1.03
Palustrate	1.05	1.17
Levopimarate	1.07	
Isopimarate	0.99	1.00
Abietate	1.08	1.15
Dehydroabietate	0.99	1.09
Neoabietate	1.12	1.31

CONCLUSIONS

The highly polar cyanosilicone liquid phases, Silar 10C and OV-275, are useful in the GLC of both methyl and *tert.*-butyl esters of diterpene resin acids, particularly in the separation of palustrate–levopimarate. No single liquid phase in a packed column, however, provides sufficient separation between the common pine resin acids for an adequate resolution of all components. Thus, the complete analysis of all the resin acids from a pine extract requires GLC on several different columns.

ACKNOWLEDGEMENT

This work was supported in part by the Pulp Chemicals Association, New York. N.Y., U.S.A.

REFERENCES

1 J. A. Hudy, *Anal. Chem.*, 31 (1959) 1754.
2 D. F. Zinkel, L. C. Zank and M. F. Wesolowski, *Diterpene Resin Acids —A Compilation of Infrared, Mass, Nuclear Magnetic Resonance, Ultraviolet Spectra and Gas Chromatographic Retention Data*, U.S.D.A., Forest Service, Forest Products Laboratory, Madison, Wis., 1971.
3 I. I. Bardyshev and A. N. Bulgakov, *Khromatogr. Anal. Khim. Drev.*, (1975) 101.
4 F. H. M. Nestler and D. F. Zinkel, *Anal. Chem.*, 35 (1963) 1747.
5 F. H. M. Nestler and D. F. Zinkel, *Anal. Chem.*, 39 (1967) 1118.
6 A. J. Sheppard and J. L. Iverson, *J. Chromatogr. Sci.*, 13 (1975) 448.

7 N. E. Hetman, H. G. Arlt, Jr., R. Paylor and R. Feinland, *J. Amer. Oil Chem. Soc.*, 42 (1965) 255.
8 D. F. Zinkel, M. B. Lathrop and L. C. Zank, *J. Gas Chromatogr.*, 6 (1968) 158.
9 J.-P. Thenot, E. C. Horning, M. Stafford and M. G. Horning, *Anal. Lett.*, 5 (1972) 217.
10 *Chromatography Lipids*, VII, No. 4 (1973), Supelco, Bellefonte, Pa.
11 *Chromatography Lipids*, VIII, No. 6 (1974), Supelco, Bellefonte, Pa.
12 D. M. Ottenstein, D. A. Bartley and W. R. Supina, *J. Chromatogr.*, 119 (1976) 401.
13 D. F. Zinkel and W. B. Clarke, unpublished results.
14 D. F. Zinkel and B. P. Spalding, *Phytochem.*, 11 (1972) 425.
15 G. Pensar and H. H. Bruun, *Suomen Kemistilehti*, B38 (1965) 223.

Journal of Chromatography, 136 (1977) 253–258

CHROM. 9948

GAS CHROMATOGRAPHIC SEPARATION OF LOWER ALIPHATIC PRIMARY AMINES AS THEIR SULPHUR-CONTAINING SCHIFF BASES USING A GLASS CAPILLARY COLUMN

YASUYUKI HOSHIKA

Aichi Environmental Research Centre, 7-6, Tsuji-machi, Kita-ku, Nagoya-shi, Aichi (Japan)

(Received December 21st, 1976)

SUMMARY

Thirteen C_1–C_7 aliphatic primary amines were quantitatively converted to the corresponding sulphur-containing Schiff bases by the reaction (60°, 1 h) with 2-thiophene aldehyde. Complete separation of the derivatives of the 13 amines was achieved on a 30 m × 0.25 mm I.D. glass capillary column (125°), packed with PEG-20M.

INTRODUCTION

The trace analysis of lower aliphatic amines is important because these amines cause odours and problems in agriculture, such as the decay of foods and fish. It is particularly important to know the components of the amines, especially the primary amines, and any carbonyl compounds additionally present, because these compounds easily produce Schiff base condensates.

In general, when packed columns such as those listed in Table I are employed, the complete separation of free amines, such as ethyl-, *n*-propyl-, isopropyl-, *tert.*-butyl- and allylamines, is very difficult. Seven columns, *viz.*, THEED + TEP*,[1], triethanolamine[2], Squalane + glycerine[3], Chromosorb 103[4,5], Pennwalt 223[5] and PEG 1500 and 20M[6] have been tested but all were unsatisfactory for the purpose of complete separation.

Gas chromatography (GC) using chemical reactions for the biologically important primary amines such as epinephrine, dopamine, catecholamines, phenethylamine and amphetamine has been reported. Several derivatives, such as trimethylsilyl ethers[7], Schiff bases[8–12], *p*-tosylamides[13], pentafluorobenzoylamides[5,14,15], 2,4-dinitrophenyl derivatives[16,17], isothiocyanates[18,19] and trifluoroacetates[20–22] have been studied for this purpose. Pentafluorobenzaldehyde, pentafluorobenzoyl chloride, 2,4-dinitrofluorobenzene and trifluoroacetic anhydride have been used as derivatization agents for the electron-capture detection of picogram amounts of several primary amines.

However, most of these techniques have been applied to high-molecular-weight

* THEED = N,N,N′,N′-Tetrakis (2-hydroxyethyl)ethylene diamine; TEP = tetraethylene pentamine.

compounds, with few applications to lower aliphatic primary amines[5,17,23,24]. The reactions of pentafluorobenzoyl chloride[5] with ammonia and lower aliphatic primary and secondary amines such as methyl-, dimethyl-, ethyl-, diethyl-, *n*-propyl- and di-*n*-propylamines gave white, curdy precipitates, which may be the hydrochlorides of the amines, so that the reactions were not quantitative. Day *et al.*'s procedure[17], involving partial separation into three distinct zones by thin-layer chromatography, is required as a preliminary treatment prior to GC analysis. The procedure for the derivative-formation reaction (2,4-dinitrophenylamines) is also sometimes complex.

In contrast, the reaction of Schiff base formation with the lower aliphatic primary amines using benzaldehyde and pentafluorobenzaldehyde take place easily and rapidly at room temperature. The by-product of these reactions is water, which does not undergo secondary reactions in the reaction systems involved. These methods resulted in sharper peaks, high precision and high selectivity with the lower aliphatic primary amines. While the minimum detectable amount of fluorine-containing Schiff bases[24] was about 0.02 ng with an electron-capture detector (ECD), that of non-fluorine-containing Schiff bases was about 60 ng with a flame-ionization detector (FID)[23]. However, in these methods, the separation of the derivatives of *n*-propyl-, allyl- and *tert.*-butylamines was poor.

This paper describes a complete GC separation of 13 lower aliphatic primary amines as their sulphur-containing Schiff bases using a glass capillary column, which has a high resolving power.

EXPERIMENTAL

Preparation of sulphur-containing Schiff base

The procedure for the preparation of sulphur-containing Schiff bases derived from lower aliphatic primary amines with 2-thiophene aldehyde (THA) was as follows. The amine ($1 \cdot 10^{-5}$ mole) and THA ($1–10 \cdot 10^{-5}$ mole) were added to 1 ml of ethanol and the mixture was allowed to react at temperatures from room temperature to 60° for 0.5–4 h. The rate of reaction was evaluated by measuring the disappearance of the amine or THA by GC using Tenax-GC as column packing.

Reagents

THA (b.p. 198°) and methyl- (40 wt.-% aqueous solution), ethyl- (70 wt.-% aqueous solution) and isopropylamine were obtained from Tokyo Kasei Kogyo, Tokyo, Japan. *n*-Propyl-, allyl-, *n*-butyl-, *sec.*-butyl- and *tert.*-butylamine and ethanol were obtained from Wako Pure Chemical Industries, Osaka, Japan. Isobutyl-, *n*-amyl-, isoamyl-, *n*-hexyl- and *n*-heptylamine were obtained from PolyScience Corp., Niles, Ill., U.S.A. All reagents were guaranteed or reagent-grade chemicals.

Apparatus

The gas chromatograph was a Shimadzu Model GC5AP$_5$FFp equipped with an FID and a flame-photometric detector (FPD); the FPD had an FID for monitoring. The large amounts of organic solvent present were detected with the latter FID. However, the sensitivity of this FID is lower than that of the normal FID, because it operates with a hydrogen-rich flame. The FPD and its FID accessory were operated with a separate electrometer (Shimadzu Model EM-5S). The detector signals were

recorded at 10 mV full scale simultaneously on a Shimadzu Model R-201 double-pen recorder.

The Shimadzu GC5AP$_5$FFp gas chromatograph was also equipped with a digital integrator (Shimadzu Model ITG-2A) for the determination of the rate of the sulphur-containing Schiff base formation reaction.

Chromatographic conditions

The analytical columns used were as follows: (a) a 3 m × 3 mm I.D. glass column packed with Tenax-GC (made by Enka, Arnhem, The Netherlands), 60–80 mesh; (b) a G-SCOT column with an SF-96 20 m × 0.28 mm I.D. glass capillary column (obtained from Gasukuro Kogyo, Tokyo, Japan); (c) a Hitachi Chemi-column with a PEG-20M 30 m × 0.25 mm I.D. glass capillary column (obtained from Hitachi, Ibaraki, Japan).

The GC conditions for each of the analytical columns were as follows. (a) carrier gas (nitrogen) flow-rate, 55 ml/min; air and hydrogen flow-rates, 1.0 l/min and 50 ml/min, respectively; column temperature (programmed), held for 1 min at 100°, heated at 10°/min to 250°, maintained at this temperature for 25 min, then cooled to the starting temperature; injection port and detector (FID) temperatures, 250°. (b) carrier gas (nitrogen) flow-rate, 0.97 ml/min; purge gas (nitrogen) flow-rate, 50 ml/min; air and hydrogen flow-rates, 1.0 l/min and 50 ml/min, respectively; column temperature (programmed), held for 1 min at 100°, heated at 4°/min to 200°; injection port and detector (FID) temperatures, 200°; splitting ratio, 1:140. (c) carrier gas (nitrogen) flow-rate, 0.7 ml/min; purge gas (nitrogen) flow-rate, 50 ml/min for FID and 140 ml/min for FPD; air flow-rate, 1.0 l/min for FID and 50 ml/min for FPD; hydrogen flow-rate, 50 ml/min for FID and 80 ml/min for FPD; column temperature, 125°; injection port temperature, 180°; detector temperature, 180° for FID and 205° for FPD; splitting ratio, 1:180.

RESULTS AND DISCUSSION

The complete GC separation of lower aliphatic primary amines, especially ethyl-, *n*-propyl-, isopropyl-, allyl-, isobutyl-, *sec.*-butyl- and *tert.*-butylamine, is very difficult in some packed columns. As listed in Table I, all columns tested were inadequate for the complete separation of the 13 amines of interest here.

The optimal conditions for the sulphur-containing Schiff base formation reaction were as follows: molar ratio of THA to amine, >2; reaction temperature, 60°; reaction time, 1 h. The reactions of THA with the 13 amines were quantitative under these conditions.

Fig. 1 shows a typical gas chromatogram obtained with the SF-96 glass capillary column and an FID. The derivatives of *n*-propyl- and *tert.*-butylamine were not separated, probably as a result of the low polarity of SF-96.

Fig. 2 shows a typical gas chromatogram obtained with the PEG-20M glass capillary column and an FID. The complete separation of the derivatives of the 13 amines was achieved. Unfortunately, when helium was used as the carrier gas at a flow-rate of 0.7 ml/min, the separation of the peaks of the derivatives of methyl-, *tert.*-butyl-, *n*-butyl- and allylamine was poor.

Fig. 3 shows a typical gas chromatogram obtained with the PEG-20M glass capillary column and an FPD. Although the sensitivity was much higher than that

TABLE I

EFFECTIVENESS OF COLUMNS TESTED USING AN FID

Packing	*Note**	*Free amines giving overlapping peaks*
THEED + TEP	1	Ethyl-, isopropyl- and *tert.*-butylamine
Triethanolamine	2	Ethyl- and isopropylamine
Squalane + glycerine	3	Allyl- and *tert.*-butylamine
Chromosorb 103	4	Isopropyl-, isobutyl- and *tert.*-butylamine
Pennwalt 223	5	*n*-Propyl- and allylamine
PEG-1500	6	Isobutyl- and *sec.*-butylamine
PEG-20M	7	Ethyl-, *n*-propyl-, isopropyl-, allyl- and *tert.*-butylamine

* Notes:

(1) 15% THEED + 5% TEP on Chromosorb W AW DMCS, 60–80 mesh, 3 m × 3 mm I.D., 60°, N_2 flow-rate 58 ml/min.

(2) 20% triethanolamine on Celite 545, 60–80 mesh, 3 m × 3 mm I.D., 70°, N_2 flow-rate 50 ml/min.

(3) 20% Squalane + 2.5% glycerine + 2.5% KOH on Diasolid L, 60–80 mesh, 3 m × 3 mm I.D., 60°, N_2 flow-rate 50 ml/min.

(4) Chromosorb 103, 60–80 mesh, 3 m × 3 mm I.D., 130°, N_2 flow-rate 57 ml/min.

(5) 28% Pennwalt 223 + 4% KOH on Gas-Chrom R, 80–100 mesh, 3 m × 3 mm I.D., 60°, N_2 flow-rate 57 ml/min.

(6) 0.5% PEG-1500 + 0.2% KOH on Carbopack B, 60–80 mesh, 1.5 m × 3 mm I.D., 75°, N_2 flow-rate 50 ml/min.

(7) 1.3% PEG-20M + 0.3% KOH on Carbopack B, 60–80 mesh, 1.5 m × 3 mm I.D., 50°, N_2 flow-rate 50 ml/min.

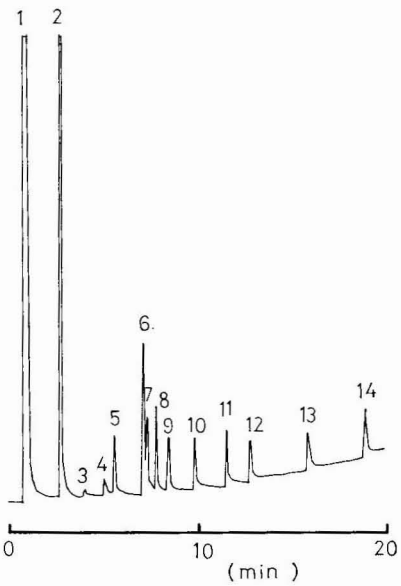


Fig. 1. Gas chromatogram of sulphur-containing Schiff bases, obtained with an SF-96 glass capillary column and an FID. GC conditions, (b). Sample concentration, $1 \cdot 10^{-5}$ mole/ml; sample injected, 1 μl. Peaks of sulphur-containing Schiff bases: 1 = ethanol (solvent); 2 = THA (excess); 3 = methylamine; 4 = ethylamine; 5 = isopropylamine; 6 = *tert.*-butylamine + *n*-propylamine; 7 = allylamine; 8 = *sec.*-butylamine; 9 = isobutylamine; 10 = *n*-butylamine; 11 = isoamylamine; 12 = *n*-amylamine; 13 = *n*-hexylamine; 14 = *n*-heptylamine.

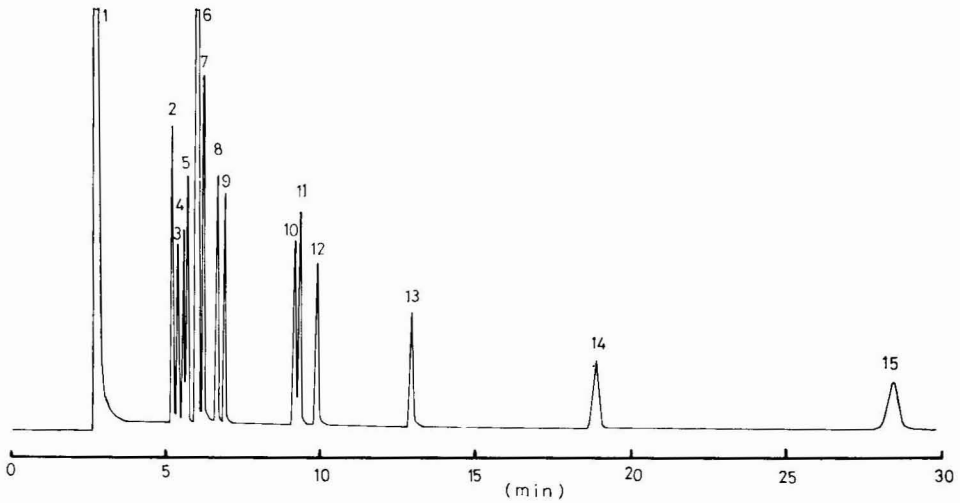


Fig. 2. Gas chromatogram of sulphur-containing Schiff bases obtained with a PEG-20M glass capillary column and an FID. GC conditions, (c). Sample concentration and volume injected as in Fig. 1. Peaks of sulphur-containing Schiff bases: 1 = ethanol (solvent); 2 = isopropylamine; 3 = methylamine; 4 = ethylamine; 5 = *tert.*-butylamine; 6 = THA (excess); 7 = *sec.*-butylamine; 8 = *n*-propylamine; 9 = isobutylamine; 10 = *n*-butylamine; 11 = allylamine; 12 = isoamylamine; 13 = *n*-amylamine; 14 = *n*-hexylamine; 15 = *n*-heptylamine.

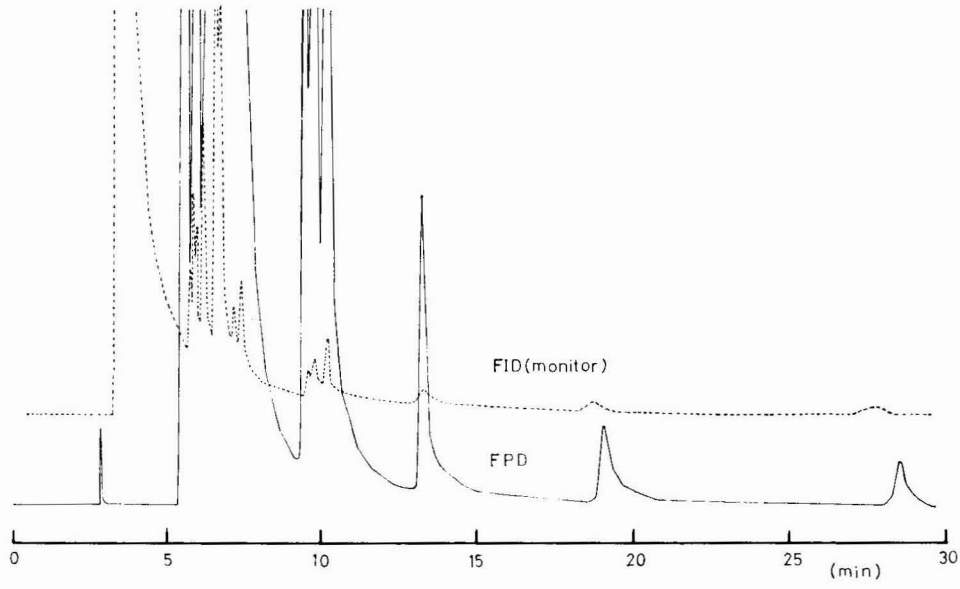


Fig. 3. Gas chromatogram of sulphur-containing Schiff bases obtained with a PEG-20M glass capillary column and an FPD and FID (monitor). GC conditions, (c). Sample concentration and volume injected as in Fig. 1.

of the FID for the derivatives of the C_1–C_4 amines, the sensitivities for the derivatives of the C_5–C_7 amines were similar. The first peak eluted from the PEG-20M column was the derivative of isopropylamine; the derivative of *n*-heptylamine was eluted within about 30 min.

The optimal flow-rates of the purge gas, hydrogen and air or oxygen in FPDs when using glass capillary columns have been described recently[25–28]. In this study of these optimal conditions the derivative of *n*-butylamine was used. At flow-rates of the purge gas from 50 to 110 ml/min, peaks with considerable tailing were obtained. At flow-rates greater than 200 ml/min the sensitivity was lower, but the tailing was only slight. Therefore, the optimal flow-rate of the purge gas was about 150 ml/min. At flow-rates of hydrogen from 40 to 70 ml/min a lower sensitivity of peak detection occurred, and the optimal flow-rate was about 80 ml/min. The flow-rate of air was maintained constant at 50 ml/min.

In the presence of 100 molar equivalents of ammonia and dimethyl-, trimethyl-, diethyl- and triethylamine, no evidence of interference was found in the gas chromatogram.

ACKNOWLEDGEMENTS

The author thanks Y. Takata, Hitachi Research Laboratory, and K. Yoshimoto, Aichi Environmental Research Centre, for useful suggestions.

REFERENCES

1 Y. L. Sze, M. L. Borke and D. M. Ottenstein, *Anal. Chem.*, 35 (1963) 240.
2 R. E. Burks, Jr., E. B. Baker, P. Clark, J. Esslinger and J. C. Lacey, Jr., *J. Agr. Food Chem.*, 7 (1959) 778.
3 Y. Kaburaki, Y. Mikami, Y. Okabayshi and Y. Saida, *Bunseki Kagaku (Jap. Anal.)*, 18 (1969) 1100.
4 C. E. Andre and A. R. Mosier, *Anal. Chem.*, 45 (1973) 1971.
5 A. R. Mosier, C. E. Andre and F. G. Viets, Jr., *Environ. Sci. Technol.*, 7 (1973) 642.
6 A. DiCorcia and R. Samperi, *Anal. Chem.*, 46 (1974) 977.
7 N. P. Sen and P. L. McGeer, *Biochem. Biophys. Res. Commun.*, 13 (1963) 390.
8 W. J. A. VandenHeuvel, W. L. Gardiner and E. C. Horning, *Anal. Chem.*, 36 (1964) 1550.
9 A. C. Moffat and E. C. Horning, *Biochim. Biophys. Acta*, 222 (1970) 248.
10 A. C. Moffat and E. C. Horning, *Anal. Lett.*, 3 (1970) 205.
11 T. Uno, T. Nakagawa and R. Toyoda, *Bunseki Kagaku (Jap. Anal.)*, 21 (1972) 993.
12 R. Toyoda, T. Nakagawa and T. Uno, *Bunseki Kagaku (Jap. Anal.)*, 22 (1973) 914.
13 H. M. Fales and J. J. Pisano, in H. A. Szymansky (Editor), *Biomedical Application of Gas Chromatography*, Plenum Press, New York, 1964, p. 39.
14 S. B. Matin and M. Rowland, *J. Pharm. Sci.*, 61 (1972) 1235.
15 A. C. Moffat, E. C. Horning, S. B. Matin and M. Rowland, *J. Chromatogr.*, 66 (1972) 255.
16 R. E. Weston and B. B. Wheals, *Analyst (London)*, 95 (1970) 680.
17 E. W. Day, Jr., T. Golab and J. R. Koons, *Anal. Chem.*, 38 (1966) 1053.
18 H. Brandenberger and H. Hellbach, *Helv. Chim. Acta*, 50 (1967) 958.
19 N. Narasimhachari and P. Vouros, *J. Chromatogr.*, 70 (1972) 135.
20 S. Kawai and Z. Tamura, *Chem. Pharm. Bull.*, 16 (1968) 699.
21 K. Imai, M. Sugiura and Z. Tamura, *Chem. Pharm. Bull.*, 19 (1971) 409.
22 J. P. Chaytor, B. Crathorne and M. J. Saxby, *J. Chromatogr.*, 70 (1972) 141.
23 Y. Hoshika, *J. Chromatogr.*, 115 (1975) 596.
24 Y. Hoshika, *Anal. Chem.*, submitted for publication.
25 W. Bertsch, F. Hsu and A. Zlatkis, *Anal. Chem.*, 48 (1976) 928
26 M. Hrivac, W. Frischknecht and L. Cechova, *Anal. Chem.*, 48 (1976) 937.
27 G. Goretti and M. Possanzini, *J. Chromatogr.*, 77 (1973) 317.
28 W. Bertsch, F. Shunbo, R. C. Chang and A. Zlatkis, *Chromatographia*, 7 (1974) 128.

Journal of Chromatography, 136 (1977) 259–270

CHROM. 9927

COMPARISON OF TWO GAS–LIQUID CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF NITRAZEPAM IN PLASMA

LAURI KANGAS

Department of Pharmacology, University of Turku, 20520 Turku 52 (Finland)

(First received September 29th, 1976; revised manuscript received December 16th, 1976)

SUMMARY

Nitrazepam in plasma was determined by gas–liquid chromatography with a nickel-63 electron-capture detector, unchanged by a direct method and also by a hydrolysis method. The extraction in the direct method was carried out with benzene–dichloromethane (90:10) and in the hydrolysis method with diethyl ether. The hydrolysis was performed with 6 *N* sulphuric acid. The hydrolysis product was extracted with toluene–*n*-heptane–ethyl acetate (80:20:5) directly from acid. Thus the commonly used change in pH was omitted.

Nitrazepam concentrations in plasma were determined in 10 healthy volunteers after two oral doses (5 and 10 mg); 0.5 ml of plasma was used for each determination and clonazepam, methylbromazepam and methylnitrazepam were used as internal standards. The recoveries of the methods are almost quantitative (>96%). The two methods are clinically comparable.

The high sensitivity and specificity make these methods useful in clinical determinations of nitrazepam in plasma. Advantages and disadvantages of both methods are discussed.

INTRODUCTION

The structures of the widely used hypnotic drug nitrazepam* and the anti-epileptic drug clonazepam are similar, they undergo the same metabolic reactions[1,2] and have related pharmacokinetic characteristics[1,3–6]. They can be analyzed by gas–liquid chromatography (GLC) by almost identical methods[7,8].

Nitrazepam and clonazepam, like many other benzodiazepines, are determined by GLC in two different ways, either unchanged or after acid hydrolysis to benzophenones. Both of these methods have been used successfully, for example, in analyzing diazepam, but the determination of nitrazepam and clonazepam is more difficult without acid hydrolysis owing to the tailing of their peaks in GLC.

* Nitrazepam = 7-nitro-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one; methylnitrazepam = 7-nitro-5-phenyl-1,3-dihydro-1-methyl-2H-1,4-benzodiazepin-2-one; clonazepam = 7-nitro-5-(2′-chlorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one; methylbromazepam = 7-bromo-1,3-dihydro-1-methyl-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one.

Direct analyses of clonazepam in plasma by GLC have been published by Næstoft and co-workers[8,9], Berlin and co-workers[6,10] and Gerna and Morselli[11], but Knop *et al.*[12] and De Silva and co-workers[13,14] could not obtain satisfactory results. Nitrazepam, with its shorter retention time, should give a better peak shape than clonazepam. However, there have been only a few reports on its direct GLC determination[15–17], and such methods are not sensitive enough to be used in clinical or pharmacokinetic analysis[18]. Methylation and trimethylsilylation[19,20] have been used to improve the characteristics of nitrazepam in GLC. However, most nitrazepam determinations have been made after acid hydrolysis[4,7].

Both methods described here give clinically identical results, but they differ in principle. Some minor metabolites of nitrazepam (3-hydroxynitrazepam and perhaps some unknown metabolites[2,21,22]) yield the same end-product as nitrazepam itself on acid hydrolysis. The amount of possible metabolites of nitrazepam is very small, and the acid hydrolysis method is regarded as being specific for nitrazepam[4,7]. In this study there is a small, statistically significant, but clinically negligible, difference between these two methods.

The aim of this study was to develop a rapid and simple direct method for determining nitrazepam in clinical and biological samples, to compare it with the hydrolysis method and to simplify the latter.

EXPERIMENTAL

Thirteen healthy volunteers, five females and eight males, aged 21–38 years and of weight 58–73 kg, received no benzodiazepines for 3 weeks and no food for 3 h prior to the test. They were first given a 5-mg nitrazepam tablet (Dumex, Copenhagen, Denmark) at 7 p.m., then 3 weeks later two 5-mg tablets (a 10-mg dose). Blood samples were collected in heparinized tubes at 0, $\frac{1}{2}$, 1, 2, 3, 4, 12 (or 14), 24, 48 and 72 h after administration of the nitrazepam. After centrifugation of the blood, the plasma was separated and stored at −20° until analyzed. Three of the volunteers received placebo tablets.

Apparatus

A Varian Aerograph Model 2100 gas chromatograph equipped with a nickel-63 electron-capture detector (ECD) (potential 90 V, specific activity 5 C/g), was used with a dual-channel Omniscribe 5211-4-2A (Houston Instruments) or dual-channel Varian Aerograph Model 20 strip-chart recorder, together with U-shaped borosilicate glass columns of length 5 or 6 ft., I.D. 2 mm and O.D. $\frac{1}{4}$ in.

A Bühler Sm-2 mechanical shaker, shaking speed 150 rpm, was employed.

GLC conditions

The carrier gas was nitrogen (AGA, Helsinki, Finland, 99.995% purity) at a flow-rate of 40 ml/min. Molecular sieve 13X was used for decontamination and drying of the carrier gas. The columns were packed with 3% OV-17 on 100–120-mesh Chromosorb W (Varian, Palo Alto, Calif., U.S.A.) or 3% SP-2250 on 100–120-mesh Supelcoport (Supelco, Bellefonte, Pa., U.S.A.). The temperatures were: injector, 275°; column, 245° (indirect method) or 275° (direct method); and detector, 340°. Silanization of the columns was effected with Silyl-8 (Pierce, Rockford, Ill., U.S.A.).

Reagents

The following analytical-grade reagents were used: dipotassium hydrogen orthophosphate (anhydrous) (J. T. Baker, Phillipsburgh, N.J., U.S.A.), diethyl ether (Merck, Darmstadt, G.F.R. or Koch-Light, Colnbrook, Great Britain), sulphuric acid, benzene, dichloromethane, toluene, *n*-heptane, ethyl acetate, acetone and *n*-hexane (all from Merck). The solvents were used without distillation.

Standard and internal standards

Pure nitrazepam, clonazepam, methylnitrazepam, methylbromazepam and 2-amino-5-nitrobenzophenone (ANB) were kindly donated by Hoffman La Roche (Basle, Switzerland) and by Dumex (Copenhagen, Denmark).

Preparation of standard solutions

All standard solutions were prepared according to De Silva *et al.*[14] using absolute ethanol, acetone and *n*-hexane–acetone (80:20) as solvents.

Statistical tests

The paired *t*-test, linear regression and correlation were used.

Analytical methods

The plasma samples were thawed at room temperature overnight. Each of two 0.5-ml portions were pipetted into 10-ml glass-stoppered conical test-tubes containing the internal standard corresponding to 50–75 ng/ml of clonazepam or 30 ng/ml of methylnitrazepam or methylbromazepam in plasma. Standards corresponding to 0, 5, 10, 25, 50, 75 and 100 ng/ml of nitrazepam in plasma were also prepared. About 50 mg of anhydrous dipotassium hydrogen orthophosphate were added in order to produce a slightly alkaline pH (about 9). The tubes were vortexed for a short period to dissolve the phosphate.

Extraction steps. (1) Direct method (unchanged nitrazepam). A 3.3-ml volume of benzene–dichloromethane (90:10) was added to the plasma sample, the tubes were shaken for 10 min in a Bühler shaker and centrifuged (800 *g* for 10 min) at room temperature. A 3-ml aliquot of the upper organic phase was transferred into a clean test-tube, evaporated to dryness in a 60° water-bath under a gentle stream of dry air and finally dried in a vacuum oven at 50° for 2–4 h.

(2) Hydrolysis method. A 3-ml volume of diethyl ether was added to the sample. After shaking for 10 min and centrifuging (800 *g* for 10 min), the ether layer was nearly completely transferred into a clean test-tube (glass stopper). Another 3-ml portion of diethyl ether was added to the plasma sample. While the steps of the second extraction (shaking and centrifuging) were being carried out, the first portion of ether was evaporated to dryness in a 50° water-bath under a gentle stream of dry air, then 1.0 ml of 6 *N* sulphuric acid was added to the residue. The second diethyl ether extract was pipetted directly on to the acid. The tubes were shaken and centrifuged as before and diethyl ether was removed by suction. The tubes containing the acid phase were placed in a 50° water-bath to remove the remainder of the diethyl ether, and finally in a 100° oil-bath for hydrolysis for 1 h (see Discussion for the optimal time of hydrolysis). The tubes were closed tightly after 10 min and cooled to room temperature in a water-bath. A 3.3-ml volume of toluene–*n*-heptane–ethyl

acetate (80:20:5) was pipetted directly on to the acid. After shaking and centrifuging as before, a 3-ml aliquot of the upper organic phase was transferred into a clean test-tube, evaporated to dryness in 60° water-bath under a gentle stream of dry air and dried in a vacuum oven at 50° for 2–4 h.

Analysis by GLC. (1) Column pre-treatment in the direct method. The determination of nitrazepam with a normally stabilized (silanized) column does not give satisfactory results because of marked tailing of the peak. Benzene–dichloromethane (90:10) extracts of plasma seem to contain some unspecific impurities, which decrease the absorption of nitrazepam (and clonazepam) on the column (see also refs. 13 and 14). These extracts are obtained by extracting 1 ml of reference plasma in the same manner as the nitrazepam samples in the direct method. Another plasma extract containing nitrazepam and clonazepam (about 200 ng/ml each) can be prepared to control the peak shape and separation of nitrazepam and clonazepam. The residue is dissolved in 150 μl of *n*-hexane–acetone (80:20) and 1–2 μl is injected into the column. Repeated silanizations and injections of extracts at high temperatures (270–285°) stabilize and inactivate the column. Decreasing the oven temperature overnight to 150° may also assist the inactivation. To avoid detector and column contamination, not more than 2 μl of extract should be injected at a time. The influence of column stabilization is illustrated in Fig. 1.

(2) Analysis of unchanged nitrazepam. The samples are dissolved in 150 μl of *n*-hexane–acetone (80:20) after the stabilization of the column and a 1.5-μl sample is injected into the gas chromatograph at 275°.

(3) Analysis of ANB (hydrolysis method). The samples are dissolved in 300 μl

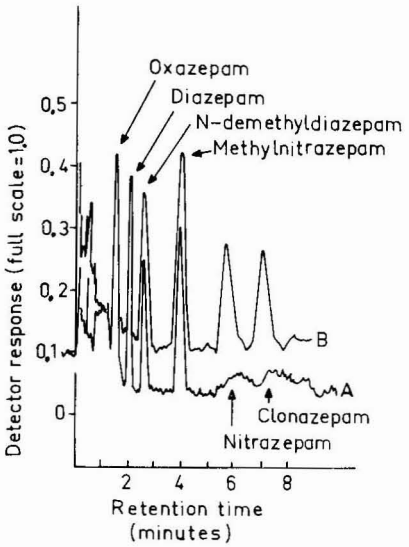


Fig. 1. Influence of column stabilization on the peak shapes of nitrazepam and clonazepam. Curve A: a sample containing 20 ng/ml of oxazepam, 10 ng/ml of diazepam and N-demethyldiazepam, 30 ng/ml of methylnitrazepam and 50 ng/ml of nitrazepam and clonazepam before column stabilization. Curve B: the same sample after column stabilization (repeated injections of reference plasma extract and silanization). Column, 3% OV-17; temperature, 275°.

of *n*-hexane–acetone (80:20) and 1.5 μl is injected into the gas chromatograph at 245°. No special column inactivation is needed. A typical gas chromatogram is shown in Fig. 2.

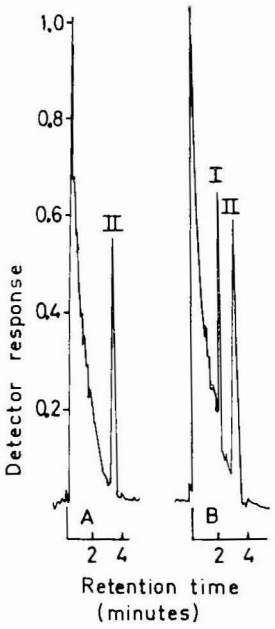


Fig. 2. Typical gas chromatograms obtained in the hydrolysis method. Curve A: a reference plasma sample containing 50 ng/ml of ANCB as internal standard (II). Curve B: a plasma sample containing 50 ng/ml of ANB (I) and ANCB (II).

Calculation of the results

The qualitative determination is based on retention times. The quantitative results are calculated from the peak heights using the ratio of the peak height of nitrazepam (or ANB) to the peak height of the internal standard. Standard graphs are presented in Fig. 3 (unchanged nitrazepam) and Fig. 4 (ANB).

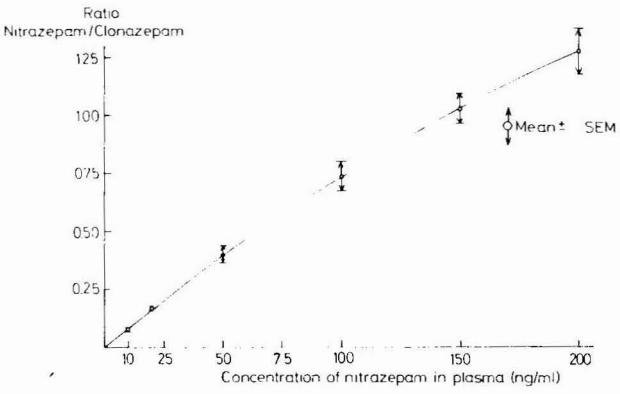


Fig. 3. Standard graph for plasma nitrazepam in the direct method. Mean ± SEM of five standard series on different days. Internal standard, clonazepam (150 ng/ml).

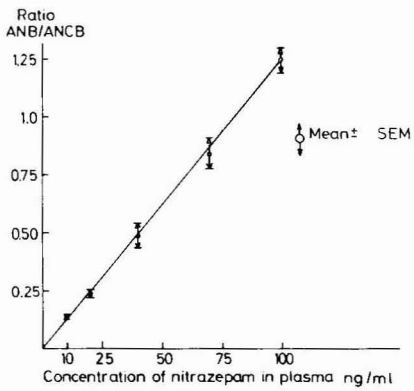


Fig. 4. Standard graph for plasma ANB (nitrazepam after acid hydrolysis). Mean ± SEM of six standard series on different days. Internal standard, ANCB (75 ng/ml).

RESULTS

Comparison of the methods by linear regression and correlation

The direct and hydrolysis methods, different internal standards in the direct method (methylnitrazepam and methylbromazepam in comparison with clonazepam) as well as the internal and external standards are compared by correlation and linear regression in Figs. 5, 6 and 7, respectively. In all instances there is a very significant correlation ($p < 0.001$) between the concentrations compared.

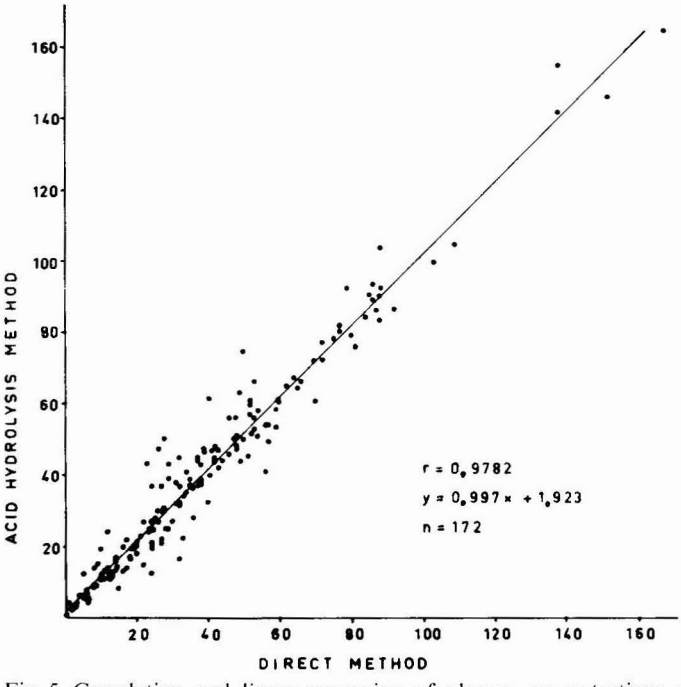


Fig. 5. Correlation and linear regression of plasma concentrations of nitrazepam (ng/ml) in the whole material measured by the direct and acid hydrolysis methods.

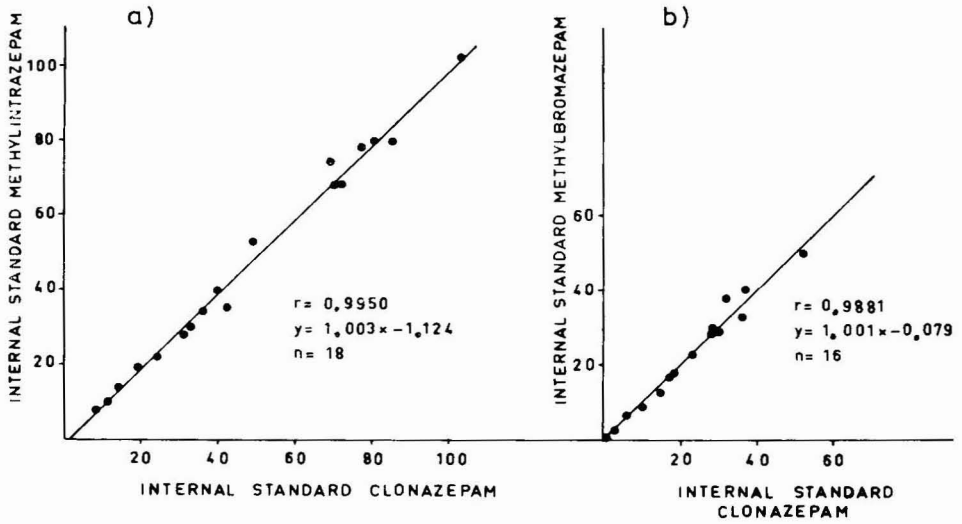


Fig. 6. Comparison of different internal standards in the direct method. The plasma concentrations of nitrazepam (ng/ml) are compared by correlation and linear regresssion by using clonazepam and methylnitrazepam (a) as well as clonazepam and methylbromazepam (b) as internal standards. In all instances a very significant correlation ($p < 0.001$) is found. For further details, see text.

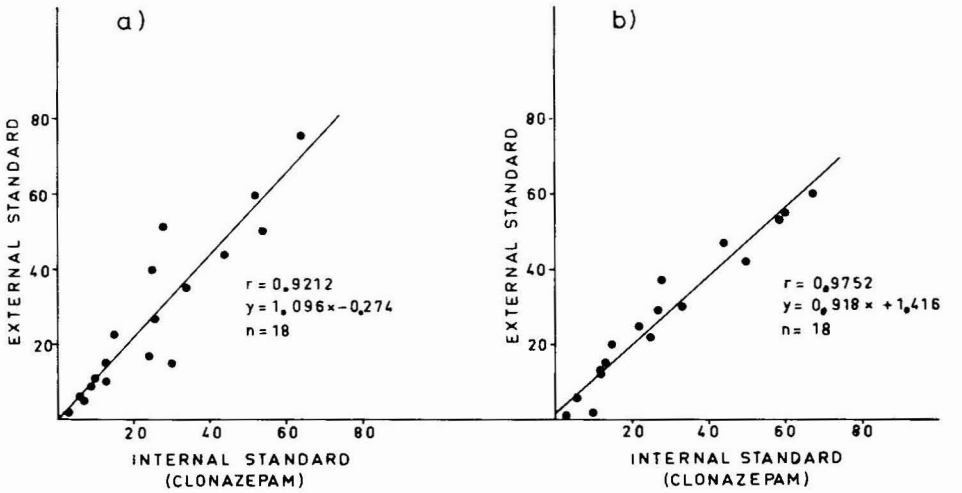


Fig. 7. Comparison of external and internal standardization. The plasma concentrations of nitrazepam (ng/ml), found with both of these systems, are compared by correlation and linear regression in the direct (a) and hydrolysis (b) methods. A very significant correlation ($p < 0.001$) is found in both instances.

Comparison of the methods by paired t-test

The same comparisons as above were made by the paired *t*-test in order to control the specificity of the methods. The comparisons are presented in Table I, which indicates the identities of all internal standards used in this study. In the direct

TABLE I

PAIRED *t*-TESTS IN DIFFERENT METHODS

A: Comparison of direct (Di) and hydrolysis (H) methods. B: Comparison of different internal standards [clonazepam (CL) and methylnitrazepam (MN)] C, Comparison of different internal standards [clonazepam (CL) and methylbromazepam (MB)]. D: Comparison of internal (INT) and external (EXT) standards in direct (Di) and hydrolysis (H) methods. SD = standard deviation; N.S. = not significant.

Parameter	*A*	*B*	*C*	*D*	
				Di	*H*
Mean nitrazepam concentration in plasma (ng/ml)	Di 39.4 H 41.2	CL 47.9 MN 46.8	CL 22.2 MB 22.1	INT 25.4 EXT 27.6	27.3 26.4
SD	0.480	0.681	0.544	2.023	1.076
t	3.737	1.632	0.115	1.721	0.774
p	<0.001	N.S.	N.S.	N.S.	N.S.
n	172	18	18	18	18

method, the calculated nitrazepam concentrations (39.4 ng/ml) are lower than those in the hydrolysis method (41.2 ng/ml) ($p < 0.001$).

Precision

Identical samples corresponding to 50 ng/ml of ANB in plasma were injected 20 times repeatedly into the gas chromatograph and the peak heights were measured. The resulting precision of the apparatus, peak-height measurement and injections on the same day are shown in Table II (column A).

One plasma sample corresponding to 50 ng/ml of ANB and 2-amino-5-nitro-2'-chlorobenzophenone (ANCB) (internal standard) was injected 10 times repeatedly and the ratio of the peak heights of ANB and ANCB was calculated. Column B in

TABLE II

PRECISION AND REPRODUCIBILITY OF THE METHODS

A: Precision on the same day by using external standard [peak height of ANB (cm) measured]. B: Precision on the same day by using internal standard (peak-height ratio of ANB to ANCB calculated). C: Reproducibility by using external standard [peak height of nitrazepam (cm) measured]. D: Reproducibility by using internal standard (peak height ratio of nitrazepam to clonazepam calculated). SD = Standard deviation; SEM = standard error of the mean; CV = coefficient of variation. For further explanations, see text.

Parameter	*A*	*B*	*C*	*D*
Mean of peak height or peak-height ratio	9.21	0.905	3.56	1.015
SD	0.78	0.013	0.49	0.064
SEM	0.17	0.004	0.11	0.014
CV	0.085	0.014	0.139	0.063
t	52.8	224.2	32.2	71.2
n	20	10	20	20

Table II illustrates the intra-day precision of the apparatus and peak-height measurement.

Reproducibility

The reproducibility was studied by preparing 20 identical plasma samples, containing 25 ng/ml of nitrazepam and clonazepam, and analyzing them on different days (direct method). Columns C and D in Table II represent the reproducibility of the method by using the external standard (the peak heights measured) and internal standard (the ratio of the peak heights of nitrazepam and clonazepam measured), respectively.

Reliability

The reliability of the methods is illustrated as percentage recovery in Table III. The tests of recovery from plasma were performed by adding known amounts of nitrazepam and clonazepam to the dried extracts of reference plasma in order to avoid peak tailing, which always occurs when these drugs are injected into the gas chromatograph in pure organic solutions. In this table, the recovery of ANB from 6 *N* sulphuric acid in comparison with the recovery from alkali[7] is also presented. The mean peak height of ANB, extracted from acid, was 7.94 cm and from alkali 7.84 cm. In the paired *t*-test the standard error of the mean (SEM) was 0.146 and $t = 0.687$ ($n = 14$). This result confirms that the change of pH is unnecessary in ANB extraction.

TABLE III

RECOVERY TESTS ON THE METHODS AND RECOVERY OF ANB FROM ACID AND ALKALI

A: Recovery (%) of nitrazepam in the direct method (internal standard clonazepam). B: Recovery (%) of nitrazepam in the hydrolysis method (internal standard clonazepam). C: Recovery (%) of ANB (ANB = hydrolysis product of nitrazepam) from 6 *N* H_2SO_4 and from alkalinized solution. For further details, see text.

Parameter	*A*	*B*	*C*	
			6 N H_2SO_4	*Alkali*[7]
n	20	10	14	14
Mean recovery (%)	97.44	96.80	95.36	95.16
SD	4.66	3.30	5.43	8.03
SEM	1.04	1.04	1.45	2.15
Largest value	106.0	103.4	109.9	113.5
Smallest value	91.0	92.1	86.4	84.5
Concentration range in plasma (ng/ml)	5–150	5–150	10–200	10–200

DISCUSSION

Many GLC methods for the determination of benzodiazepines are inconvenient owing to the large volumes of distilled analytical-grade organic solvents and many working steps involved[7,12–14,17,18]. Aggarwal *et al.*[23], Horning *et al.*[24] and Kangas *et al.*[25] successfully used salt–solvent systems for separating drugs and drug

metabolites from biological samples. In our work, these rapid systems were modified: solid dipotassium hydrogen orthophosphate is added to buffer the pH of the samples to about 9. The volume of the samples is kept to the minimum and the amount of organic solvent may be diminished. Handling of the small samples is convenient and the evaporation rapid. The validity of the extraction systems used was discussed by Møller Jensen[4] and Beharrell *et al.*[7] (nitrazepam), Næstoft *et al.*[8] (clonazepam), Berlin and co-workers[6,10] (diazepam, its metabolites and clonazepam), De Silva and co-workers[13,14] (clonazepam and flunitrazapam) and Kangas *et al.*[25] (diazepam and its metabolites). In the direct method, the working steps are minimized. In the hydrolytic method, relatively numerous steps remain in spite of omitting the regularly used but unnecessary change of pH in extracting ANB.

The problem with the direct method is the tailing of the nitrazepam peak in GLC analysis. The inactivation of the column is sometimes difficult and time consuming. However, once the column is inactivated, it can be used continuously and successfully for the analysis of plasma samples for nitrazepam and clonazepam. It is necessary to use standards that are prepared through the method in order to avoid the peak tailing. OV-17 is a better liquid phase than SE-30; the latter does not separate nitrazepam and clonazepam completely.

The time of acid hydrolysis is not critical. The optimal time in 6 *N* sulphuric acid and at 100° seems to be 30 min, but no significant decrease in recovery was found with longer time periods up to 2 h (recovery better than 90% between $\frac{1}{2}$ and 2 h).

As Figs. 6 and 7 indicate, the use of an internal standard in comparison with an external standard results in smaller errors, no matter which internal standard is used. Methylbromazepam is of special value in the direct method, because it can also be used as the internal standard in determining diazepam and its metabolites. In clinical studies, it is important not to use any benzodiazepine drug or *invivo* metabolite as the internal standard because of the wide use of these drugs. If any doubts about the validity of an internal standard exist, the use of an external standard should be considered.

The difference found between the methods may be explained by the minor metabolites of nitrazepam[2,21,22]. These metabolites are not determined in plasma as such by GLC. The difference, although it is statistically very significant, is too small to be detected reliably in individual samples, at least after an acute dose of nitrazepam. Both of the methods may be considered to be specific for nitrazepam.

The reduced metabolites of nitrazepam (7-aminonitrazepam and 7-acetamidonitrazepam) are not measurable by the described methods with sufficient sensitivity. These metabolites are perhaps clinically inactive[26,27] and their determination has only a slight value for clinical purposes. They can be determined by fluorimetric methods[2,5,28,29].

The following advantages and disadvantages may help in selecting suitable methods for nitrazepam determinations.

The advantages of the direct method are that extraction is easy and rapid, the sensitivity (about 1 ng/ml) and specificity are good and simultaneous quantitative analysis of other benzodiazepines in the same sample is possible. Its disadvantages are the toxicity of benzene (ventilation needed), absorption of nitrazepam occurs in the GLC column and special inactivation may be needed, a high temperature is required in GLC and the peaks, although symmetrical, are not sharp.

The advantages of the acid hydrolysis method are the very good sensitivity (0.2–0.5 ng/ml), GLC analysis is easy and the peaks are sharp and symmetrical, purification of the samples occurs in the extraction steps and no special column stabilization is needed. Its disadvantages are that extraction is time consuming (many steps), selection of an internal standard may be difficult if clonazepam is not to be used and some metabolites of nitrazepam are hydrolyzed to the same benzophenone as nitrazepam itself.

In the clinical monitoring of nitrazepam in plasma and when rapid analyses of, for instance, toxicological plasma samples are required, the direct method is to be preferred, because it is fairly rapid and simple. In more urgent cases, the drying of the extracts in the vacuum can be omitted. On the other hand, if samples contain many impurities (*e.g.*, tissue samples) or extremely low concentrations of nitrazepam, the hydrolysis method is to be preferred because it is more sensitive and the samples are purified during the extraction. Improved specificity and reliability of the qualitative analysis of unknown samples are achieved with parallel use of the methods.

In conclusion, the two methods for the determination of nitrazepam at therapeutic concentrations in plasma described here do not measure the main metabolites of nitrazepam. The results of the methods are comparable and both methods are suitable for clinical and pharmacokinetic studies of nitrazepam. The direct method can be used for analysing concentrations of clonazepam and diazepam and its metabolites in plasma.

ACKNOWLEDGEMENTS

Hoffman la Roche (Basle) and Dumex (Copenhagen) are thanked for generous supply of all of the standards and internal standards required in this work, and Mrs. Sirkka Kiiskilä is thanked for technical assistance.

REFERENCES

1 E. Eschenhof, *Arzneim.-Forsch.*, 23 (1973) 390.
2 Y. Yanagi, F. Haga, M. Endo and S. Kitagawa, *Xenobiotica*, 5 (1975) 245.
3 J. Rieder, *Arzneim.-Forsch.*, 23 (1973) 212.
4 K. Møller Jensen, *J. Chromatogr.*, 111 (1975) 389.
5 J. Rieder, *Arzneim.-Forsch.*, 23 (1973) 207.
6 A. Berlin and H. Dahlström, *Eur. J. Clin. Pharmacol.*, 9 (1975) 155.
7 G. P. Beharrell, D. M. Hailey and M. K. McLaurin, *J. Chromatogr.*, 70 (1972) 45.
8 J. Næstoft, M. Lund, N.-E. Larsen and E. Hvidberg, *Acta Neurol. Scand.*, 49, Suppl., No. 53 (1973) 103.
9 J. Næstoft and N.-E. Larsen, *J. Chromatogr.*, 93 (1974) 113.
10 A. Berlin, B. Siwers, S. Agurell, Å. Hiort,F. Sjöqvist and S. Ström, *Clin. Pharmacol. Ther.*, 13 (1972) 733.
11 M. Gerna and P. L. Morselli, *J. Chromatogr.*, 116 (1976) 445.
12 H. J. Knop, E. van der Kleijn and L. C. Edmunds, *Pharm. Weekbl.*, 110 (1975) 297.
13 J. A. F. de Silva and I. Bekersky, *J. Chromatogr.*, 99 (1974) 447.
14 J. A. F. de Silva, C. V. Puglisi and N. Munno, *J. Pharm. Sci.*, 63 (1974) 520.
15 A. Forgione, P. Martelli, F. Marcucci, R. Fanelli, E. Mussini and G. C. Jommi, *J. Chromatogr.*, 59 (1971) 163.
16 Á. Viala, J. P. Cano and A. Angeletti-Philippe, *Eur. J. Toxicol.*, 3 (1971) 109.
17 P. Lafargue, P. Pont and J. Meunier, *Ann. Pharm. Fr.*, 28 (1970) 477.
18 D. M. Hailey, *J. Chromatogr.*, 98 (1974) 527.

19 H. Ehrsson and A. Tilly, *Anal. Lett.*, 6 (1973) 197.
20 M. S. Greaves, *Clin. Chem.*, 20 (1974) 141.
21 J. Rieder and G. Wendt, in S. Garattini, E. Mussini and L. O. Randall (Editors), *The Benzodiazepines*, Raven Press, New York, 1973, p. 99.
22 K.-H. Beyer and W. Sadée, *Arzneim.-Forsch.*, 19 (1969) 1929.
23 V. Aggarwal, R. Bath and I. Sunshine, *Clin. Chem.*, 20 (1974) 307.
24 M. G. Horning, P. Gregory, J. Nowlin, M. Stafford, K. Lertratanangkoon, C. Butler, W. G. Stillwell and R. M. Hill, *Clin. Chem.*, 20 (1974) 282.
25 L. Kangas, A. Pekkarinen, C. Sourander and E. Raijola, *Ann. Clin. Res.*, 6, Suppl., No. 11 (1974) 12.
26 O. Sjö, E. F. Hvidberg, J. Næstoft and M. Lund, *Eur. J. Clin. Pharmacol.*, 8 (1975) 249.
27 L. O. Randall and B. Kappell, in S. Garattini, E. Mussini and L. O. Randall (Editors), *The Benzodiazepines*, Raven Press, New York, 1973, p. 27.
28 P. Haefelfinger, *J. Chromatogr.*, 111 (1975) 323.
29 H. Schütz, *J. Chromatogr.*, 94 (1974) 159.

Journal of Chromatography, 136 (1977) 271–277

CHROM. 9973

SEPARATION AND QUANTITATIVE DETERMINATION OF TRACES OF CARBONYL COMPOUNDS AS THEIR 2,4-DINITROPHENYLHYDRAZONES BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

SAMI SELIM

FMC Corporation, Agricultural Chemical Division 100 Niagara Street, Middleport, N.Y. 14105 (U.S.A.)

(Received December 21st, 1976)

SUMMARY

A method for the quantitative conversion of traces of aldehydes and ketones to their 2,4-dinitrophenylhydrazones at room temperature is presented. The 2,4-dinitrophenylhydrazones of a number of aliphatic and aromatic carbonyl compounds have been prepared. The compounds were separated on a reversed-phase μBondapak C_{18} column. The method is valuable in the quantitative determination of traces of low-molecular-weight aldehydes and ketones. Derivatives of identical molecular weight can be easily separated by high-pressure liquid chromatography but not by gas chromatography under the conditions tried.

INTRODUCTION

High-pressure liquid chromatography (HPLC) is becoming a powerful technique for the analysis of trace components. Liquid chromatography (LC) has many of the advantages of gas chromatography (GC) such as speed and accurate quantitative analysis, and some added advantages such as capability to analyze high-molecular-weight or thermally unstable compounds. However the applicability of LC to trace analysis has been limited because of the few types of detectors commercially available. Introduction of the variable-wavelength detector and the development of totally porous small-particle packings are allowing separations at least as good as those obtained by GC, with the needed sensitivity.

Nanogram quantities of certain groups of compounds such as aliphatic alcohols, aldehydes, ketones, acids, etc., cannot be selectively detected by GC or LC. One approach to overcoming the detection problem is to tag the compound by adding a group that renders it detectable. This approach has been used extensively in GC and is gaining in importance in HPLC.

Derivatives that absorb strongly in UV light have been prepared for HPLC. Hydroxy steroids have been benzoylated[1], the 3,5-dinitrobenzoates of glycols have been formed[2], and hexachlorophene has been detected via its *p*-methoxybenzoate

derivatives[3]. The HPLC response of fatty acids has been improved by the formation of benzyl[4], *p*-nitrobenzyl[5], 2-naphthacyl[6], and phenacyl[7,8] esters.

Carbonyl compounds have received much attention recently because of an increasing realization of their importance when present in trace amounts in air pollution, vegetable flavoring, cigarette smoke, and the aroma of commercial beverages. The method most commonly used to determine traces of these compounds is to form the 2,4-dinitrophenylhydrazone and determine that derivative by GC or HPLC. The detectability of 17-keto steroids has been enhanced by the formation of the 2,4-dinitrophenylhydrazone[9] such that as little as 10 ng of steroids was detected[10]. The 2,4-dinitrophenylhydrazones of a number of aliphatic and aromatic carbonyl compounds have been synthesized and successful separation of C_1–C_5 2,4-dinitrophenylhydrazones was obtained on pellicular LC columns[2,11]. It was reported[2] that the retention times of the 2,4-dinitrophenylhydrazones of carbonyl compounds with six or more carbons tend to merge because the solubilities in the hydrocarbon mobile phase are nearly the same. Adsorption chromatography was used in all the reported HPLC separations.

The quantitative conversion of aldehydes and ketones to their corresponding 2,4-dinitrophenylhydrazones on a microscale has not been investigated.

In this article, the quantitative conversion of aldehydes and ketones to their corresponding 2,4-dinitrophenylhydrazones on a microscale at room temperature is reported. The separation of same-molecular-weight aldehydes and ketones could not be accomplished by GC but was achieved by HPLC. The separation of the 2,4-dinitrophenylhydrazones of aldehydes and ketones has been extended to higher molecular weights, aldehydes and ketones (above 6 carbon) by using reversed-phase HPLC.

EXPERIMENTAL

Preparation of 2,4-dinitrophenylhydrazine reagent

The 2,4-dinitrophenylhydrazine reagent was prepared by adding 0.25 g of 2,4-dinitrophenylhydrazine to 100 ml of 6 *N* hydrochloric acid.

Preparation of 2,4-dinitrophenylhydrazones

The 2,4-dinitrophenylhydrazones were prepared by standard procedures[12] and purified by recrystallization from ethanol.

Conversion of propionaldehyde to its 2,4-dinitrophenylhydrazone on a microscale

To each of six Erlenmeyer flasks, 25 ml of water, 0.2 ml of the 2,4-dinitrophenylhydrazine reagent, 20 μg of propionaldehyde and 10 ml of isooctane were added. The mixtures were stirred on a magnetic stirrer. At intervals, the two-phase mixture was transferred to a separatory funnel and the two phases separated. The aqueous phase was extracted with 10 ml of isooctane, and the two isooctane fractions were combined and extracted twice with 10 ml of acetonitrile. The acetonitrile extract was concentrated and injected onto the liquid chromatograph (system II, see below).

High-pressure liquid chromatography

A Waters Assoc. ALC 202 liquid chromatograph equipped with a U6K

injector and a Schoeffel spectrophotometer was used in this study. Two HPLC systems were studied.

System I. Adsorption chromatography on a prepacked 30-cm Microporasil column (Waters Assoc.) using ethyl acetate–hexane (1:49) as the mobile phase.

System II. Reversed-phase chromatography on a 30-cm prepacked μBondapak C_{18} (Waters Assoc.) column using different ratios of acetonitrile–water as the mobile phase.

Gas chromatography

A Hewlett-Packard 5700A gas chromatograph equipped with a Ni 63 electron capture detector was used. This instrument features on-column injection and direct connection of the column to the detector. Glass columns (4 ft. × 6 mm O.D. × 2 mm I.D.) packed with various liquid phases on Chromosorb W AW DMCS (80–100 mesh) were used. The flow-rate of the argon–methane (95:5) gas was 65 ml/min.

RESULTS AND DISCUSSION

The reaction between propionaldehyde and 2,4-dinitrophenylhydrazine was studied on a microscale at room temperature. Propionaldehyde was added to an excess of the 2,4-dinitrophenylhydrazine reagent. The solution was extracted at intervals and injected onto the HPLC. After 30 min, 67% of propionaldehyde was converted to the corresponding 2,4-dinitrophenylhydrazone. Allowing the reaction to proceed for 15 h did not substantially improve the yield (Table I). These results indicate that an equilibrium was reached in the aqueous phase when 70.6% of the derivative had been formed:

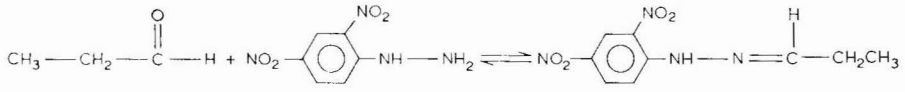


TABLE I

CONVERSION OF PROPIONALDEHYDE TO ITS 2,4-DINITROPHENYLHYDRAZONE AT ROOM TEMPERATURE IN A ONE-PHASE REACTION MEDIUM

Time (h)	*Added (μg)*	*Found (μg)*	*Recovery (%)*
½	20	13.4	67
1	20	12.4	62
2	20	13.5	67.7
4	20	12.6	63.3
6	20	12.4	62.2
15	20	14.1	70.6

When the same reaction is carried out with gram quantities, the 2,4-dinitrophenylhydrazone of propionaldehyde precipitates immediately due to its low solubility in the aqueous phase. The removal of the derivative from the aqueous phase by precipitation shifts the equilibrium toward the formation of more derivative and the reaction is almost quantitative. The water-insolubility of the 2,4-dinitrophenylhydra-

TABLE II

CONVERSION OF PROPIONALDEHYDE TO ITS 2,4-DINITROPHENYLHYDRAZONE AT ROOM TEMPERATURE IN A TWO-PHASE REACTION MEDIUM

Time (min)	*Added (μg)*	*Found (μg)*	*Recovery (%)*
5	20	10.7	53.5
10	20	15.1	75.3
15	20	17.6	88.1
20	20	19.8	99
25	20	20	100.4
30	20	20	100.7

zone of propionaldehyde indicates that the reaction could be made quantitative at the microscale by using an aqueous–organic two-phase reaction medium. Propionaldehyde and the 2,4-dinitrophenylhydrazine reagent are water-soluble. Propionaldehyde reacted with the 2,4-dinitrophenylhydrazine reagent in the aqueous phase. Partitioning of the derivative into the organic phase shifted the equilibrium of the reaction to the right and allowed the reaction to go to completion. Propionaldehyde was quantitatively converted to its 2,4-dinitrophenylhydrazone in 20 min (Table II, Fig. 1).

The HPLC separation of the 2,4-dinitrophenylhydrazones of carbonyl compounds offers many advantages over the GC separation. The reported GC methods capable of separating different aldehyde derivatives and ketone derivatives are not capable of separating aldehyde derivatives from ketone derivatives of the same molecular weight (Fig. 2). GC columns having liquid phases ranging in polarity from OV-1 to OV-25 were tried in this laboratory to separate the 2,4-dinitrophenylhydrazones of acetone and propionaldehyde without success. Base-line separation of the 2,4-di-

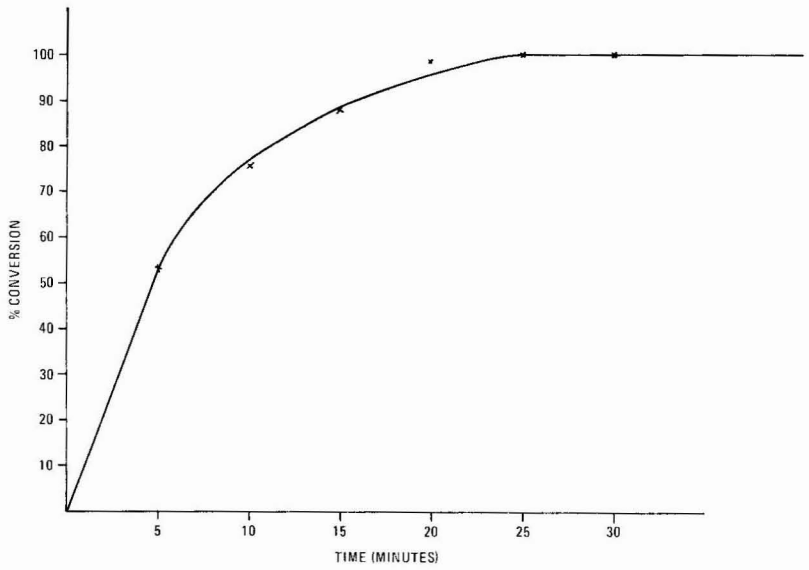


Fig. 1. Rate of conversion of propionaldehyde to propionaldehyde 2,4-dinitrophenylhydrazone using a two-phase reaction medium.

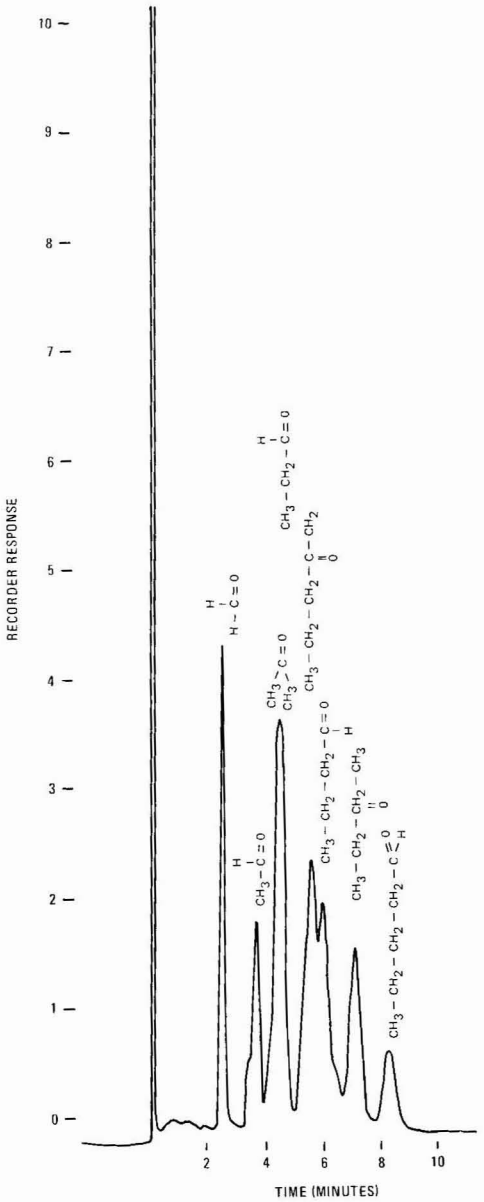


Fig. 2. Gas chromatogram of a mixture of 2,4-dinitrophenylhydrazones of carbonyl compounds on a 2% OV-17 column.

nitrophenylhydrazone of propionaldehyde from the 2,4-dinitrophenylhydrazone of acetone was achieved on reversed-phase HPLC using a acetonitrile–water (3:2) mobile phase (Fig. 3). Aldehydes and ketones with four or five carbons were also separated (Fig. 3). As expected, higher-molecular-weight aldehyde and ketone 2,4-dinitrophenylhydrazones were retained longer on the reversed-phase column. The 2,4-dinitrophenylhydrazone of the aromatic aldehydes α-tolualdehyde and salicyl-

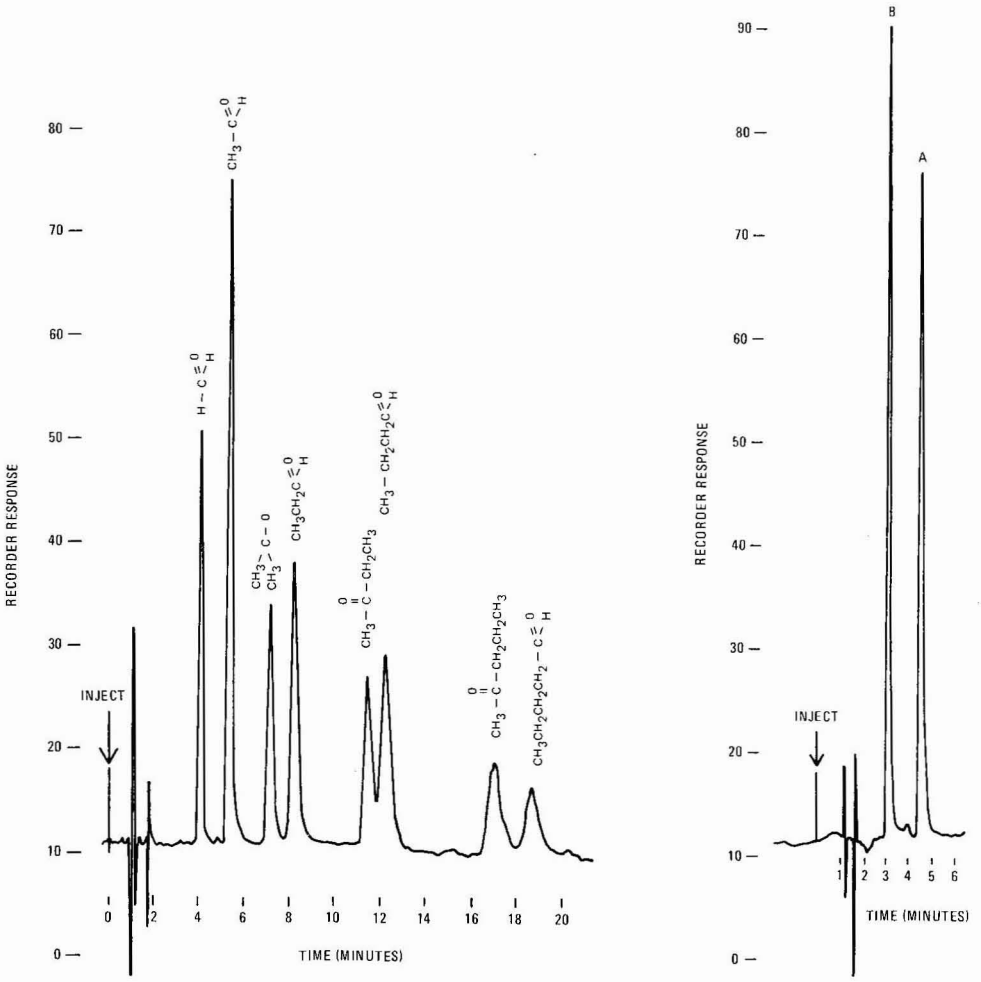


Fig. 3. LC of a mixture of 2,4-dinitrophenylhydrazones of aldehyde and ketone. Mobile phase acetonitrile–water (3:2). Flow-rate, 3 ml/min; pressure, 1000 p.s.i.; column temperature ambient; UV detection at 336 nm, 0.04 a.u.f.s.

Fig. 4. LC of a mixture of the 2,4-dinitrophenylhydrazone of (A) tolualdehyde and (B) salicylaldehyde. Conditons are the same as in Fig. 3, except a acetonitrile–water (13:7) mobile phase was used.

aldehyde were easily separated on the same column using a acetonitrile–water (13:7) mobile phase (Fig. 4). 2,4-Dinitrophenylhydrazones of aldehydes with eight, nine or ten carbons were separated on the same column using acetonitrile–water (3:1). 2,4-Dinitrophenylhydrazones of even higher aldehydes and ketones should not be difficult to separate but would probably require a higher concentration of acetonitrile. Shorter columns could also be used effectively.

Reversed-phase LC offers some advantages over adsorption chromatography for the separation of non-polar derivatives. When adsorption chromatography is used, the retention of a compound on the column depends on the interaction of the compound with the packing and on the solubility of the compound in the mobile

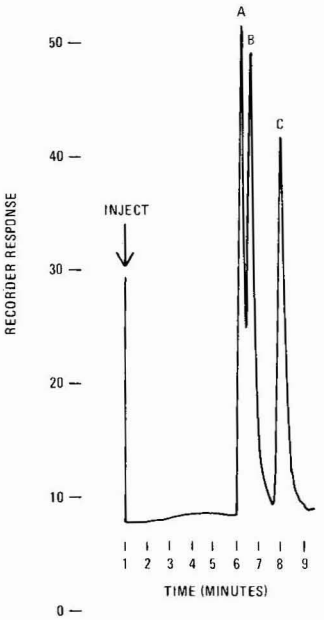


Fig. 5. LC of a mixture of the 2,4-dinitrophenylhydrazone of (A) 4-heptanone, (B) 3-heptanone and (C) 2-heptanone on a 1 ft. × $\frac{1}{4}$ in. microporasil column; mobile phase, ethyl acetate–hexane (2:98). Flow-rate, 3 ml/min; pressure, 1000 p.s.i.; column temperature, ambient; UV detection at 336 nm, 0.04 a.u.f.s.

phase. It has been found[2] that compounds containing six or more carbon atoms could not be separated on a Corasil II column using ethyl acetate–hexane (3:97) as mobile phase. The solubilities of these compounds in the mobile phase were nearly the same. Decreasing the ethyl acetate concentration to 2% and using a Microporasil column allowed separation of the 2,4-dinitrophenylhydrazones of three heptanones (Fig. 5). However, the 2,4-dinitrophenylhydrazones of aldehydes with eight or nine carbons could not be separated.

REFERENCES

1 F. A. Fitzpatrick and S. Siggia, *Anal. Chem.*, 45 (1973) 2310.
2 M. A. Carey and A. F. Persinger, *J. Chromatogr. Sci.*, 10 (1972) 537.
3 P. J. Porcaro and P. Shubiak, *Anal. Chem.*, 44 (1972) 1865.
4 I. R. Plitzer, G. W. Griffin, B. J. Dowty and J. L. Laseter, *Anal. Lett.*, 6 (1973) 539.
5 W. Morozowich, *APLA Acad. Pharm. Sci. Abstr.*, 69 (1972).
6 M. J. Cooper and M. W. Anders, *Anal. Chem.*, 46 (1074) 1849.
7 H. D. Durst, M. Milano, E. J. Kikta, Jr., S. A. Connelly and E. Grushka, *Anal. Chem.*, 47 (1975) 1797.
8 R. F. Borch, *Anal. Chem.*, 47 (1975) 2437.
9 F. A. Fitzpatrick, S. Siggia and J. Dingman, Sr., *Anal. Chem.*, 44 (1972) 2211.
10 R. A. Henry, J. A. Schmit and J. F. Diekman, *J. Chromatogr. Sci.*, 9 (1971) 513.
11 L. S. Papa and L. P. Turner, *J. Chromatogr. Sci.*, 10 (1972) 744.
12 R. L. Shriner, R. C. Fuson and D. Y. Curtin, *The Systematic Identification of Organic Compounds*, Wiley, New York, 5th ed., 1965.

Journal of Chromatography, 136 (1977) 279–287

CHROM. 9881

SENSITIVE DETERMINATION OF DERIVATIZED CARBOHYDRATES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

F. NACHTMANN and K. W. BUDNA

Department of General Chemistry, Micro- and Radiochemistry, Technical University, Graz (Austria)

(Received December 17th, 1976)

SUMMARY

The separation and determination of the 4-nitrobenzoates of some monosaccharides, disaccharides and trisaccharides by high-performance liquid chromatography are described. The relatively apolar derivatives permit rapid isocratic separations on polar adsorbents such as silica gel and alumina. The sugar derivatives are detectable with high sensivity, detection limits being in the nanogram region for all sugars tested. The extinction maximum of 260 nm permits the use of low-cost UV detectors (254 nm). A complete analysis takes 70–75 min, 10–15 min of this time being needed for the chromatographic separation. The practical aspects of the method are demonstrated by some examples.

INTRODUCTION

The use of high-performance liquid chromatography (HPLC) for the analysis of trace amounts of sugars has previously been almost impossible owing to the low sensitivity of detection. Hitherto, HPLC separations of carbohydrates with various stationary phases have been described[1–5]. The absence of suitable chromophores demands the use of refractive index (RI) detectors, and the detection limit obtained by these detectors is about 20 μg of sugar[5].

Classical liquid chromatographic methods involve the use of cation exchangers[6] or sugar–borate complexes on anion exchangers with gradient elution for separation, which is the principle of commercial sugar analyzers[7–9]. The detection is effected by colour reactions after elution[7,8] and detection limits in the microgram region are achieved. Better results can be obtained by using an oxidative detector that relies on the reduction of cerium(IV) to fluorescent cerium(III) by the eluted carbohydrates[10]. Significant disadvantages of all classical liquid chromatographic methods are that they require several hours for one analysis and no anomer separations are effected.

The separation of perbenzoylated carbohydrates into their anomers by HPLC using gradient elution was described by Lehrfeld[11]. Another possibility for anomer separations is offered by gas chromatography (GC) following silylation. The detection

* This paper is part of a "poster-presentation at the International Symposium on Microchemical Techniques 1977, Davos, Switzerland.

limit of such procedures using a flame-ionization detector is about 10 ng of sugar[12].

Recently, the esterification of digitalis glycosides with 4-nitrobenzoyl chloride (4-NBCl) was described[13]. The derivatives showed excellent chromatographic characteristics and detection limits were in the nanogram region[14]. Nitrobenzoates are more suitable than the corresponding benzoates for trace determinations of carbohydrates. The incorporation of an additional chromophoric group into the aryl portion of the derivative results in a shift of the wavelength of maximum absorption from 230 to 260 nm, and the detection limit is lowered by a factor of ten[15].

In this paper, the possibilities of the determination of carbohydrates by HPLC are described.

EXPERIMENTAL

Reagents

Sugars and sugar alcohols were provided by Merck (Darmstadt, G.F.R.), Fluka (Buchs, Switzerland) and Serva (Heidelberg, G.F.R.). LiChrosorb SI 60 silica gel and Alox T alumina (Merck) of particle size 5 μm were used as stationary phases in HPLC. All solvents used were of analytical-reagent grade (Merck). The reagents used for derivatization were described elsewhere[13,14].

Instruments

For HPLC determinations, a Siemens S200 liquid chromatograph equipped with a Zeiss PM-4 spectrophotometric detector (260 nm) was used. The injection systems were a Siemens pneumatic syringe injector (10 μl) and a Siemens loop injector (50 μl).

Derivatization

The reaction is carried out in pyridine. The derivatization was studied with digitalis glycosides and can be used unchanged with all carbohydrates except fructose, for which room temperature (20–22°) must not be exceeded during all reaction and isolation steps. An exact description of the procedure was given elsewhere[13].

HPLC separations

Steel columns, 15 or 20 cm long and 3 mm I.D., were filled by means of the slurry technique using the mixture tetrabromoethane–dioxane–tetrachloromethane (1 : 1 : 1) as solvent for the slurry and *n*-hexane as the pressure solvent[16]. For Alox T, a suspension in methanol, saturated with *n*-hexane, was pressed into the column with *n*-hexane, saturated with methanol. This filling technique resulted in better columns than the "balanced slurry" technique. On applying a pressure of 300 atm, the packing of one column is completed within 10 min.

The wavelength of detection was 260 nm, which is the extinction maximum of 4-NB derivatives of sugars[17]. All separations were performed isocratically at room temperature (20–22°), thermostating being unnecessary.

RESULTS AND DISCUSSION

A problem in the determination of monosaccharides is the equilibrium of at least six compounds (the two pyranoses, the two furanoses, the aldehydo or keto

form and its hydrate) in solution. Hitherto, good separations were possible by the GC of silylated derivatives[12,18–20].

4-Nitrobenzoates permit similar separations by HPLC. D-Glucose and D-mannose, which in aqueous solution exist only in the pyranose form at room temperature[21], result in the expected double peaks of the α- and β-pyranose. The same is true for D-maltose, maltotriose and D-lactose, while D-saccharose, D-raffinose and the sugar alcohols D-mannitol, D-xylitol and D-sorbitol result in a single peak. These findings, together with the results of NMR and UV investigations[17], indicate the absence of decomposition products originating from the esterification reaction and extraction of the derivatives.

Analogous to the silylation in pyridine[18], anomerizations that occur in pyridine during the preparation of the 4-nitrobenzoates for slowly mutorotating sugars such as glucose are minimal, but only if the sugar is not dissolved in pyridine before the derivatization is carried out. If the sugars are kept in pyridine, other anomerization equilibria occur. Similar results were found for the benzoylation reaction of carbohydrates[11]. The method described can be used for the determination of proportions of anomers in the nanogram range for slowly mutorotating sugars (see Fig. 3). However, these separations are disadvantageous for the determination of complex mixtures of monosaccharides as they result in increased peak overlapping between the anomers of different monosaccharides.

Polar adsorbents such as silica gel and alumina are the most suitable for effective separations. They have the advantages that solvents of low viscosity can be used as the mobile phase and it is possible to inject the derivatives, dissolved in chloroform, directly on to the column[14]. For alumina, a strictly reproducible adjustment of the water content is of great importance. This extreme dependence of the k' values on water content for a series of sugar derivatives is shown in Fig. 1. *n*-Hexane was saturated with water, ethyl acetate dried by molecular sieve 4A and a defined volume of water subsequently added. In general, a considerable decrease in the k' values with increasing water content is obvious, tending asymptotically towards a limit. For the derivative of α-D-mannose, this limit is reached in the range investigated. Disaccharides can be eluted from the column only after the addition of water. The peak resolution decreases continuously with increasing water content, except for the pair of derivatives of α-D-glucose and β-D-glucose. It is also interesting that the k' values do not conform with the number of 4-NB groups in the sugar molecules, *e.g.*, the trisaccharide raffinose (with eleven 4-NB groups) is eluted before the disaccharides maltose, saccharose and lactose (each with eight 4-NB groups).

As an example of a separation, Fig. 2 shows the mono-, di- and trisaccharide composition of malt extract (Oxoid, London, U.K.; Code No. L. 39). The malt extract can be derivatized directly, with no pre-treatment. The solvents for the mobile phase, a three-component mixture consisting of *n*-hexane–chloroform–acetonitrile (10:2.5:3) with the addition of 280 ppm of water, were pre-treated as described above: *n*-hexane was saturated with water and chloroform and acetonitrile dried with molecular sieve 4A. The first peaks, unmarked in Fig. 2, originate from chloroform, the solvent for the derivatives, and reagent contaminants.

Glucose, maltose, saccharose and maltotriose could be identified by comparing the retention values with those for reference substances. Whereas the peaks of α- and β-D-glucose were not separated, good separations of the anomers of maltose and

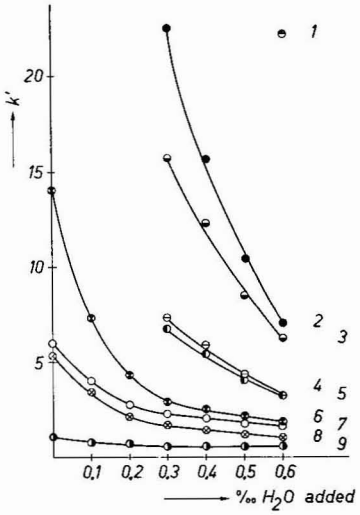


Fig. 1. k' values as a function of the water content of the mobile phase. 1 = α-D-Lactose (8); 2 = D-saccharose (8); 3 = β-D-maltose (8); 4 = α-D-maltose (8); 5 = D-raffinose (11); 6 = D-sorbitol (6); 7 = β-D-glucose (5); 8 = α-D-glucose (5); 9 = α-D-mannose (5). The number of OH groups esterified is given in parentheses. Solvent system: *n*-hexane–ethyl acetate (2:1), with different amounts of water added. Column: Alox T, 5 μm, 20 cm × 3 mm I.D. Flow-rate: 1.8 ml/min, p = 93 atm. Injection volume: 10 μl.

maltotriose were achieved. This considerable improvement in separation with increasing size of the sugar molecules (higher degree of substitution) was also observed on the 4-NB derivatives of digitalis glycosides[14]. LiChrosorb SI 60 has a better selectivity than Alox T for anomer separations. Fig. 3 shows an anomer separation of the

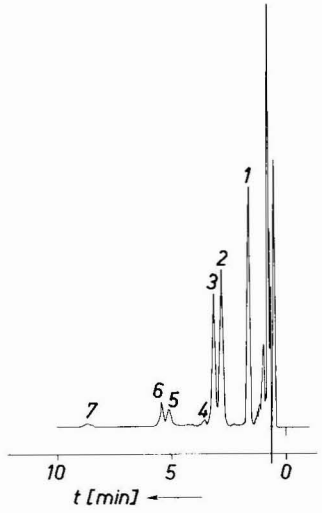
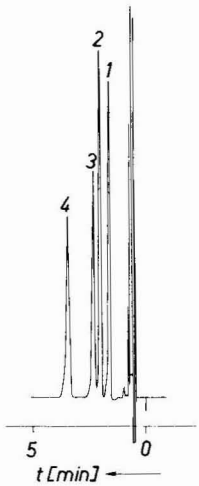


Fig. 2. HPLC of sugars (4-NB derivatives) in malt extract. 1 = D-Glucose (α- + β-); 2 = α-D-maltose; 3 = β-D-maltose; 4 = D-saccharose; 5 = α-maltotriose; 6 = β-maltotriose; 7 = unknown. Solvent system: *n*-hexane–acetonitrile–chloroform (10:3:2.5), 280 ppm of water added. Column: Alox T, 5 μm, 20 cm × 3 mm I.D. Flow-rate: 1.64 ml/min, p = 120 atm. Injection volume: 50 μl.



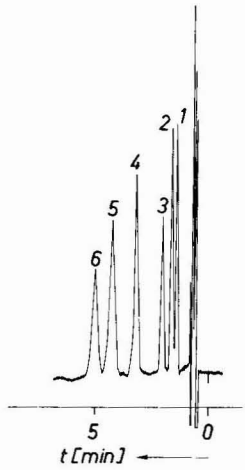


Fig. 3. HPLC of the anomers of D-glucopyranose and 1-methyl-D-glucopyranose (4-NB derivatives). 1 = α-D-Glucopyranose; 2 = 1-methyl-α-D-glucopyranose; 3 = β-D-glucopyranose; 4 = 1-methyl-β-D-glucopyranose. Solvent system: *n*-hexane–ethyl acetate (3:1), with 5% dioxane. Column: LiChrosorb SI 60, 5 μm, 15 cm × 3 mm I.D. Flow-rate: 2.1 ml/min, p = 155 atm. Injection volume: 10 μl.

Fig. 4. HPLC of four carbohydrates (4-NB derivatives). 1 = α-D-Glucose; 2 = β-D-glucose; 3 = D-sorbitol; 4 = D-saccharose; 5 = α-D-lactose; 6 = β-D-lactose. Solvent system: *n*-hexane–chloroform–acetonitrile–tetrahydrofuran (10:5:1:0.5). Column: LiChrosorb SI 60, 5 μm, 15 cm × 3 mm I.D. Flow-rate: 1.46 ml/min, p = 116 atm. Injection volume: 10 μl.

4-NB derivatives of D-glucopyranose and 1-methyl-D-glucopyranose. The methyl groups are not split off during the derivatization. On the chromatogram it can also be seen that the sequence of peaks does not necessarily correspond with the number of 4-NB groups in the molecule.

Quantitation

The different anomer ratios of some sugars in the sample and standard in most instances prevent quantitation by comparison of peak heights. It is possible, as previously reported[17], to define a specific extinction coefficient (ε_{OH}): the extinction coefficient of one esterified hydroxy group for the 4-NB derivatives of carbohydrates. This ε_{OH} value is valid for all aldoses tested, but not for fructose. Quantitation by applying the ε_{OH} value after calculation of the peak areas gives good results. For the determination of the peak areas, the peak height was multiplied by the peak width at half-height. For standardization, all carbohydrates that do not form isomers are suitable, *e.g.*, sugar alcohols.

A quantitative determination was carried out with a synthetic mixture of D-glucose, D-sorbitol, D-saccharose and D-lactose, using D-sorbitol as the internal standard. Eight test mixtures were derivatized under identical conditions and 10 μl (syringe injector) or 50 μl (loop injector) of the derivatized solutions injected on to the column. A chromatogram is shown in Fig. 4, and corresponds to the second largest

sugar composition tested (see also Table II, $E = 8$). For the calculation of the sugar content, the following equation was used:

$$C_{sugar} = \frac{A_{sugar}}{A_{standard}} \cdot \frac{A_{standard}}{f} \quad (1)$$

the conversion factor, f, being

$$f = \frac{MW_{standard}}{MW_{sugar}} \cdot \frac{n \cdot OH_{sugar}}{n \cdot OH_{standard}} \quad (2)$$

where

C (ng) = amount (concentration) reacted;
A (mm^2) = peak area;
MW = molecular weight;
$n \cdot OH$ = number of hydroxy groups reacted.

The conversion factors for the components of the test mixture are presented in Table I and quantitative results are given in Table II. For a mixture containing C ng of sugar, one calculates C_s ng after HPLC determination and standardization with sorbitol. The given values are averages of eight determinations. For glucose and lactose, the sum of the concentrations of the α- and β-forms are given. An increase in the injection volume from 10 to 50 μl results in an increase in the height of the signal by the same factor. Only the derivatives of α-D-glucose ($k' = 2.20$) and β-D-glucose ($k' = 2.62$) show a small degree of peak broadening. A comparison of the standard deviations of the means at the smallest sugar content ($E = 32$) for injection volumes of 10 and 50 μl shows the advantage of using the greater injection volume. The detection limit is between 0.75 ng (α-D-glucose) and 2.5 ng (β-D-lactose) for an injection volume of 50 μl. The criterion for the calculation of the detection limit is a signal-to-noise ratio of 3. Applying a filter photometer (DuPont 842 UV detector, 254 nm), the detection limit can be decreased by a factor of 4.

Quantitation by external standardization is also possible. This is shown for the determination of D-sorbitol in a confectionery for diabetic patients (Fig. 5). A 1-mg amount of the pulverized confectionery was dissolved in 1 ml of pyridine and 50 μl of this solution was used for derivatization, as described earlier[13]. D-Sorbitol exists in only one isomeric form and results in one peak in the chromatogram. Therefore, quantitation by calculating the peak height is possible. In addition to D-sorbitol,

TABLE I

CONVERSION FACTORS

$n \cdot OH$ = number of substituted OH groups in the molecule; f = conversion factor.

Derivative	$n \cdot OH$	f
Glucose monohydrate	5	0.7661
Sorbitol	6	1.000
Saccharose	8	0.7099
Lactose monohydrate	8	0.6741

TABLE II

QUANTITATIVE DETERMINATION OF SACCHARIDES BY INTERNAL STANDARDIZATION WITH SORBITOL

C = Actual concentration; C_s = concentration found using sorbitol as internal standard; s = standard deviation (8 determinations); V = volume injected; E = amplification factor.

Saccharide	C (ng)	C_s (ng)	s (%)	V (µl)	E
Glucose (α- + β-)	199.0	195.3	1.7	10	2
	39.8	40.4	1.7	10	8
	49.8	52.0	0.6	50	16
	10.0	9.6	4.5	10	32
	10.0	12.0	4.0	50	32
Saccharose	209.0	218.6	2.6	10	2
	41.8	43.4	2.3	10	8
	52.3	55.4	1.3	50	16
	10.5	11.2	7.4	10	32
	10.5	11.2	2.1	50	32
Lactose (α- + β-)	200.0	190.0	1.1	10	2
	40.0	35.7	2.3	10	8
	50.0	43.9	2.0	50	16
	10.0	9.6	9.1	10	32
	10.0	10.2	2.5	50	32

a significantly smaller amount of some unidentified monosaccharides is detectable in the chromatogram (small peaks before sorbitol). The amount of sorbitol found was 90.3% with a relative standard deviation of 2.3% (seven determinations), compared with a value of 91% stated by the manufacturer. A reference analysis was carried out by another laboratory using polarimetry, and 86.6% of sorbitol was found.

Quantitative determinations of more complex sugar mixtures by external standardization are also possible, as shown by the analysis of a pharmaceutical syrup

Fig. 5. HPLC of diabetic confectionery after derivatization with 4-NBCl. 1 = D-Sorbitol. Solvent system: *n*-hexane–chloroform–acetonitrile (10:3:2.5). Column: Alox T, 5 µm, 20 cm × 3 mm I.D. Flow-rate: 1.7 ml/min, p = 130 atm. Injection volume: 50 µl.

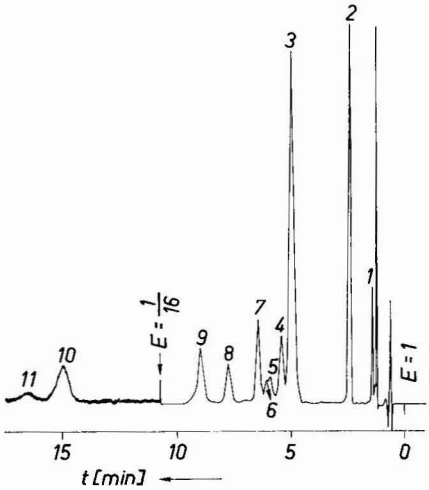


Fig. 6. HPLC determination of mono- and disaccharides (4-NB derivatives) in a pharmaceutical syrup. 1 = Propylene glycol; 2 = glycerine; 3 = D-glucose (α- + β-); 4–7, 9 = isomers of D-fructose; 8 = D-sorbitol; 10 = D-saccharose; 11 = unknown. Solvent system: *n*-hexane–chloroform–acetonitrile (10:3:1.9), 0.1% of water added. Column: LiChrosorb SI 60, 5 μm, 15 cm × 3 mm I.D. Flow-rate: 1.4 ml/min, p = 135 atm. Injection volume: 50 μl.

in Fig. 6. A 10-μl volume of the syrup was pipetted into a test-tube and the water removed by a water suction vacuum within 10 min; the residue was dissolved in 2 ml of pyridine and 25 μl of the solution were derivatized as described above. If fructose is present, all operations must be carried out at room temperature (20–22°); the use of a temperature of 50° for isolation of the derivatives[13] results in artefacts of the fructose derivatives. The derivatives of α- and β-D-glucose are eluted in one peak with the chosen separation system, but D-fructose-4-NB produces five peaks. NMR investigations showed that these five peaks are not artefacts but isomers, which is in agreement with the work of Curtius *et al.*[20], who were able to separate persilylated D-fructose into five components by means of GC; two compounds represent the pyranoside, two the furanoside and one the open-chain form.

The quantitation is made more difficult by the presence of five peaks. It is essential to calculate the peak areas, because the isomer ratio can hardly be kept constant. The peak areas were calculated by cutting the peaks from copies of the chromatograms and weighing the cuttings. This explains the relatively high standard deviation (Table III) in relation to the determination of glucose, which was quantified by cal-

TABLE III

DETERMINATION OF MONO- AND DISACCHARIDES IN A PHARMACEUTICAL SYRUP

s = Relative standard deviation (7 determinations).

Sugar	*Content (%)*	*s (%)*	*Method of calculation*	*Content (%) by reference analysis*
Glucose	21.3	2.4	Peak height	19.9
Fructose	21.5	6.8	Peak area	
Sorbitol	2.7	4.0	Peak height	
Saccharose	0.36	7.2	Peak height	0.36

culating the peak height. As already mentioned, the ε_{OH} value is not valid for fructose-4-NB and external standardization with a fructose standard is therefore necessary. The results of the quantitation are summarized in Table III. The agreement with a reference analysis, made by another laboratory using classical chemical procedures[22], is satisfactory. The finding of the same amounts of fructose and glucose in the syrup permits the conclusion that the monosaccharides were formed during storage by the hydrolysis of saccharose.

CONCLUSIONS

Nitrobenzoates of sugar and sugar alcohols are suitable for the determination of small amounts of these substances by HPLC. Separations on LiChrosorb SI 60 and Alox T are possible within a few minutes. The increase in sensivity by this method, compared with RI detectors, is four orders of magnitude.

Excellent separations of anomers can be carried out. On the other hand, these anomer separations can cause difficulties, especially in the determination of monosaccharides, because of the overlapping of peaks originating from the isomers of different saccharides. Quantitation can be effected by internal and external standardization; for complex mixtures, the latter is more favourable.

ACKNOWLEDGEMENTS

The "Jubiläumsfonds" of the Austrian National Bank is thanked for an instrument grant in support of this research. Thanks are due to E. Schindler for carrying out the reference analysis.

REFERENCES

1 J. L. Rocca and A. Rouchouse, *J. Chromatogr.*, 117 (1976) 216.
2 R. Schwarzenbach, *J. Chromatogr.*, 117 (1976) 206.
3 G. P. Belue and G. D. McGinnis, *J. Chromatogr.*, 97 (1974) 25.
4 J. C. Linden and C. L. Lawhead, *J. Chromatogr.*, 105 (1975) 125.
5 J. K. Palmer, *Anal. Lett.*, 8 (1975) 215.
6 R. W. Goulding, *J. Chromatogr.*, 103 (1975) 229.
7 A. M. C. Davies, D. S. Robinson and R. Couchman, *J. Chromatogr.*, 101 (1974) 307.
8 H. Bauer, *Z. Naturforsch., B*, 29 (1974) 7.
9 W. Voelter, *Chem. Ztg.*, 98 (1974) 493.
10 S. Katz, W. W. Pitt, Jr., J. E. Mrochek and S. Dinsmore, *J. Chromatogr.*, 101 (1974) 193.
11 J. Lehrfeld, *J. Chromatogr.*, 120 (1976) 141.
12 B. Müller and G. Göke, *Deut. Lebensm.-Rundsch.*, 68 (1972) 222.
13 F. Nachtmann, H. Spitzy and R. W. Frei, *Anal .Chem.*, 48 (1976) 1576.
14 F. Nachtmann, H. Spitzy and R. W. Frei, *J. Chromatogr.*, 122 (1976) 293.
15 F. A. Fitzpatrick and S. Siggia, *Anal. Chem.*, 45 (1973) 2310.
16 W. Strubert, *Chromatographia*, 6 (1973) 50.
17 F. Nachtmann, *Z. Anal. Chem.*, 282 (1976) 209.
18 C. C. Sweeley, R. Bentley, M. Makita and W. W. Wells, *J. Amer. Chem. Soc.*, 85 (1963) 2497.
19 K. Zürcher and H. Hadorn, *Deut. Lebensm.-Rundsch.*, 70 (1974) 425.
20 H. Ch. Curtius, M. Müller and J. A. Völlmin, *J. Chromatogr.*, 37 (1968) 216.
21 S. J. Angyal and V. A. Pickles, *Aust. J. Chem.*, 25 (1972) 1695.
22 K. Rauscher, B. Engst and U. Freimuth, *Untersuchung von Lebensmitteln*, VEB Fachbuchverlag Leipzig, 1972, pp. 129–135.

Journal of Chromatography, 136 (1977) 289–300

CHROM. 9864

LONG-CHAIN PHENOLS

VIII*. QUANTITATIVE ANALYSIS OF THE UNSATURATED CONSTITUENTS OF PHENOLIC LIPIDS BY THIN-LAYER CHROMATOGRAPHY–MASS SPECTROMETRY

J. H. P. TYMAN

School of Chemistry, Brunel University, Uxbridge, Middlesex (Great Britain)

(First received November 2nd, 1976; revised manuscript received December 6th, 1976)

SUMMARY

A thin-layer chromatographic–mass spectrometric procedure has been devised for the quantitative analysis of phenolic lipids. Correction for different response factors of the constituents of each component phenol and for isotopic abundances is necessary and leads to results showing excellent agreement with those from gas chromatographic analysis. No special instrumental requirements are needed and the method is successful due to the reproducible scanning obtainable.

INTRODUCTION

The composition of the unsaturated constituents of the complex phenolic shell liquid from *Anacardium occidentale* has engaged considerable attention since the finding of the inhomogeneity of so-called cardanol diene and the separation by adsorption chromatography of its methyl ether into saturated, monoene, diene and triene constituents**,[1] followed by structural elucidation. The constituents of cardol were similarly treated[2], and of those anacardic acid by low-temperature crystallisation[3]. Later, argentation thin-layer chromatography (TLC)–ultraviolet spectrophotometry was used quantitatively[4]. More recently, quantitative separations of the constituents[5] of the component phenols of *Anacardium occidentale*[6] and of *Rhus toxicodendron*[7] have been effected by TLC–gas–liquid chromatography (GLC). The composition of the unsaturated constituents in the latter case differs from a reported analysis[8] for *Rhus vernicifera* and the comparative results are interesting in view of the dermatological properties[9] of the active substance, urushiol (I; $n = 0, 2, 4, 6$), and studies made in the Rhus and Anacardium species. In both also, quantitative knowledge of un-

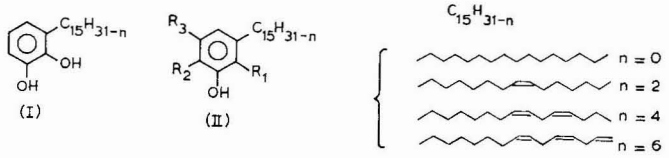
* Part VI, see ref. 6; Part VII, *J. Org. Chem.*, 41 (1976) 894.

** The term constituent is used for the individual saturated, monoene, diene and triene substances and component phenol for the natural mixture of the four constituents.

saturation is necessary for an understanding of certain industrial polymerization processes involving the component phenols.

During the course of work in 1966–67 on argentation TLC and on the identification of 2-methylcardol[10] in cashew nut-shell liquid (*Anacardium occidentale*) it became clear that the saturated, monoene, diene and triene constituents gave well defined mass spectra and, moreover, those of mixtures were remarkably reproducible. Opportunity was not then available for examining the potentiality of a mass spectral method of analysis.

Subsequently in a study of regional variations in composition of *Anacardium occidentale*, the examination of a large number of samples by the TLC–GLC method[5] was considered to be too lengthy on the limited stationary phases available. Attention was turned to TLC–mass spectrometry and it has been found that quantitative analysis can be both rapid and accurate. The results for the constituents of the component phenols in the natural product, namely anacardic acid (II; $R_1 = CO_2H$, $R_2 = R_3 = H$, $n = 0, 2, 4, 6$), cardol (II; $R_3 = OH$, $R_1 = R_2 = H$, $n = 0, 2, 4, 6$), cardanol (II; $R_1 = R_2 = R_3 = H$, $n = 0, 2, 4, 6$) and 2-methylcardol (II; $R_3 = OH$, $R_2 = CH_3$, $R_1 = H$, $n = 0, 2, 4, 6$) have been found to agree closely with those obtained by the TLC–GLC procedure[5].



The applicability of the method depends firstly on the almost identical vapour pressures of the saturated, monoene, diene and triene constituents and secondly on the difference of two mass units between each successive constituent with the effect that the P+2 peak contribution of the preceding constituent makes a minimal contribution to the peak height of the next. No correlation with the results by GLC could be obtained, however, unless sensitivity factors (response factors) were determined for each constituent.

Generally, there has been more interest in the fragmentation than the separatory aspect of mass spectrometry. Quantitative analysis of mixtures of known structure by mass spectrometry[11] has to some extent been overlooked. It has been somewhat displaced by GLC in recent years although there are numerous examples to which the latter procedure can be applied only with difficulty. Thus, a quantitative mass spectral procedure[12] for the analysis of component glycerides in natural oils and fats has been described in which various corrective rather than sensitivity response factors were employed. TLC–mass spectrometry is in our experience a reliable quantitative analytical procedure for the unsaturated constituents of long-chain phenolic lipids.

EXPERIMENTAL

Materials

On ordinary silica gel G plates the saturated, monoene, diene and triene

constituents of each component phenol migrate as a single band and separate only on argentated silica gel G into four bands.

Whole cashew nuts were obtained from Mozambique and were extracted as described earlier[10]. The component phenols were separated by preparative TLC with ammoniated ethyl acetate–chloroform (5:95, v/v) or, as described[4], ammoniated diethyl ether–light petroleum (b.p. 40–60°) (30:70, v/v). The bands made visible with rhodamine 6G were eluted (approximately 25 cm^3 of eluting solvent per gram of adsorbent) with diethyl ether–methanol (90:10, v/v) containing 0.1% of 2,6-di-*tert.*-butyl-4-methylphenol, and examined for purity on analytical plates. Solvent of the above composition was found to remove all of the adsorbed constituents without co-extraction of the detection reagent (in early experiments[4], pure methanol was employed and after a short period both the phenolic material and dye passed into solution). After standing for 1–2 h with occasional swirling, the mixture was filtered to remove the silica gel G that was washed with the eluting solvent. The combined filtrates were evaporated (rotary evaporator) *in vacuo* to remove the solvents. As only 1-mg amounts were required for mass spectral examination, the TLC separation could be effected on analytical plates (10 cm × 8 cm × 0.25 mm) but generally preparative plates and a sample size in the range 0.1–0.25 g were employed. Within 3 h the component phenols were available for mass spectral examination. All phenolic materials isolated were stored in closed flasks at −20° under nitrogen. Anacardic acid in ethereal solution at 0° was converted into the methyl ester by treatment at 0° with ethereal diazomethane[5].

An alternative TLC separation of cashew nut-shell liquid involved treatment with ethereal diazomethane at 0°, to esterify anacardic acid, followed by initial development with light petroleum to obtain methyl anacardate near the solvent front and subsequently use of a more polar solvent, chloroform-ethyl acetate (95:5, v/v), to separate cardanol, 2-methylcardol and cardol. Recoveries were effected as previously described[4].

For the preparation of standards the component phenols were each separated into their saturated, monoene, diene and triene constituents by argentation TLC[4] with chloroform–ethyl acetate in the proportions 90:10 (v/v) for methyl anacardate, 80:20 (v/v) for cardanol, 75:25 (v/v) for 2-methylcardol and 50:50 (v/v) for cardol. Eluted and recovered materials freed from trace amounts of silver nitrate were examined on ordinary and argentation analytical plates and were re-purified when necessary on ordinary plates. All materials purified by this sequence were stored at −20° in the dark under nitrogen. As the natural product contained insufficient of the saturated (15:0) component phenol for the standard, in each instance it was supplemented by hydrogenation of the side-chain[10] of the appropriate component phenol. All standards comprising the four constituents of each component phenol were made up in benzene solution (2 ml) from the relevant material weighed on a five-place balance.

*Mass spectra**

Mass spectra were determined on an AEI MS9 and on a Hitachi–Perkin-

* Mass spectra on the MS9 instrument were carried out under the ULIRS scheme at the School of Pharmacy, University of London; the RMS4 spectra were obtained at Brunel University.

Elmer RMS4 instrument by direct insertion as for solids under standard conditions (70 eV, temperature in the range 160–180°). Samples were used at a concentration of 20% in diethyl ether or chloroform. A standard capillary mounted in the probe was used for the RMS4 and the quartz tip of the probe for the MS9. Scans were made at $\frac{1}{2}$, 1, 2, 3, 4 and 5 min after injection (and sometimes longer) until the sample was exhausted. As much of the width of the chart paper as possible was used to obtain the maximum peak height on scale in the first scan. Scale expansion could be used also but generally was not required. In every instance after the final scan no background material was found in the instrument. In order to find the reproducibility, several runs were carried out on a further sample.

The mass spectra of the individual constituents of each component phenol, the natural component phenol and the relevant standard mixture were all examined.

The chart records were sprayed with a stabilising lacquer. The peak heights were accurately measured by inspection under powerful magnification. The percentage composition was then obtained for both the natural component phenol and the standard mixture. From the weights taken of the consituents of each component phenol, sensitivity factors (relative response factors) were obtained in each instance. These were applied to correct the results obtained for the natural component phenol. The peak heights for the diene, monoene and saturated consituents were corrected for the theoretical P+2 peak contribution from the triene, diene and monoene, respectively. The theoretical P+2 peak contribution was calculated from the usual binomial expression[13,14] according to the molecular formula of the relevant phenol, taking into consideration the carbon, hydrogen and oxygen atoms. The observed P+2 peak contribution was also used as a basis for calculation. In all instances standard deviations were calculated in the usual way.

The RMS4 instrument was used for the analysis of cardanol in order to find the performance of an instrument with more limited resolution than the MS9.

RESULTS AND DISCUSSION

General procedure

The analytical method depends for its applicability on a preliminary TLC separation on ordinary silica gel G plates of the component phenols each containing saturated, monoene, diene and triene constituents. In this way materials of comparable volatility were then available for mass spectral examination. Attempts to run unseparated natural cashew nut-shell liquid in which the anacardic acid had been converted into methyl anacardate were not as successful.

The volatility of the phenols at the temperature attained by the probe and at the operative vacuum in the mass spectrometers used was adequate and it was unnecessary to use volatile derivatives. The dimethyl ether of urushiol[8] has been used for the analysis of its constituents but in our work this did not prove to be a requirement. Anacardic acid could be used without conversion into methyl anacardate but its known decarboxylation at temperatures slightly above that of the probe, possibly also catalyzed by the metal surface, led to the use of the derivative[5].

Reproducibility of results

In the early experiments, the variation in results was greater than in the final

procedure. The standard deviations for the percentage composition of the triene, diene and monoene constituents of cardol (65.14 ± 1.26%, 24.91 ± 1.07% and 9.91 ± 0.88%) and of cardanol (29.94 ± 1.61, 21.99 ± 0.77 and 40.87 ± 2.70) were higher than those given in Table II. The reproducibility of results on a given sample examined at widely different times was excellent. Thus, for cardol two sets of results for the triene, diene and monoene were 65.38%, 24.31% and 10.30%, and 65.65%, 24.60% and 10.09%.

Determination of relative response factors

The mass spectral analytical procedure depended on the absence of P−6, P−4 and P−2 ions in the mass spectrum of the 15:0 constituent, P−4, P−2 ions in that of the monoene and a P−2 ion in that of the diene. The four separated pure constituents of each component phenol were found not to contain interfering peaks at the above masses, nor did P+2 ions constitute more than a few per cent, which would not have been the case had the purified constituents contained vinylogous impurities. Formation of P−1, P−2, P−3 and P−4 ions has been observed with some compounds[15] and makes the examination of pure constituents necessary. The molecular ion peaks were strong but not base peaks[10]. The basis for quantitative mass spectral analysis has been generally discussed[11] and the molecular ion intensities for the 15:0 (s), 15:2 (m), 15:2 (d) and 15:3 (t) constituents are proportional to $S_s \cdot p_s$, $S_m \cdot p_m$, $S_d \cdot p_d$, and $S_t \cdot p_t$, respectively, where S represents the sensitivity (relative response factor) of the detector and p the vapour pressure of the relevant constituent. It was not expected that relative response factors would be similar but the vapour pressures are known to be comparable and the small standard deviations clearly demonstrate this. No further correction for minor differences in volatility was made and the relative response factors obtained from the calibration experiments were used to correct the results from the natural product.

The peak heights for the four constituents were accurately measured, normalised to give the percentage composition and the standard deviations calculated. The results were corrected for the theoretical contribution of the P+2 peak and the relative response factor calculated as in grams per unit peak height. The results for the standards, methyl anacardate, cardol and cardanol are shown in Table I.

Results for the unsaturated constituents of natural cashew nut shell liquid

The mass spectral interpretative aspects of the spectra obtained are the subject of a forthcoming report on the synthesis of the unsaturated constituents of cardanol[16] and are therefore not discussed here. Fig. 1 shows the mass spectrum of methyl anacardate. The molecular ion peaks for the triene (*m/e* 356), diene (*m/e* 358), monoene (*m/e* 360) and saturated (*m/e* 362) constituents appear at the right-hand side of the spectrum. In Fig. 2 the molecular ion peaks of the four constituents resulting from scans at ½, 1-, 2-, 3- and 4-min intervals are given. The total results for the constituents of the natural component phenols are summarised in Table II. The uncorrected results (i), their correction for the P+2 peak contribution (ii) and for the differing relative response factors (iii), the final normalised percentage (iv) and a comparison with the GLC values are given. Close similarity is apparent between the two methods for methyl anacardate, cardol and cardanol. For 2-methylcardol, the purity of the constituents of the material was doubtful owing to the long period that

TABLE I

RELATIVE RESPONSE FACTORS FOR THE FOUR CONSTITUENTS OF THE COMPONENT PHENOLS

Phenol	*Parameter*	*15:0 (saturated)*	*15:1 (8′z) monoene*	*15:2 (8′z, 11′z) diene*	*15:3 (8′z, 11′z, 14′) triene*
Methyl anacardate	(i) Standard (%)	7.29 ± 0.52	50.66 ± 3.19	13.47 ± 1.02	28.576 ± 1.91
	(ii) P + 2 peak (% of P)	3.17	3.17	3.17	3.17
	(iii) Corrected standard (%)	5.86	51.76	12.95	29.43
	(iv) Wt. (g)	0.00391	0.06647	0.02720	0.09078
	(v) Relative response factor (RRF):(iv) × 10^3/(iii)	0.66736	1.2842	2.1004	3.0843
Cardol	1st standard (%)	47.19 ± 0.89	16.84 ± 0.38	11.50 ± 0.37	24.46 ± 0.64
	P + 2 peak (% of P)	2.63	2.63	2.63	2.63
	Corrected standard (%)	47.41	16.77	11.01	24.81
	Wt. (g)	—	0.00469	—	0.00636
	Relative response factor (RRF)	—	2.7964	—	2.5636
	2nd standard (%)*	54.708	—	20.582	24.703
	Corrected standard (%)	55.070	—	19.436	24.867
	Wt. (g)	0.01245	—	0.01958	0.02840
	Relative response factor (RRF)	0.2261	—	1.0074	1.1421
	Interrelation of the two sets	0.5074	2.7964	2.2612	2.5636
Cardanol	Standard (%)	29.04 ± 0.65	45.98 ± 0.84	13.45 ± 0.33	11.53 ± 0.23
	P + 2 peak (% of P)	2.63	2.63	2.63	2.63
	Corrected standard (%)	28.359	46.492	13.397	11.752
	Wt. (g)	0.00278	0.0104	0.00340	0.00756
	Relative response factor (RRF)	1.95730	2.2621	2.5279	6.5557

* For a number of reasons it became necessary to use separate standards and to interrelate the two sets of results.

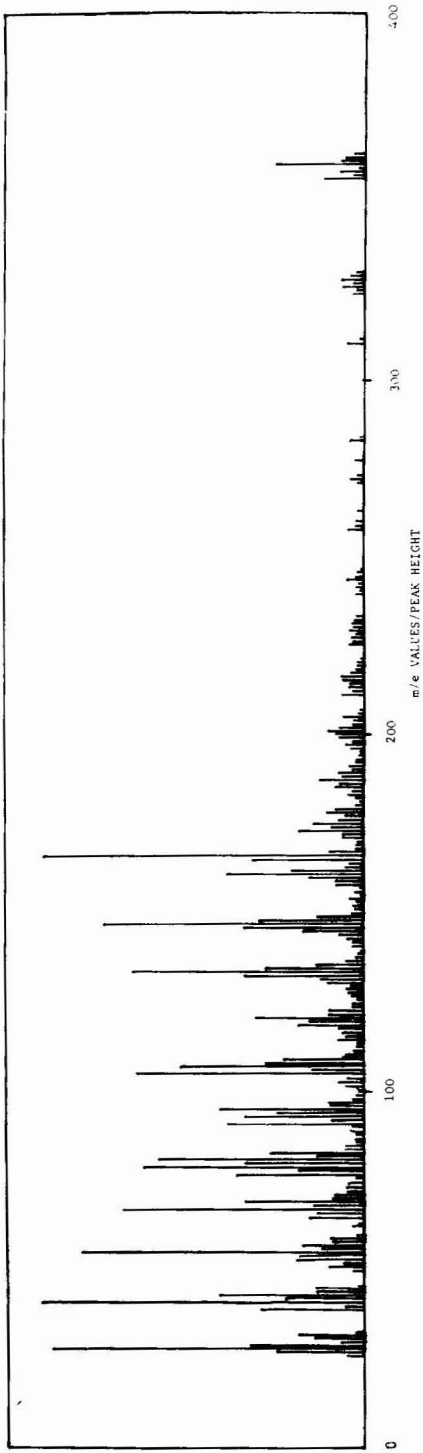


Fig. 1. Mass spectrum of methyl anacardate (triene, diene, monoene and saturated constituents).

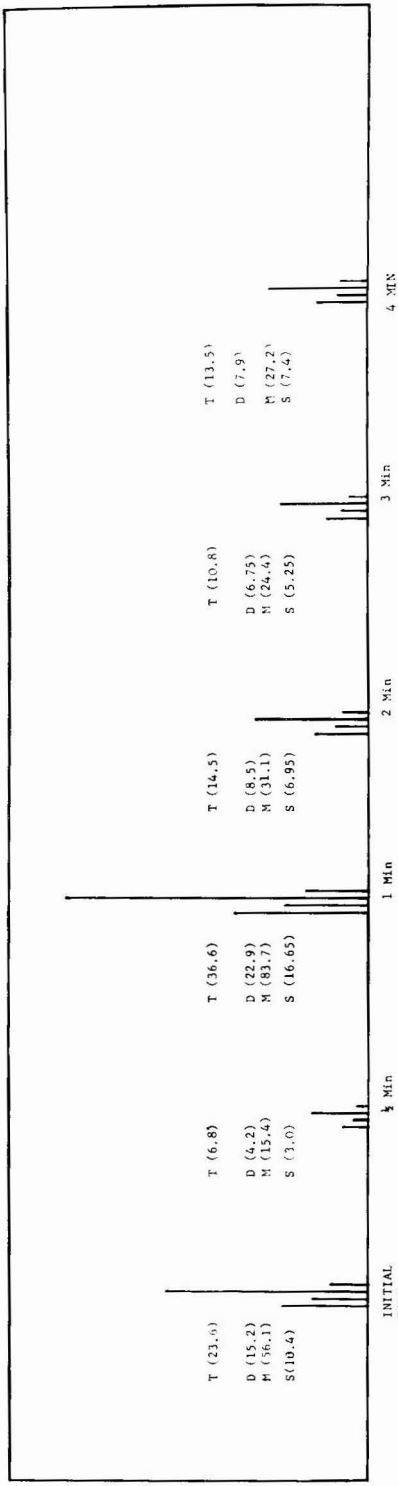


Fig. 2. Peak heights of the molecular ions from mass spectral scans of methyl anacardate at intervals up to 4 min.

TABLE II

COMPOSITION OF OLEFINIC CONSTITUENTS OF COMPONENT PHENOLS IN NATURAL CNSL BY MASS SPECTROMETRY

Phenol	*Parameter*	*Saturated*	*Monoene*	*Diene*	*Triene*
Methyl anacardate	(i) Composition of natural product (uncorrected) (%)	11.04 ± 1.15	51.54 ± 1.65	14.23 ± 0.18	23.20 ± 0.62
	(ii) Corrected (for P + 2 peak)	9.40	51.99	13.49	23.20
	(iii) Corrected for relative response factor [(ii) × RRF]	6.2733	65.605	28.341	71.544
	(iv) Normalized (%)	3.65	38.19	16.50	41.65
	(v) GLC analysis (%)	4.05	38.3 ± 2.33	17.3 ± 0.4	40.4 ± 1.6
Cardol	(i) Composition of natural product (uncorrected) (%)	1.41 ± 0.11	9.95 ± 0.56	23.93 ± 0.11	64.74 ± 0.51
	(ii) Corrected (for P + 2 peak)	1.15	9.32	22.22	64.74
	(iii) Corrected for relative response factor [(ii) × RRF]	0.598	26.742	51.403	170.353
	(iv) Normalized (%)	0.24	10.74	20.64	68.39
	(v) GLC analysis (%)	0.31	8.1. ± 1.41	21.9 ± 3.01	69.7 ± 3.74
Cardanol	(i) Composition of natural product (uncorrected) (%)	7.79 ± 0.6	46.03 ± 1.25	20.19 ± .41	25.99 ± 0.41
	(ii) Corrected (for P − 2 peak)	6.57	45.49	19.50	25.99
	(iii) Corrected for relative response factor [(ii) × RRF]	6.604	104.264	50.726	171.412
	(iv) Normalized (%)	1.98	31.31	15.23	51.47
	(v) GLC analysis (%)	2.68	29.5 ± 1.03	16.6 ± 0.64	51.2 ± 1.39
2-Methylcardol*	(i) Composition of natural product (uncorrected) (%)	4.36 ± 0.57	18.54 ± 0.88	21.21 ± 0.51	55.91 ± 1.17
	(ii) Corrected (for P + 2 peak)	3.81	17.93	19.59	55.91
	(iii) Normalized (%) (uncorrected)	3.92	18.43	20.15	57.50
	(iv) GLC analysis (uncorrected)	1.63	18.08	19.55	60.74

* Relative response factors for the constituents of this component phenol to be reported in later work.

had elapsed between isolation and examination and corrected results have not been obtained in this instance. Nevertheless, the uncorrected mass spectral and GLC results show reasonable similarity.

Further experiments with technical CNSL, the product of industrial decarboxylation of the natural product, have indicated the applicability of the mass spectral procedure to that product.

Results for different mass spectrometers

In Table III, the results for experiments carried out with a Hitachi–Perkin-Elmer RMS4 and with an AEI MS9 are given for cardanol.

The agreement was acceptable although the reproducibility was not so good with the former instrument. Mass spectral tracings for the standard followed by the natural product could usually be obtained in a few minutes, and the measurement of peak heights and calculation of corrections occupied most of the procedure. No attempt was made to enhance the molecular ion by reduction of the ionization voltage or by the use of the field ionization technique, and both instruments were used essentially as set up for standard mass spectral examination at 70 eV. The consistency of the results obtained was to some extent unexpected in view of the proverbial variation sometimes associated with mass spectra. It would have been advantageous to have a digital display of maximum peak height and numerous other improvements suggest themselves.

Comparison of results obtained with different extents of correction

It was clear that, apart from gross correction for the differing relative response factors of the constituents, different extents of correction for the P+2 ion peak could be applied. Where no correction was used the percentages of monoene, diene and triene constituents agreed tolerably well with those obtained by GLC but the percentage of saturated constituents was too high as shown in Table IV for methyl anacardate. Consequently, it was generally desirable to correct both the standard and the natural product for the P+2 peak contribution.

At first it seemed more logical to correct the results in either instance by the use of the observed P+2 peak contribution, obtained from the mass spectral examination of the individual constituents, rather than the theoretical value. The values for the observed P+2 peak were always slightly higher than the theoretical value but no simple explanation is evident as each constituent was checked analytically for purity by argentation TLC and by GLC, to ensure homogeneity. For quick results and certain applications where the percentage of unsaturated constituents only is of primary interest the uncorrected standard and natural product results may be acceptable.

Influence of homologous phenols on the analytical procedure

GLC examination has shown that homologous phenols are present particularly in the case of anacardic acid in natural CNSL and cardanol in technical CNSL. The C-17 contribution is the greatest, although constituents with chain lengths of C-9, C-11 and C-13 are also present. For cardol, 2-methylcardol and cardanol in natural CNSL, C-17 homologues are present in smaller proportions. The detection of the C-17 homologue is laborious by GLC but straightforward by mass spectroscopy. In the adsorption TLC separation used in the initial stage, homologues migrate together.

TABLE III

COMPARISON OF RESULTS FOR THE CONSTITUENTS OF CARDANOL DETERMINED ON HITACHI–PERKIN-ELMER RMS4 AND AEI MS9 INSTRUMENTS

Instrument	*Parameter*	*Saturated*	*Monoene*	*Diene*	*Triene*
RMS4	Standard (%)	26.60 ± 0.51	45.24 ± 0.59	14.76 ± 0.48	13.46 ± 0.58
	Corrected standard (for P + 2)	25.411	44.851	14.403	13.458
	Normalized (%)	25.897	45.709	14.679	13.715
	Wt. (g)	0.00278	0.0104	0.00340	0.00756
	RRF ($g \times 10^4$) (%)	1.0735	2.2752	2.3162	5.5122
	Natural product (%)	8.995 ± 1.230	42.996 ± 1.057	20.446 ± 2.043	27.347 ± 1.267
	Corrected (for P + 2)	7.862	42.457	19.725	27.347
	Normalized (%)	8.073	43.594	20.254	28.079
	RRF × normalized (%)	8.666	98.959	46.911	154.773
	Normalized (%)	2.81	32.04	15.19	50.12
MS9	Totally corrected (%)	1.98 ± 0.66	31.31 ± 1.25	15.23 ± 0.41	51.47 ± 0.41

TABLE IV

COMPARISON OF RESULTS FOR CONSTITUENTS OF COMPONENT PHENOLS WITH DIFFERING EXTENTS OF CORRECTION

Phenol	*Parameter*	*Saturated*	*Monoene*	*Diene*	*Triene*
Methyl anacardate	Standard (corrected with observed P + 2)	4.07	49.99	11.79	28.57
	Natural product (%) (corrected with observed P + 2)	4.19	37.88	16.62	41.31
	Standard (uncorrected)	7.29	50.66	13.47	28.57
	Natural product (%) (uncorrected)	3.36	38.42	16.32	41.89
	Standard (uncorrected)	7.29	50.66	13.47	28.57
	Natural product (%) (corrected with observed P + 2)	1.02	39.90	14.56	44.52
	Standard (corrected with theoretical P + 2)	5.86	51.76	12.95	29.43
	Natural product (%) (corrected with theoretical P + 2)	3.65	38.19	16.50	41.65
	% by GLC	4.05	38.3	17.3	40.4
Cardol	1st standard (uncorrected)	47.19	16.84	11.50	24.46
	2nd standard (uncorrected)	54.71	—	20.58	24.70
	Natural product (%) (uncorrected)	0.30	11.16	20.74	67.80
	% by GLC	0.31	8.1	21.9	69.7
Cardanol	Standard (uncorrected)	29.04	45.98	13.45	11.53
	Natural product (%) (uncorrected)	2.24	31.26	15.32	51.18
	% by GLC	2.68	29.5	16.6	51.2

The importance of precise TLC separation of the component phenols is shown by reference to cardol (molecular weight of 15:0 constituent = 320) where the presence of anacardic acid (molecular weight of 15:0 constituent = 348) would contribute a mass coincident with the C-17 homologue of cardol. In the method used in this work, the methylation of anacardic acid clearly avoids this occurrence. In Table V the molecular weights of the C-13, C-15 and C-17 homologues of the saturated constituents of the component phenols are shown. This indicates that homologues do not interfere in the analytical procedure and furthermore, the examination of the pure constituents of each component phenol ensures the validity of the method.

TABLE V

MOLECULAR WEIGHTS OF SATURATED HOMOLOGOUS COMPONENT PHENOLS

Chain length	*Cardanol*	*Cardol*	*2-Methylcardol*	*Methyl anacardate*
C-13	276	292	306	334
C-15	304	320	334	362
C-17	332	348	362	390

Table V indicates the difficulties inherent in a direct mass spectral examination with no initial TLC separation. An attempt to analyze technical CNSL is being examined but much further work remains to be done. While the different proportions of the component phenols present and their different volatilities might be overcome by the use of appropriate standard mixtures, the reproducibility of repeated scans remains a difficulty. Another possibility with a combined TLC–MS method would be to use direct recovery[17] from the chromatographic plate rather than the present indirect technique involving elution of bands and recovery of the component phenol containing its four constituents. Experimental work is also in progress to develop a GLC–MS procedure by means of which the component phenols could be analyzed quantitatively[5] simultaneously with the unsaturated constituents.

General observations

The close similarity of analytical results obtained by the GLC and TLC–MS procedures, despite wide differences in relative response factors between individual unsaturated constituents, is a strong recommendation for the latter approach. Without the application of relative response factors the results show no such conformity. The success of the mass spectral method is inherently associated with the comparable vapour pressure of the four constituents of each component phenol. Hites[12], in his work on glycerides, employed corrective rather than relative response factors and this entailed using information obtained from molecular distillation characteristics to allow for differing volatilities.

In biological, biosynthetic and technological areas[18], where the rapid analysis of methyl esters of polyethenoid fatty acids may be required, and indeed GLC is readily applicable, the quantitative usefulness of mass spectrometry has to some extent been overlooked. It is our experience that, like the phenolic lipids, the polyethenoid C-18 methyl esters with similar vapour pressures may be rapidly and quantitatively determined provided that relative response factors are employed. The ex-

treme rapidity of multiple scanning in mass spectrometry from a single sample has much in its favour compared with the repeated injection and time-consuming equilibration–elution process in GLC. The stability and reproducibility of the detector response in the mass spectral procedure are further strong recommendations for this technique.

REFERENCES

1 W. F. Symes and C. R. Dawson, *J. Amer. Chem. Soc.*, 75 (1953) 4952.
2 W. F. Symes and C. R. Dawson, *Nature (London)*, 171 (1953) 841.
3 V. J. Paul and L. M. Yeddanapalli, *Nature (London)*, 174 (1954) 604.
4 J. H. P. Tyman and N. Jacobs, *J. Chromatogr.*, 54 (1974) 83.
5 J. H. P. Tyman, *J. Chromatogr.*, 111 (1975) 277 and 285.
6 J. H. P. Tyman, *Anal. Chem.*, 48 (1976) 30.
7 J. H. P. Tyman and C. H. Khor, *Chem. Ind. (London)*, (1974) 526.
8 M. Sato and S. Syoti, *Nippon Kagaku Zasshi*, 89 (1968) 814.
9 A. P. Kurtz and C. R. Dawson, *J. Med. Chem.*, 14 (1971) 729.
10 J. H. P. Tyman, *J. Chem. Soc., Perkin Trans. I*, (1973) 1639.
11 J. A. Benyon, R. A. Saunders and A. E. Williams, *Mass Spectrometry of Organic Compounds*, Elsevier, Amsterdam, 1968, p. 33.
12 R. A. Hites, *Anal. Chem.*, 42 (1970) 1736.
13 I. Fleming and D. H. Williams, *Spectroscopic Methods in Organic Chemistry*, McGraw-Hill, Maidenhead, 1973, p. 154.
14 D. W. Mathieson, *Interpretation of Organic Spectra*, Academic Press, London, 1965, p. 147.
15 I. Fleming and D. H. Williams *Spectroscopic Methods in Organic Chemistry*, McGraw-Hill, Maidenhead, 1973, p. 161.
16 J. H. P. Tyman and J. Caplin, *Chem. Ind. (London)*, 40 (1973) 953 (preliminary communication).
17 R. Kaiser, *Chem. Brit.*, (1969) 54.
18 J. Boldingh, *Chem. Ind. (London)*, (1975) 984.

Journal of Chromatography, 136 (1977) 301–305

CHROM. 10,023

Note

Non-dispersive atomic fluorescence spectroscopy, a new detector for chromatography

JON C. VAN LOON, J. LICHWA and BERNARD RADZIUK
Institute for Environmental Studies, University of Toronto, Toronto M5S 1A1, Ontario (Canada)
(Received March 2nd, 1977)

Metal pollution and related health problems are becoming increasingly common. The incidents involving mercury have shown that total metal data is insufficient and often misleading. Metal compounds must be identified and determined quantitatively. The many types of chromatography provide the most powerful approach to the separation of these compounds. Conventional chromatographic detectors, however, show poor selectivity to the metal compounds of interest.

Gonzalez and Ross[1] and Longbottom[2] described the interfacing of an atomic absorption spectrophotometer with a gas chromatograph for the detection of alkylated mercury compounds. This detector is metal specific and hence will only record peaks for the metal used as the radiation source. A number of researchers have used this system for the study of compounds of lead, mercury and chromium.

In 1973, Manahan and Jones[3] used atomic absorption spectroscopy as a detector for high-pressure liquid chromatography in the study of chromium compounds. As in the case of gas chromatography, the metal specificity of this detector greatly simplifies chromatograms compared to those obtained using less specific, conventional detectors.

Since the above pioneering work, atomic absorption spectroscopy has become a routinely used detector for chromatography in metal speciation studies. In this regard, it has made an invaluable contribution to the study of metal compounds.

It has been found by the present authors that atomic fluorescence spectroscopy can be used as a metal specific detector with column chromatography. If non-dispersive atomic fluorescence spectroscopy is employed, two distinct advantages over atomic absorption can be obtained. Firstly, simultaneous multielement detection can be achieved. Secondly, the detection limits for most elements can be improved by 1 to 3 orders of magnitude compared to atomic absorption.

Non-dispersive atomic fluorescence spectroscopy has been described in detail, as a technique for trace metal analysis by Larkins[4]. As with flame atomic absorption, interfacing of atomic fluorescence as a column chromatographic detector is simple. For liquids, the effluent drain of the column is connected directly to the nebulizer capillary of the burner. When gas chromatography is used, the effluent can be introduced into the flame through a port at the base of the burner.

Non-dispersive atomic fluorescence equipment is inexpensive to assemble.

This is another advantage compared to atomic absorption spectroscopy. If the detection limit advantage mentioned above is to be realized, then high intensity hollow-cathode or electrodeless discharge lamps are essential. The latter can be obtained commercially for many elements at about the price of ordinary hollow-cathode lamps.

The unique capabilities of this detector can be demonstrated in the difficult separation and detection, simultaneously, of three different metal–glycines (amino acids) and in the same sample the three metal–EDTA compounds. This combination occurs in clinical samples obtained during the diagnosis and treatment of metal poisoning.

EXPERIMENTAL

Equipment and reagents

A 3-channel non-dispersive atomic fluorescence spectrometer was assembled as diagrammed. Two AA4 amplifiers, one modified to modulate at 325 Hz and the other capable of modulating at the normal 285 Hz were employed. Two Techtron AA4 lamp power supplies were used with these amplifiers. A home-made amplifier and lamp power supply with a modulation frequency of 80 Hz were used for the third channel. R-166 and R-106 photomultipliers (Hamamatsu TV) were used. The former, a solar blind tube, was used for Ni and Zn and the latter for Cu.

Optics consisted of 50 mm focal length by 4 cm diameter silica lenses, arranged as shown in Fig. 1. A 9.2-mm aperature iris was placed in front of the photomultiplier.

Perkin-Elmer intensitron hollow-cathode lamps were run at 15 mA for all elements. When better detection limits are required, electrodeless discharge lamps or high intensity hollow-cathode lamps can be employed.

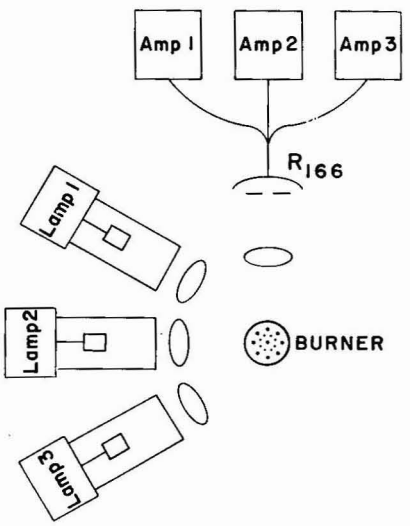


Fig. 1. Schematic of a 3-element non-dispersive atomic fluorescence instrument.

A nitrogen shielded, air–acetylene flame was used. The home-made burner was 15.8 mm in diameter with three concentric rings of 1.09 mm holes. This burner fits inside a SB-E sheathing device (R11C, London, Great Britain) and was used with a Perkin-Elmer Model 303, pre-mix chamber-nebulizer system.

To record the output from the three amplifiers, it was necessary to employ Model 56 double-pen and Model 165 single-pen recorders (Perkin-Elmer). The chart speeds of these were equal allowing superimposition of the output.

A Perkin-Elmer Model 601 high-pressure liquid chromatograph was used. The cation-exchange column was packed with Perkin-Elmer Partisil-10 SCX. The column temperature was maintained at 55°. Interfacing is simple. The outflow from the column is connected directly to the nebulizer capillary of the burner (*cf.* Fig. 2). A column flow-rate of 4 ml/min was maintained to be compatible with the nebulizer flow-rate of the fluorescence burner. A pressure of 100 p.s.i. was used.

The amino acid– and EDTA–metal compounds were prepared by the method of Lau[5].

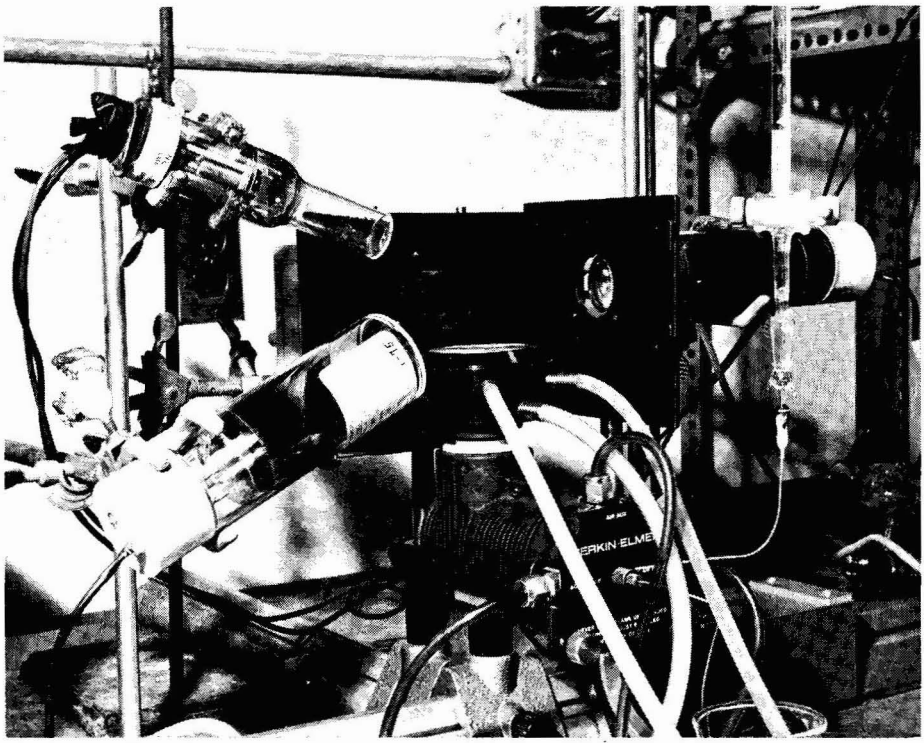


Fig. 2. Non-dispersive atomic fluorescence detector for chromatography.

Procedure

The column is rinsed and equilibrated with water. The column temperature is adjusted to 55°. A 25-μl sample is injected and the column eluted with pure water. After the first peaks (*E* on Fig. 3) come over, a 5-min convex gradient, curvature 999, to 100% 1 *M* NH_4NO_3 is used.

RESULTS AND DISCUSSION

Fig. 3 is a chromatogram of the three elements Zn, Ni and Cu. Peaks designated *E* are metal–EDTA complexes, those marked *G* are metal–glycines, and peak T is Cu-Trien. Because of their physical and chemical similarities, the glycine peaks have almost identical retention times. The same is true for the EDTA peaks. A conventional UV–visible detector would fail to resolve individual glycines or EDTA's. The automatic fluorescence detector, on the other hand, gives excellent resolution of these compounds.

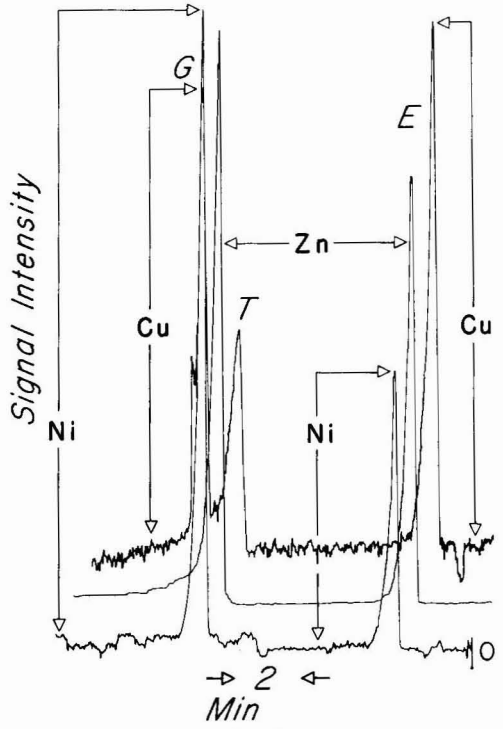


Fig. 3. Chromatogram for Cu–, Zn– and Ni–glycines, –EDTA's and Cu-Trien.

CONCLUSIONS

Until recently, it has been impossible to provide metal speciation data in quantity. This has been due to the difficulty in detecting trace metal compounds in complex samples. Atomic absorption spectroscopy used as detector for chromatography has provided the first breakthrough toward solving this problem. Nondispersive atomic fluorescence spectroscopy has all the advantages of atomic absorption spectroscopy in this application while providing, in addition, better detection limits and a simultaneous multielement capability.

Future work

Now that the technique has been shown to work a better multielement spectrometer, such as that described by Larkins and Willis[6], will be constructed. This instrument will contain 7 channels and will be used with a 7-pen recorder.

ACKNOWLEDGEMENTS

Financial support was provided by the National Research Council. Thanks are also due Mr. P. L. Larkins and Dr. S. Lau.

REFERENCES

1 J. E. Gonzalez and R. T. Ross, *Anal. Lett.*, 5 (1972) 683.
2 J. E. Longbottom, *Anal. Chem.*, 44 (1972) 111.
3 S. E. Manahan and D. R. Jones, *Anal. Lett.*, 6 (1973) 745.
4 P. L. Larkins, *Spectrochim. Acta*, 26B (1971) 477.
5 S. Lau, Department of Biochemistry, University of Toronto, Toronto, personal communication.
6 P. L. Larkins and J. B. Willis, *5th International Conference on Atomic Spectroscopy, Melbourne, Aug. 25–29, 1975*, Abstract B22.

Journal of Chromatography, 136 (1977) 306–307

CHROM. 10,001

Note

Simple device for sampling from vacuum systems to gas chromatographs

BRUCE A. COSGROVE and IAN D. GAY
Department of Chemistry, Simon Fraser University, Burnaby, B.C. V5A 1S6 (Canada)
(Received January 11th, 1977)

A frequently occurring problem in many types of investigation is the withdrawal of gas samples of known size from a vacuum system at sub-atmospheric pressure, and their injection into a gas chromatograph. While there are several types of commercial sampling valves which will accomplish this, these are moderately expensive, not easily compatible with glass vacuum systems, and often leaky.

The special stopcock shown in Fig. 1 performs the necessary sampling function. It is constructed of borosilicate glass and can be sealed directly to a glass vacuum system. In the position shown, the carrier gas enters at A and leaves at B, bypassing the sampling loop. At the same time, the sampling loop D is connected to tube C which is sealed to the vacuum system for evacuation and filling. Rotation of the stopcock through 90° isolates C and connects A and B via the sampling loop, flushing the contents of the latter into the gas chromatograph. An absolute volume calibration is obtained by filling the sample tube with mercury and weighing. The dead volume in the stopcock plug is irrelevant, since this will contain only carrier gas after operation of the valve.

The valve we have tested is constructed from 2-mm I.D. capillary tubing, and

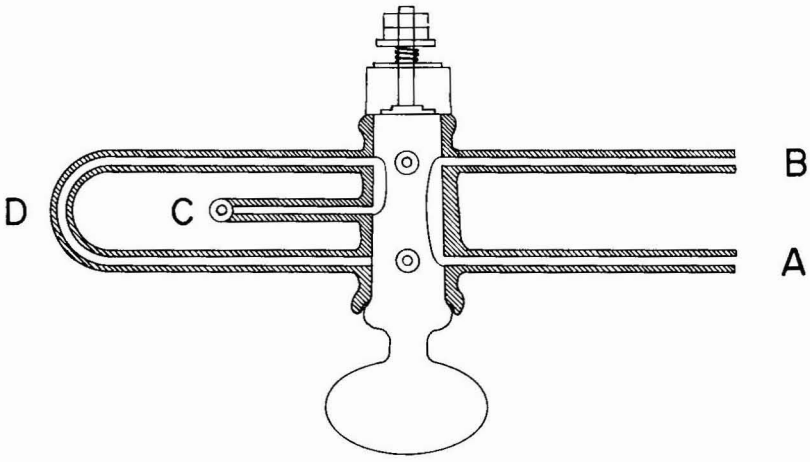


Fig. 1. Special stopcock. A, gas inlet; B, gas outlet; C, tube to vacuum system; D, sampling loop.

has a loop volume of 0.708 ml. We have tested the valve for reproducibility and linearity using various permanent gases and molecular sieve or silica gel columns in the chromatograph. Over the range of 10^{-6} to 10^{-5} moles injected, corresponding to sample pressures of 26 to 260 torr, reproducibility and linearity were within $3 \cdot 10^{-8}$ moles, which was within the uncertainty of our measurements of peak areas. The small peak caused by the pressure pulse on injection had an area corresponding typically to $4 \cdot 10^{-8}$ moles, which did not cause any significant errors.

While the present apparatus could not be used for experiments which preclude stopcock grease, it is simple, cheap and effective for those which do not. An upper limit to carrier gas pressure will be set by possible displacement of the plug at higher pressures. Using apiezon grease, and helium carrier pressures of 2.0 atm, we have not experienced any problems of this nature, or any peaks due to air leakage.

ACKNOWLEDGEMENT

We are indebted to Peter Hatch and Tony Cutteridge of the Simon Fraser University Glass Workshops for their skillful construction of the device.

Journal of Chromatography, 136 (1977) 308–310

CHROM. 9991

Note

Dynamic coating of glass capillaries with polar phases and Silanox*

R. G. McKEAG** and F. W. HOUGEN
Plant Science Department, University of Manitoba, Winnipeg, Manitoba R3T 2N2 (Canada)
(Received January 27th, 1977)

The potential benefits of glass capillary columns in gas chromatography have not been widely exploited, presumably because of technological problems and irreproducibility associated with their production. The nonuniformity and instability of liquid films, especially polar liquid films, when coated on smooth glass surfaces constitute a major limitation to high chromatographic efficiency[1]. To overcome these difficulties, many procedures have been developed for surface modification of the glass prior to coating. These include deposition of a layer of carbon[2] or of a polymer[3], gas phase etching[4], surface deactivation[5], and the formation of selective monomolecular layers[6].

German and Horning[7] have reported a simple two-step dynamic method of coating glass capillary columns using Silanox, a silanized silica powder. Their columns coated with SE-30 exhibited excellent thermostability and efficiency. Their method has been reported unsatisfactory, however, for the preparation of polar phase columns[8,9], as has also been our experience. Blakesley and Torline[9] have successfully produced polar columns by a modification of German and Horning's method[7], by coating Silanox on glass with a surface-active material. The use of surfactants, however, will change the retention and possibly the order of elution of compounds[10]. We wish to report the preparation of efficient polar-phase glass capillaries by a modification in the solvent system used in the German–Horning dynamic two-step coating procedure.

EXPERIMENTAL

Duran 50 tubing was cleaned prior to extrusion by immersion for 2 h in 4% aqueous NaOH saturated with potassium permanganate. The tubing was drained and successively rinsed with water, conc. HCl, water, and abs. methanol. After drying, the tubing was drawn with a Hupe-Busch 1045A glass drawing and coiling machine. From a 1.50-m Duran tube (7.0 mm O.D. × 5.0 mm I.D.), a 135-m capillary (1.0 mm O.D. × 0.62 mm I.D.) was obtained. Capillaries were coiled with a 12-cm diameter.

* Department of Plant Science contribution No. 471.

** Present address: The Griffith Laboratories Ltd., 757 Pharmacy Ave., Scarborough, Ontario M1L 3J8, Canada.

The coiled capillary tubing was evenly spaced and affixed to a support cylinder (1.25 m × 12 cm O.D.). With the cylinder erect, the top of the column was connected to a 30-ml reservoir and the bottom to a flow restrictor consisting of a 10-m length of tubing identical to that being coated. Connections were made with standard 1/16-in. unions. Strips of teflon tape were wound around the capillary tubing between the back ferrule and nut. When the nut was tightened, compression caused the teflon to flatten into a washer which sealed the connection.

Capillaries were coated by a dynamic two-step process. The solvent used in both steps of the coating process was chloroform–acetone (10:1). In the first step, 0.10 g stationary phase was dissolved in 10.0 ml solvent, 0.25 g Silanox (Grade 101) was added, and the suspension was sonicated for 2 min. A plug of this suspension (20% of column length), preceded by a plug of solvent (5% of column length), was passed through the tubing at 18 cm/sec. After the liquid had been expelled, the coating was dried with carrier gas flow for 10–12 h. In the second step, additional liquid phase was dynamically coated on the Silanox bed utilizing the same apparatus as in the first step. A plug (20% of column length) of a solution of 0.25 g stationary phase in 10.0 ml solvent was passed through the tubing at 12 cm/sec. When the main plug of liquid had been expelled, the gas pressure was reduced in stages to promote smooth flow of the viscous secondary plugs formed by solution draining from the column walls. After the last plugs of solution had been expelled, the column was dried with gas flow for 10–12 h.

The column was conditioned in the chromatographic oven by purging with carrier gas at a temperature programmed from 20 to 250° at 2°/min and maintained at 250° for 12 h. The column was then silylated at 200° with four injections of 2.5 μl Silyl-8 at 5-min intervals and maintained at 200° for an additional hour.

DISCUSSION

The chloroform–acetone solvent combination had adequate density to stabilize the Silanox suspension and sufficient polarity to prevent the formation of a thixotropic gel[11] during the coating process. Irregularities in the thickness of the deposited Silanox bed could be seen after the first coating step; these irregularities, however, were eliminated during the second coating step. The localized patches of excess Silanox were apparently washed away, leaving a uniform thin layer of Silanox, which was estimated by scanning electron microscopy to be 2.5 μm thick.

Columns prepared by this technique with the polar liquid phases Silar-5CP, OV-225, OV-210, and Carbowax 20M TPA, have shown excellent efficiency and thermostability. A 135-m Silar-5CP column (0.62 mm I.D.) yielded 151,000 theoretical plates and 122,000 effective plates, as tested with methyl linolenate at 175° column temperature and 4.0 ml/min helium gas flow. Only minimal column bleed was evident during gas chromatographic–mass spectrometric application. Columns have displayed high durability, having tolerated splitless injection of flavour volatiles and fatty acid methyl esters for 6 months before appreciable losses in efficiency have occurred. The same coating procedure has been used with success using the apolar liquid phase OV-101.

The solvent mixture reported in this work has extended the German–Horning procedure[7] for dynamic two-step coating of glass capillary columns to include the

use of polar liquid phases. Whether all polar phases can be used may depend on their solubility in the employed solvent mixture.

Caution: Chloroform–acetone mixtures may be explosive. In the presence of basic substances, a catalytic, highly exothermic, condensation reaction takes place[12].

ACKNOWLEDGEMENTS

This work was supported by the National Research Council of Canada and the Rapeseed Association of Canada. Samples of Silanox were provided by Cabot Carbon of Canada, Ltd.

REFERENCES

1 M. Novotný and A. Zlatkis, *Chromatogr. Rev.*, 14 (1971) 1.
2 K. Grob, *Helv. Chim. Acta*, 48 (1965) 1362.
3 A. L. Gordon, P. J. Taylor and F. W. Harris, *J. Chromatogr. Sci.*, 14 (1976) 428.
4 G. Alexander and G. A. F. M. Ruttan, *Chromatographia*, 6 (1973) 231.
5 L. Blomberg, *J. Chromatogr.*, 115 (1975) 365.
6 M. Novotný and K. Grohmann, *J. Chromatogr.*, 84 (1973) 167.
7 A. L. German and E. C. Horning, *J. Chromatogr. Sci.*, 11 (1973) 76.
8 S.-N. Lin, C. D. Pfaffenberger and E. C. Horning, *J. Chromatogr.*, 104 (1975) 319.
9 C. N. Blakesley and P. A. Torline, *J. Chromatogr.*, 105 (1975) 385.
10 W. G. Jennings, K. Yabumoto and R. H. Wohleb, *J. Chromatogr. Sci.*, 12 (1974) 344.
11 M. Blumer, *Anal. Chem.*, 45 (1973) 980.
12 H. K. King, *Chem. Ind. (London)*, (1970) 185.

Journal of Chromatography, 136 (1977) 311–317

CHROM. 9961

Note

SP 2340 in the glass capillary chromatography of fatty acid methyl esters

H. HECKERS, F. W. MELCHER and U. SCHLOEDER
Department of Internal Medicine, University Hospitals, Justus Liebig University, Giessen (G.F.R.)
(Received January 10th, 1977)

The development of the cyanopropylsiloxane stationary phases Silar 10C, Silar 9CP, SP 2340 and OV-275, which have high polarity and temperature resistance, made it possible to separate *cis/trans* isomeric fatty acids on packed columns[1]. Of course, a comparison of the retention data[2] indicated that some fatty acids which commonly occur in biological material show considerable overlapping in complex acid mixtures. However, gas–liquid chromatographic (GLC) analysis of these highly polar phases is useful when the complex sample is additionally analyzed on an EGA or DEGA packed column. Further, the cyanopropylsiloxanes gave small but constant differences in retention data[2] for many positional isomeric fatty acids on packed columns, suggesting that SP 2340 is as suitable as FFAP for the separation of fatty acid mixtures in glass capillary chromatography[4]. In this paper, we report on some GLC characteristics of a 30-m glass capillary column coated with SP 2340 used to re-evaluate the fatty acid composition of red cell phospholipids in young and old people[5].

EXPERIMENTAL

All of the standards of fatty acid methyl esters tested were more than 99% pure and were purchased from Nu-Check-Prep (Elysian, Minn. 56028, U.S.A.) and from Analabs (North Haven, Conn., U.S.A.). Octadecadienoate and octadecatrienoate mixtures containing the *cis/trans* isomers were prepared from pure linoleic and linolenic standards according to Litchfield and co-workers[6,7]. Pure standards of 9-*trans*-12-*cis*- and 9-*cis*-12-*trans*-octadecadienoic fatty acids were a generous gift from Th. Wieske, Union Deutsche Lebensmittelwerke (Hamburg, G.F.R.).

The quantitative extraction of lipids from human red cell ghosts, the separation of the pure phospholipid fraction[8] and the preparation of the fatty acid methyl esters derived from this lipid fraction were performed by a modification of the method of Dodge and Phillips[5], details of which are reported elsewhere[9]. The results of the fatty acid composition of the human red cell phospholipids are given in Table I as means ± standard deviation from 25 individuals of both sexes and of two age groups (twelve between 15 and 30 years of age and thirteen older than 70 years). All subjects were on a normal diet. The blood donors were free from haematological and liver diseases, had red cell counts and haematocrits in the normal range, and were not taking any drugs.

GC analysis

A Hewlett-Packard Model 5830 A gas chromatograph, equipped with a dual flame-ionization detector and an integrator, was used for all analyses. The chromatograph was fitted with a 30-m glass capillary column of 0.3 mm I.D., coated with the cyanopropylsiloxane SP 2340 of average molecular weight 2800. The oven temperature was programmed from 100° to 190°, heating for 5 min at the rate of 5°/min, followed by 0.5°/min up to 35.5 min and finally 1°/min up to the maximum temperature of 190°, which was maintained for a further 10 min before cooling. The recorded analysis time for the fatty acid mixture from red cell phospholipids was 95 min; longer runs could not rule out later peaks. The splitting ratio was 1:70, the column was operated with nitrogen as the carrier gas at a flow-rate of 2.5 ml/min and the injection and detector temperatures were 240°. The sample size was 0.1–0.2 μl (solutions in chloroform) and the peaks were identified by means of known standards. Additionally, samples were run a second time after catalytic hydrogenation over PtO_2 for identification purposes.

RESULTS AND DISCUSSION

In order to characterize the SP 2340 column performance, the following numerical values were established. Using *n*-hexadecanoic and *n*-heptadecanoic fatty acid methyl esters (recorder chart speed 3 cm/min), the separation number of the column was calculated with $n_{sep}{}^{*} = 7.76$ (mean value of three recordings) and $n_{sep}/m = 0.26$. The measurements were performed under the abovementioned temperature-programmed conditions. The separation number expressed as the number of theoretical plates gave $n^{*} = 7674$ or $n/m = 256$. The capacity ratio for decanoic acid methyl ester, measured under isothermal conditions at 90° with the methyl esters of (a) octanoic, (b) nonanoic and (c) decanoic acid (mean value of three runs) was $K^{*} = 4.39$. The loadability of the column for decanoic acid methyl ester could be calculated with $l^{*} = 1.80 \cdot 10^{-6}$ g.

Although the separation number and the specific separation number are low, probably indicating insufficient impregnation with stationary liquid, the separation efficiency for fatty acid methyl esters, especially for geometric isomeric acids and for positional isomers of acids, was much better than expected with the tested material. This aspect is demonstrated by the capillary gas–liquid chromatograms depicted in Figs. 1–3. All five pairs of geometric isomeric monoethylenic fatty acids and the all-*cis versus* all-*trans* components of octadecadiethylenic and octadecatriethylenic acids

* The following equations were used[10]:

$$n_{sep} = \frac{\Delta T}{b_{0.5} + \bar{b}_{0.5}} - 1$$

$$n = 100\,(n_{sep} + 1)^2$$

$$t_d = \frac{t_{dr(a)} \cdot t_{dr(c)} - t_{dr(b)}^2}{t_{dr(a)} + t_{dr(c)} - 2t_{dr(b)}}$$

$$K = \frac{t_s}{t_d}$$

$$l = 0.05\, M\, d^3 (1 + K) \cdot 10^{-6}\ \text{g}$$

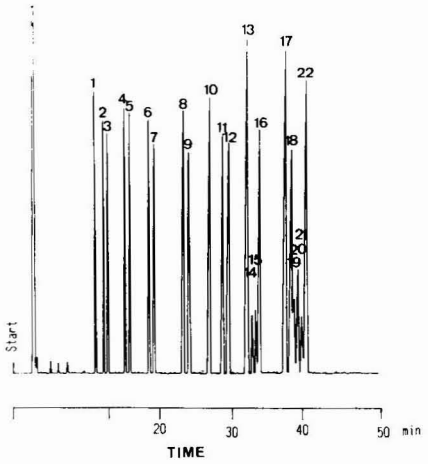


Fig. 1. Chromatogram of a mixture of five pairs of *cis/trans* isomeric monounsaturated fatty acids completed by the methyl esters derived from nitrous acid isomerized linoleic and linolenic acids. Peaks: 1 = C_{14}; 2 = $C_{14:1tr\omega5}$; 3 = $C_{14:1c\omega5}$; 4 = $C_{15:1tr\omega5}$; 5 = $C_{15:1c\omega5}$; 6 = $C_{16:1tr\omega7}$; 7 = $C_{16:1c\omega7}$; 8 = $C_{17:1tr\omega7}$; 9 = $C_{17:1c\omega7}$; 10 = C_{18}; 11 = $C_{18:1tr\omega9}$; 12 = $C_{18:1c\omega9}$; 13 = $C_{18:2tr,tr\omega6}$; 14 = $C_{18:2c,tr\omega6}$; 15 = $C_{18:2tr,c\omega6}$; 16 = $C_{18:2c,c\omega6}$; 17 = $C_{18:3tr,tr,tr\omega3}$; 18, 19, 20, 21 = mixed geometric isomers of $C_{18:3\omega3}$ (individual peaks were not definitely identified); 22 = $C_{18:3c,c,c\omega3}$. The composition of the diethylenic and triethylenic acid mixtures corresponds to the final products resulting from the nitrous acid isomerization, with the exception of the individual all-*cis* components, which were added, because they occurred only in minimal amounts. Operating conditions were identical for all presented fractograms. For details see *GC analysis*.

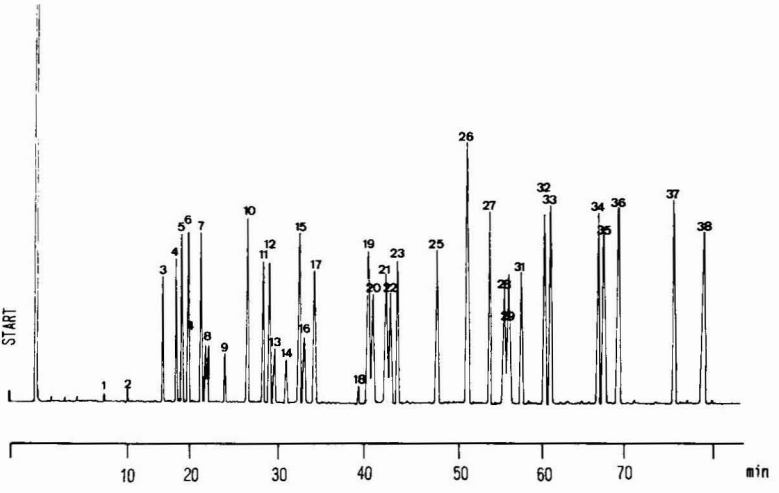


Fig. 2. Capillary gas chromatogram of a standard mixture of fatty acid methyl esters on an SP 2340 capillary column. Peaks: 1 = C_{12}; 2 = C_{14}; 3 = C_{16}; 4 = $C_{16:1tr\omega7}$; 5 = $C_{16:1c\omega7}$; 6 = anteiso-C_{17}; 7 = C_{17}; 8 = phytanic acid; 9 = iso-C_{18}; 10 = C_{18}; 11 = $C_{18:1tr\omega9}$; 12 = $C_{18:1c\omega9}$; 13 = $C_{18:1c\omega7}$; 14 = anteiso-C_{19}; 15 = $C_{18:2tr,tr\omega6}$; 16 = C_{19}; 17 = $C_{18:2c,c\omega6}$; 18 = $C_{18:3tr,tr,tr\omega3}$; 19 = C_{20}; 20 = $C_{18:3c,c,c\omega3}$; 21 = $C_{20:1c\omega12}$; 22 = $C_{20:1c\omega9}$; 23 = $C_{20:1c\omega7}$; 25 = $C_{20:2c,c\omega6}$; 26 = $C_{20:3c,c,c\omega6}$; 27 = $C_{20:4c,c,c,c\omega6}$ + C_{22} + $C_{20:3c,c,c\omega3}$; 28 = $C_{22:1tr\omega9}$; 29 = $C_{22:1c\omega9}$; 31 = $C_{22:2c,c\omega9}$; 32 = $C_{20:5all\text{-}cis\omega3}$; 33 = $C_{22:2c,c\omega6}$; 34 = $C_{22:3c,c,c\omega3}$ + C_{24}; 35 = $C_{22:4c,c,c,c\omega6}$; 36 = $C_{24:1c\omega9}$; 37 = $C_{22:6all\text{-}cis\omega3}$; 38 = C_{26}. For operating conditions see *GC analysis*.

TABLE I

FATTY ACID COMPOSITION OF TOTAL PHOSPHOLIPID FROM RED CELL GHOSTS OF YOUNG (LESS THAN 30 YEARS) AND OLD (MORE THAN 70 YEARS) SUBJECTS

Values (area %) are given as means ± standard deviation. Numbers in parentheses correspond to the numbers in Fig. 3.

Component	*Young subjects*	*Old subjects*	*Component*	*Young subjects*	*Old subjects*
C_8	(1) tr*	tr		0.02 ± 0.01	0.02 ± 0.02
C_{10}	(2) 0.03 ± 0.03	0.06 ± 0.04		0.02 ± 0.01	0.03 ± 0.01
C_{12}	(3) 0.36 ± 0.43	0.34 ± 0.53		(33) 0.07 ± 0.04	0.11 ± 0.15
C_{14}	(4) 1.33 ± 1.14	1.31 ± 0.92	C_{20}	(34) 0.48 ± 0.11	0.50 ± 0.14
	0.22 ± 0.33	0.22 ± 0.25	$C_{18:3c,c,c\omega3}$	(35) 0.26 ± 0.38	0.25 ± 0.29
	0.14 ± 0.13	0.35 ± 0.24		(36) 0.11 ± 0.11	0.10 ± 0.09
	(5) 0.07 ± 0.05	0.08 ± 0.06	$C_{20:1c\omega9}$	(37) 0.35 ± 0.16	0.39 ± 0.26
$C_{14:1tr\omega5}$	(6) 0.19 ± 0.23	0.14 ± 0.10	$C_{20:1c\omega7}$	(38) 0.02 ± 0.01	0.03 ± 0.03
$C_{14:1c\omega5}$	(7) 0.13 ± 0.15	0.12 ± 0.08		(39) 0.14 ± 0.23	0.34 ± 0.48
C_{15}	(8) 0.43 ± 0.35	0.39 ± 0.18		(40) 0.19 ± 0.22	0.16 ± 0.10
iso-C_{16} + $C_{15:1tr\omega5}$	(9) 0.16 ± 0.20	0.14 ± 0.13	$C_{20:2c,c\omega6}$	(41) 0.15 ± 0.07	0.11 ± 0.05
anteiso-C_{16}	(10) 0.06 ± 0.04	0.12 ± 0.12		(42) 0.03 ± 0.03	0.04 ± 0.04
C_{16}	(11) 23.16 ± 2.44	22.56 ± 3.53		(43) 0.03 ± 0.02	0.04 ± 0.05
	(12) 0.04 ± 0.03	0.19 ± 0.27	$C_{20:3c,c,c\omega6}$	(44) 0.95 ± 0.35	0.85 ± 0.39
	(13) 0.07 ± 0.08	0.16 ± 0.16		(45) 0.06 ± 0.08	0.16 ± 0.15
$C_{16:1tr\omega7}$	(14) 1.04 ± 1.32	0.67 ± 0.48	$C_{20:4\omega6}$*** + C_{22} + $C_{20:3\omega3}$***	(46) 10.53 ± 3.51	9.08 ± 2.95
$C_{16:1c\omega7}$**	(15) 0.47 ± 0.20	0.68 ± 0.29		(47) 0.03 ± 0.02	0.03 ± 0.02
anteiso-C_{17}	(16) 0.12 ± 0.10	0.16 ± 0.10		(48) 0.03 ± 0.01	0.03 ± 0.02
	0.02 ± 0.02	0.02 ± 0.01	$C_{22:1c\omega9}$	(49) 0.06 ± 0.04	0.12 ± 0.10
C_{17}	(17) 0.56 ± 0.06	0.62 ± 0.13		(50) 0.06 ± 0.09	0.04 ± 0.04
Phytanic acid	tr	tr	$C_{20:5\omega3}$***	(51) 0.57 ± 0.26	0.58 ± 0.32
	(18) 0.07 ± 0.10	0.09 ± 0.18		(52) 0.04 ± 0.02	0.11 ± 0.25
$C_{17:1tr\omega7}$	(19) Internal standard			(53) 0.07 ± 0.03	0.96 ± 1.33
$C_{17:1c\omega7}$	(20) tr	tr		(54) 0.08 ± 0.11	0.28 ± 0.32
iso-C_{18}	tr	tr		(55) 0.04 ± 0.06	0.12 ± 0.21
	(21) 0.06 ± 0.05	0.11 ± 0.11		(56) 0.08 ± 0.12	0.22 ± 0.19
	(22) 0.07 ± 0.06	0.08 ± 0.04	$C_{22:3\omega3}$*** + C_{24}	(57) 5.15 ± 1.65	5.15 ± 1.81
C_{18}	(23) 15.16 ± 1.96	14.23 ± 1.18	$C_{22:4\omega6}$***	(58) 2.20 ± 0.60	1.65 ± 0.77
$C_{18:1tr\omega6-12}$	(24) 0.38 ± 0.28	0.75 ± 0.42	$C_{24:1\omega9}$***	(59) 5.55 ± 1.90	6.40 ± 2.29
$C_{18:1c\omega9}$	(25) 12.73 ± 2.79	14.61 ± 2.86		(60) 0.39 ± 0.19	0.37 ± 0.20
$C_{18:1c\omega7}$	(26) 0.92 ± 0.32	1.12 ± 0.26		(61) 0.03 ± 0.01	0.04 ± 0.04
	(27) 0.10 ± 0.04	0.13 ± 0.03		(62) 0.04 ± 0.02	0.09 ± 0.21
	(28) 0.07 ± 0.08	0.06 ± 0.05	$C_{22:5\omega3}$***	(63) 1.80 ± 0.82	1.65 ± 0.77
$C_{18:2tr,tr\omega6}$	tr	tr	$C_{22:6\omega3}$***	(64) 2.30 ± 1.31	1.99 ± 1.01
C_{19}	(29) 0.13 ± 0.21	0.21 ± 0.38		(65) 0.23 ± 0.11	0.28 ± 0.12
$C_{18:2c,c\omega6}$	(30) 9.13 ± 2.87	7.75 ± 4.41		(66) 0.14 ± 0.05	0.17 ± 0.07
	(31) 0.40 ± 0.31	0.25 ± 0.15		(67) 0.03 ± 0.02	0.15 ± 0.17
	(32) 0.04 ± 0.07	0.09 ± 0.09			

* tr (trace) means less than 0.01% of total acids.

** This peak contains an artifact of BHT, *ca.* 20–30% (see Fig. 4).

*** All double bonds are in a *cis* configuration.

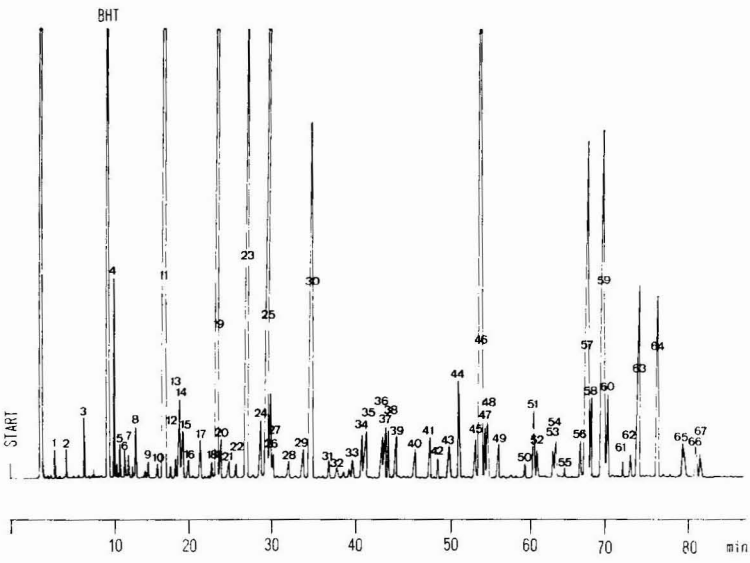


Fig. 3. Typical GLC pattern of human red cell total phospholipid (glycolipids separated) fatty acid methyl esters including the antioxidant BHT and $C_{17:1tr\omega7}$ as internal standard. Peak numbers correspond to the numbers in Table I.

show a separation of more than 4.6 σ, thus being very suitable for correct automatic integration (Fig. 1). Whereas $C_{18:2c,tr\omega6}$ and $C_{18:2tr,c\omega6}$ show more than baseline separation, the latter peak overlapped somewhat with that of elaidolinoleic acid. The GLC elution pattern of the genuine nitrous acid isomerization mixture of linolenic acid showed seven peaks out of the eight theoretically possible isomerization components. After addition of a pure linolenic acid standard, this substance occurred in the true isomerization mixture in only spurious amounts, the latest eluted combined *cis/trans* isomeric compound becoming apparent only as a slight shoulder on the all-*cis*-octadecatriethylenic acid peak (Fig. 1).

As was assumed from the characteristics of the cyanopropylsiloxane stationary phase in GLC on packed columns[2], the SP 2340 capillary column made it possible to separate even positional isomeric acids. Consequently, $C_{18:1}$ fatty acid originating from isolated human red cell phospholipids gave three peaks (Fig. 3), which could be identified by means of known standards as elaidic (or isoelaidic), oleic and *cis*-vaccenic acids (Fig. 2) when enumerated in their elution order. A further *cis*-isooleic acid peak (see peak 27 in Fig. 3) possibly occurs in the biological material, but we had no standard ($C_{18:1c\omega5}$) for definite identification. Figs. 2 and 3 further outline sufficient or baseline separations for the all-*cis* isomers of $C_{18:3}$ ($\omega6$; $\omega3$), $C_{20:1}$ ($\omega12$; $\omega9$; $\omega7$), $C_{20:3}$ ($\omega6$; $\omega3$) and $C_{22:2}$ ($\omega9$; $\omega6$). Unfortunately, arachidonic acid eluted together with behenic and Δ17-eicosatrienoic acids, and Δ19-docosatrienoic acid had almost the same retention time as lignoceric acid. As individual components were recognizable by small shoulders in all of these combined peaks, we believe that it will be possible in future to separate these individual compounds on a 50-m Silar 10C or SP 2340 coated capillary column with a normal, *i.e.*, higher separation number than on our column. It is worth mentioning that the commercially available phytanic acid standard

gave two peaks with poor resolution, providing evidence of an unidentified phytanic acid impurity[3]. Identical behaviour of phytanic acid was observed on a 50-m FFAP capillary column[4] which, although characterized by a high separation number and consequently many more theoretical plates than ours, did not give a better overall column performance for the resolution of isomeric fatty acids in complex mixtures.

The complexity that can occur in biologically derived fatty acids is best demonstrated for crude human red cell phospholipids in Fig. 3. The numbers on top of the peaks are repeated and as far as possible identified in Table I, which gives the quantitative results derived from young people in comparison with those from old people. Further identification requires a combination of GLC with mass spectrometry. To avoid autoxidation during the analytical procedure, the antioxidant BHT (butylated hydroxytoluene; 2,6-di-*tert.*-butyl-*p*-cresol) was added. The capillary chromatogram in Fig. 4 demonstrates an artifact[5] produced by the permethylation procedure with a retention time identical with that of $C_{16:1c\omega7}$. As the artifact comprises 1.6 area-% of the BHT standard, we were able to calculate that the mean *cis*-hexadecenoic acid concentration was increased by 20–30%. Re-evaluating the work of Dodge and Phillips[5], we found 67 different peaks of greater quantitative importance, plus many additional acids in smaller amounts. This is more than double the number of peaks known hitherto and demonstrates the efficiency of our SP 2340 column.

Provided that the technical problems encountered in coating capillaries with SP 2340 or Silar 10C, even by very experienced manufacturers, can be satisfactorily

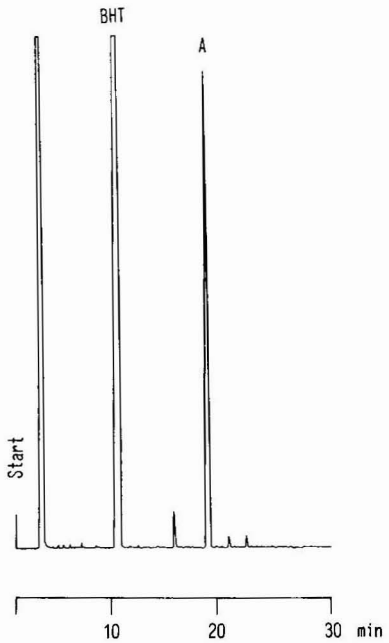


Fig. 4. Chromatogram of the antioxidant BHT following the boron trifluoride methylation procedure[11]. The additional peak A demonstrates an artifact produced by the methylation procedure. The artifact comprises 1.6 area% of the chromatogram. The remaining minute peaks were present in commercial BHT (Merck, Darmstadt, G.F.R.). The retention time for peak A was found to be identical with $C_{16:1c\omega7}$.

solved in the future, we believe that such columns will be highly selective for the separation of many types of isomeric fatty acids. Such high-performance capillary columns will probably obviate the laborious and time-consuming ozonization technique for the evaluation of the positions of double bonds in fatty acid analysis.

ACKNOWLEDGEMENT

This work was supported by the Deutsche Forschungsgemeinschaft.

LIST OF SYMBOLS

n_{sep} = separation number, *i.e.* separated peaks within the one carbon number range of C_{16} and C_{17}
ΔT = total retention time of C_{17} − total retention time of C_{16}
$b_{0.5}$ = peak width at half height
t_d = (gas) hold-up time
t_s = adjusted retention time
K = capacity ratio
l = loadability
M = molecular weight of decanoic acid methyl ester
d = inner diameter of capillary column

REFERENCES

1 *Gas-Chrom Newsl., Vol. 14, No. 5*, Applied Science Labs., State College, Pa., 1973.
2 H. Heckers, K. Dittmar, F. W. Melcher and H. O. Kalinowski, *J. Chromatogr.*, 135 (1977) 93.
3 R. P. Hansen, *J. Dairy Res.*, 33 (1966) 333.
4 H. Jaeger, H. W. Klör, G. Blos and H. Ditschuneit, in R. E. Kaiser (Editor), *Glass Capillary Chromatography*, Institut für Chromatographie, Bad Dürkheim, 1975, p. 255.
5 J. T. Dodge and G. B. Phillips, *J. Lipid Res.*, 8 (1967) 667.
6 C. Litchfield, J. E. Lord, A. F. Isbell and R. Reiser, *J. Amer. Oil Chem. Soc.*, 40 (1963) 553.
7 R. D. Harlow, C. Litchfield and R. Reiser, *J. Amer. Oil Chem. Soc.*, 40 (1963) 505.
8 D. E. Vance and C. C. Sweeley, *J. Lipid Res.*, 8 (1967) 621.
9 H. Heckers, H. Hegner, D. Platt and U. Schloeder, *Mech. Ageing Dev.*, submitted for publication.
10 R. Kaiser, in R. Kaiser (Editor), *Chromatographie in der Gasphase*, Bibliographisches Institut, Mannheim, Vienna, Zürich, 1973, p. 22.
11 W. R. Morrison and L. M. Smith, *J. Lipid Res.*, 5 (1964) 600.

Journal of Chromatography, 136 (1977) 318–322

CHROM. 9978

Note

Gas chromatographic determination of cyclic amines, ketones and alcohols, possible metabolites of sweet sulphamates

G. ANTHONY BENSON and WILLIAM J. SPILLANE
Chemistry Department, University College, Galway (Ireland)
(Received January 20th, 1977)

Cyclamate was first discovered to be metabolized to cyclohexylamine in animals by Kojima and Ichibagase[1]. The cyclohexylamine in the urine was determined initially using thin-layer[1] and visible spectrophotometry[2]. These workers subsequently developed a gas chromatographic (GC) procedure for the determination of cyclohexylamine in the urine of rats which were fed cyclamate[3]. Two other metabolites namely cyclohexanol and cyclohexanone were subsequently identified in the urine of rats[4,5].

We were interested in studying the metabolic products (if any) of certain sweet-tasting cyclic and aliphatic sulphamates similar to cyclamate. This necessitated the development of an analytical procedure to identify and quantitatively determine amines, ketones and alcohols, possible metabolites which could arise due to metabolic breakdown of these sulphamates. The procedure of Kojima and Ichibagase[3] which is summarized in Fig. 1 was considered to be tedious and time consuming and we considered it desirable to have GC conditions and suitable column material which would allow the simultaneous separation of amine, ketone and alcohol from a single injection.

GC determination of amines has been a problem in the past due to strong adsorption of the compounds on the column resulting in badly tailed elution peaks. Modification of column materials by various groups of workers with sodium and potassium hydroxides has resulted in excellent peak shapes and resolution of aliphatic amines[6–8]. O'Donnell and Mann[9] have compared a Dowfax 9N9 column with 2.5% sodium hydroxide as partition liquid against Carbowax 400 and 20M columns for the separation of a series of aliphatic amines. They concluded that the Dowfax 9N9–NaOH column is the most effective for separating a wide range of basic organic compounds. Further Derse and Daun[10] have determined cyclamate by first converting it to cyclohexylamine, this amine was then estimated by GC using 20% Dowfax 9N9 and 2.5% NaOH on 60–80 mesh Gas-Chrom R.

This paper describes a GC separation on a Dowfax 9N9 with 2.5% NaOH on 60–80 mesh Diatomite C AW of aliphatic and cyclic amines from their corresponding ketones and alcohols and a procedure for the microdetermination of cyclic amines, ketones and alcohols in the urine of rats.

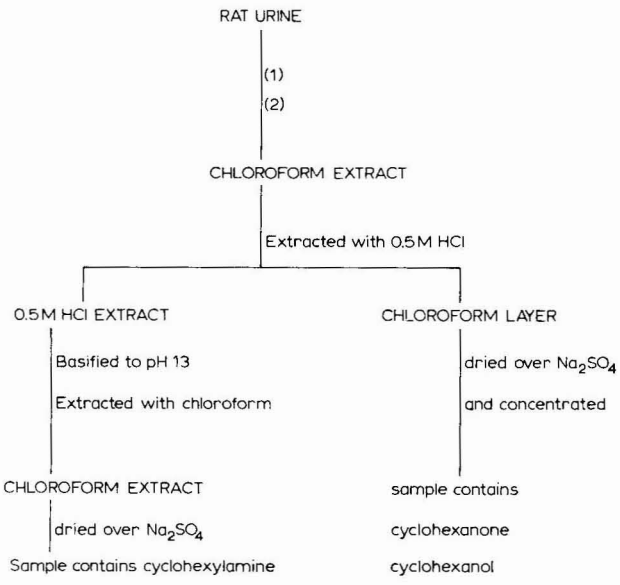


Fig. 1. Scheme for the separation of cyclohexylamine and cyclohexanone–cyclohexanol from urine according to Kojima and Ichibagase.

EXPERIMENTAL AND RESULTS

Chemicals and reagents

All amines, ketones and alcohols (supplied by Aldrich, Milwaukee, Wisc., U.S.A.) were distilled before use. *n*-Nonane, *n*-decane, *n*-dodecane and *n*-tetradecane (BDH, Poole, Great Britain) were used as obtained. Dichloromethane and sulphosalicylic acid (Analar; May & Baker, Dagenham, Great Britain) were used as obtained.

Rats and metabolism cages

Female Wistar albino rats were supplied by Trinity College (Dublin, Ireland) and were housed in individual metabolism cages supplied by Bowman Accessories (London, Great Britain). The urine from these animals was collected daily and refrigerated prior to being used.

Gas chromatograph and column

Chromatographic measurements were made on a Pye-Unicam 104 gas chromatograph with dual flame ionization detectors. The column was 5 ft. $\times$ $\frac{1}{4}$ in. glass. The column packing used in all measurements was 20% Dowfax 9N9 with 2.5% NaOH on 60–80 mesh Diatomite C AW.

Operating conditions for the separation of a cyclic or aliphatic amine from its corresponding ketone or alcohol

Table I summarizes the operating conditions and retention times for the separation of aliphatic and cyclic amines from their corresponding ketones or alcohols. The internal standard present in each separation was at a concentration of 0.02–

TABLE I

OPERATING CONDITIONS AND RETENTION TIMES FOR THE SEPARATION OF ALKYL AND CYCLIC AMINES, KETONES AND ALCOHOLS EXTRACTED FROM URINE USING A DOWFAX 9N9–2.5% NaOH ON 60–80 MESH DIATOMITE C AW COLUMN

Group	*Temp. (°C)*	*Nitrogen flow-rate (ml/min)*	*Retention time (min)*			*Internal Standard**
			Amine	*Ketone*	*Alcohol*	
Cyclopentyl	120	30	4.2	6.2	9.2	*n*-Dodecane (12.2)
Cyclohexyl	125	60	3.2	5.2	7.2	*n*-Tetradecane (16.4)
Cycloheptyl	130	75	6.3	8.4	13.0	*n*-Dodecane (4.6)
Cyclooctyl	138	75	8.4	9.5	17.0	*n*-Dodecane (3.2)
Cyclopentyl-methyl	120	60	4.8	–	12.4	*n*-Dodecane (7.8)
Isobutyl**	75	21	6.0	–	21.6	*n*-Nonane (13.0)
Isoamyl**	92	20	7.2	–	20.4	*n*-Decane (13.0)

* The number in parentheses is the retention time (min) for the internal standard.
** Determined in dichloromethane only.

0.04 ml per 250 ml dichloromethane. Separations were carried out isothermally and the carrier gas used was nitrogen.

Analytical procedure for the determination of cyclic amines, ketones and alcohols in rat urine

Calibration graphs were prepared for each of the separations outlined in Table I. A plot of peak height ratios (amine, ketone or alcohol/internal standard) *vs.* concentration, was linear over the range 0.006–0.2 mg/ml for each compound.

The calibration curves for the determination of cyclopentylamine, cyclopentanone and cyclopentanol are shown in Fig. 2. The analytical procedure employed for the setting up of the calibration graphs was as follows. Microliter quantities of cyclopentylamine, cyclopentanone and cyclopentanol were injected into 5-ml samples of rat urine. 5 ml of 20% sulphosalicylic acid (w/v) were added and the pH of the solution was adjusted to 12–13 with 1 ml of 10 *M* NaOH. Samples (2 ml) were then

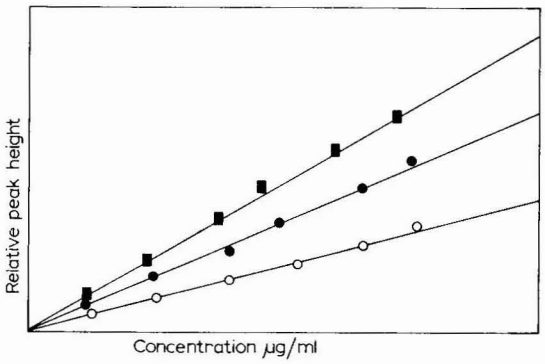


Fig. 2. Determination of cyclopentylamine (■), cyclopentanone (●) and cyclopentanol (○) by plotting relative peak heights (peak height of amine, ketone or alcohol divided by the peak height of *n*-dodecane) against concentration.

taken and extracted with 2 × 3 ml of dichloromethane (containing 0.04 ml *n*-dodecane per 250 ml dichloromethane). The samples were then centrifuged and 2-ml portions were transferred to 5-ml volumetric flasks containing approximately 2–4 mg of anhydrous sodium sulphate and 0.5 μl was injected on to the column. A similar procedure was employed to set up calibration curves for the other separations outlined in Table I.

For the purposes of estimating a percent recovery from urine cycloheptyl compounds were used and a calibration curve was prepared according to the procedure described above for the cyclopentyl compounds. The percent recoveries obtained for varying amounts of cycloheptylamine, cycloheptanone and cycloheptanol are given in Table II.

TABLE II

PER CENT RECOVERY OF CYCLOHEPTYLAMINE, CYCLOHEPTANONE AND CYCLOHEPTANOL FROM URINE

— — Not determined.

Amine		*Ketone*		*Alcohol*	
μg	%	μg	%	μg	%
60.9	92.69	67.8	90.41	67.5	96.17
121.3	90.90	135.6	98.01	135.0	96.03
151.3	101.33	175.3	87.84	166.2	93.86
225.0	105.87	254.3	101.08	249.0	106.49
303.6	94.90	338.7	97.77	333.0	111.11
456.1	109.25	508.1	115.55	499.8	115.55
874.8	104.2	950.7	99.97	947.8	—
874.8	99.4	950.7	96.97	947.8	97.6
1020.6	103.4	1109.2	98.44	1105.8	84.5
1020.6	98.7	1109.2	99.52	1105.8	—
1166.4	94.6	1267.6	95.42	1263.2	94.9
1166.4	94.6	1267.6	104.91	1263.2	—
Mean ± S.E.	99.15 ± 4.75		98.82 ± 4.48		99.57 ± 7.64

DISCUSSION

Kojima and Ichibagase were the first workers to discover that the artificial sweetener cyclamate was metabolically cleaved in animals and humans. Following a series of further tests both for metabolites and toxicological effects the artificial sweetener was banned in 1969. We have been interested in other cyclic and aliphatic sulphamates, which are sweet tasting, from a structure–activity correlation point of view[11]. It occurred to us that if the structure of the sweet sulphamate was altered, on feeding it to animals its metabolic pattern might be different to that of cyclamate. The procedures outlined, and summarized in Table I, show that three metabolites can be determined directly by one injection on to the column in a very convenient time. Excellent calibration curves (see Fig. 2) and percentage recovery (see Table II) from urine were obtained. Fig. 3 shows the separations which were obtained for the cyclopentyl group.

n-Isoamyl and *n*-isobutyl sulphamates are sweet tasting compounds. Table I

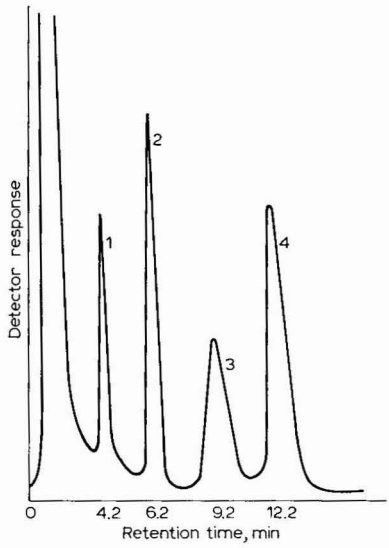


Fig. 3. Chromatographic separation of the urine metabolites of cyclopentyl sulphamate. Peaks: 1 = cyclopentylamine; 2 = cyclopentanone; 3 = cyclopentanol; 4 = *n*-dodecane (internal standard).

shows that we were able to separate the corresponding amines and ketones. However we failed to extract these compounds from rat urine quantitatively. The solvent was changed from dichloromethane to dimethyl ether but the extraction again failed. Work is continuing to obtain a satisfactory method for extracting aliphatic amines and alcohols from rat urine in order that the metabolism of *n*-isoamyl and *n*-isobutyl sulphamates can be studied.

ACKNOWLEDGEMENT

Thanks are due to the Chemical Society, London, and the Van't Hoff Fund, Amsterdam, for research grants. One of us (G.A.B.) thanks the Department of Education, Dublin, for a State Maintenance grant.

REFERENCES

1 S. Kojima and H. Ichibagase, *Chem. Pharm. Bull.*, 14 (1966) 971.
2 S. Kojima and H. Ichibagase, *Yakugaku Zasshi*, 83 (1963) 1108.
3 S. Kojima and H. Ichibagase, *Chem. Pharm. Bull.*, 16 (1968) 1851.
4 A. G. Renwick and R. T. Williams, *Biochem. J.*, 129 (1972) 857.
5 M. Asahina, T. Yamaha, K. Watanabe and G. Sarrazin, *Chem. Pharm. Bull.*, 19 (1971) 628.
6 J. R. Lindsay Smith and D. J. Waddington, *Anal. Chem.*, 40 (1968) 522.
7 A. Di Corcia and R. Samperi, *Anal. Chem.*, 46 (1974) 977.
8 S. R. Dunn, M. L. Simenhoff and L. G. Wesson, Jr., *Anal. Chem.*, 48 (1976) 41.
9 J. F. O'Donnell and C. K. Mann, *Anal. Chem.*, 36 (1964) 2097.
10 P. H. Derse and R. J. Daun, *J. Ass. Offic. Agr. Chem.*, 49 (1966) 1098.
11 G. A. Benson and W. J. Spillane, *J. Med. Chem.*, 19 (1976) 869.

Journal of Chromatography, 136 (1977) 323–327

CHROM. 9958

Note

Gas chromatographic retention characteristics of ω-alicyclic fatty acids*

TOSHI KANEDA

Research Council of Alberta 11315 - 87th Avenue, Edmonton, Alberta T6G 2C2 (Canada)

(Received January 5th, 1977)

Gas–liquid chromatography has been used extensively for the tentative identification of fatty acids prior to their confirmatory identification by mass spectrometry. In most instances the retention time can conveniently be measured, but the net retention volume adjusted for the air peak is the basic parameter. Although the net retention can be expressed in several ways, the equivalent chain length is perhaps the most useful parameter to use for identification purposes. Identification is usually accomplished by comparing the equivalent chain lengths of the unknown fatty acid obtained on two columns of different polarities with those of an authentic sample. Even without an authentic sample, the differences between the two equivalent chain lengths of the unknown compound permit the prediction of the presence or absence of unsaturation and, if present, the number of unsaturated bonds in the fatty acid molecules[1,2]. This approach has been used routinely in this laboratory for the identification of bacterial fatty acids with 13–18 carbon atoms.

It recently became necessary to identify various ω-alicyclic fatty acids synthesized by a bacterium. The behavior of these acids in gas chromatographic analysis is significantly different from that of normal or methyl-substituted acids. This paper reports findings obtained during the identification of alicyclic acids on the basis of their equivalent chain lengths.

EXPERIMENTAL

Fatty acids

Methyl esters of lauric, myristic, palmitic and stearic acids were purchased from Applied Science Labs. (State College, Pa., U.S.A.). Bacterial fatty acids were prepared from cells of *Bacillus subtilis* (ATCC 7059) grown on a culture medium containing glucose (1%), yeast extract (0.1%) and inorganic nutrients as described previously[3]. The fatty acids have previously been identified belonging to the *iso* series (*iso*-C_{14}, -C_{15}, -C_{16} and -C_{17}), *anteiso* series (*anteiso*-C_{15} and -C_{17}) and normal series (*n*-C_{14} and -C_{16}) by their physical and chemical properties, as well as by gas–liquid chromatography and mass spectrometry.

ω-Alicyclic fatty acids were isolated from the cells of *B. subtilis* grown on glucose–yeast extract medium to which one of four cyclic acid substrates (cyclopropyl,

* Contribution No. 798 from the Research Council of Alberta, Edmonton, Canada.

cyclobutyl, cyclopentyl and cyclohexyl acids) was added. The ω-alicyclic fatty acids with 14–18 carbon atoms cyclo-3-C_{14} (ω-cyclopropylundecanoic acid), cyclo-3-C_{16}, cyclo-4-C_{15}, cyclo-4-C_{17}, cyclo-5-C_{16}, cyclo-5-C_{18}, cyclo-6-C_{15}, and cyclo-6-C_{17}, were identified by gas–liquid chromatography and mass spectrometry[4].

Esterification

Fatty acids were esterified by diazomethane in diethyl ether[5].

Gas–liquid chromatography

The gas chromatograph used was a Hewlett-Packard Model 5830A equipped with a dual hydrogen flame-ionization detector. The reproducibility of temperature programming judged by the measured retention time of a given fatty acid sample, was found to be within 2%.

Three columns were used: one was a support-coated open-tubular (SCOT) column with an ethylene glycol adipate (EGA) polymer coating (50 ft. × 0.02 in.) purchased from Perkin-Elmer (Norwalk, Conn., U.S.A.) and the others were 6 ft. × $\frac{1}{8}$ in. stainless-steel tubes filled with either 2% Silar 5CP or 2.5% SE-30 on Gas-Chrom G AW DMCS (Applied Science Labs.).

The SCOT column was operated isothermally at 180° or 190° with a carrier gas (helium) flow-rate of 3 ml/min. The other packed columns were maintained at 120° for 2 min and then the temperature was increased at the rate of 2°/min up to 240°. The flow-rate of the carrier gas (helium) was 13 ml/min.

RESULTS AND DISCUSSION

Fig. 1 shows retention times on the EGA column at 180°, plotted on a logarithmic scale against total carbon number for seven series of fatty acids. All of these plots have identical slopes. The equivalent chain length (ECL) of an acid is determined by finding the point on the normal series line corresponding to the measured retention time for that acid. The corresponding ECL are listed in Table I. The ΔECL value is the difference between the actual total number of carbon atoms of the fatty acid and its ECL. Because the lines in Fig. 1 are parallel, ΔECL is constant for a given series and, once known for one member, can be assumed for any other member. For example, ΔECL for cyclo-3-C_{14} acid is +0.97. Hence the equivalent chain length of cyclo-3-C_{16} acid is expected to be 16.97, which is almost identical with the measured value of 17.00. ECLs measured at 190° were identical with the listed values obtained at 180° (maximum deviation 0.03, except for *iso*-C_{14}, which gave a deviation of 0.09).

The retention times of the fatty acids were also measured under programmed temperature conditions. Plots of retention time (on a linear scale) against total number of carbon atoms for the seven series of fatty acids gave parallel straight lines for the SE-30 column. On the Silar 5CP column under the same conditions, however, the normal, *iso*, and *anteiso* series gave parallel straight lines, whereas the plots of the four ω-alicyclic series showed a larger and parallel slope. Under the conditions used here, the difference in slopes between the two groups corresponded to 0.17 ECL unit per 10°. This difference is small enough that, to a first approximation and over the limited range of carbon numbers of interest, ΔECL can be considered to be constant (see Table I). (Table I also includes literature values for three alicyclic C_{15}

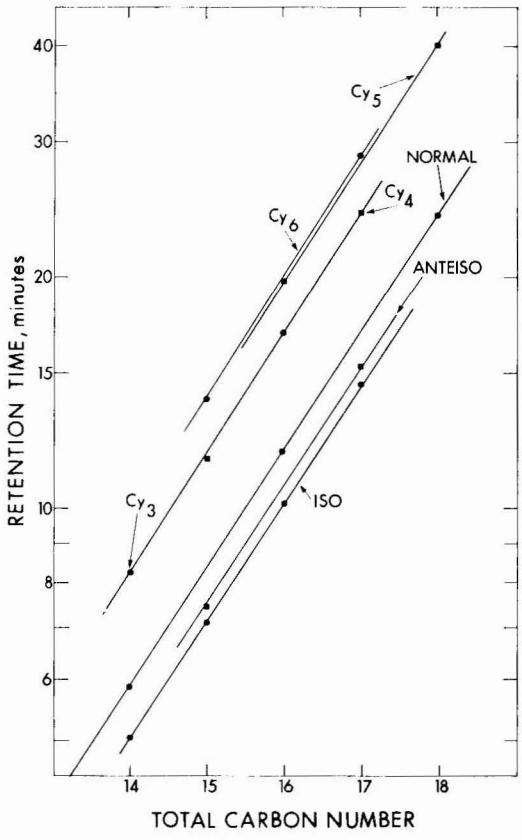


Fig. 1. Retention times of methyl esters of six series of fatty acids in relation to number of carbon atoms in the fatty acids (including the carboxyl carbon). The abbreviations used are: NORMAL, ANTEISO, ISO, Cy_3, Cy_4, Cy_5 and Cy_6 for normal, *anteiso*, *iso*, ω-cyclopropyl, ω-cyclobutyl, ω-cyclopentyl and ω-cyclohexyl acids, respectively.

acids from isothermal runs on columns of similar characteristics[6] and which agree fairly well with the values found in the present work). Attempts were made to measure ECLs on a 6-ft. packed EGA (7%) column under the same conditions of temperature programming but the maximum operational temperature of the column (190°) was not high enough for such analyses to be completed.

The average ΔECL values are listed at the bottom of Table I. It can be seen that all ΔECL values of the methyl branched series (*iso* and *anteiso*) are negative, whereas all ΔECL values of the ω-alicyclic series are positive. Thus, with respect to gas chromatographic retention characteristics, carbon atoms in an ω-alicyclic ring behave differently from alkyl-substituent carbons.

A fatty acid with a polar function, such as unsaturation or a hydroxyl group, can be recognized by chromatographing it on polar and non-polar columns, the resultant ECL being significantly larger on the polar than on the non-polar column. The differences between the ECLs for the ω-alicyclic series measured on the EGA and SE-30 columns, as listed in Table II, range from 0.45 to 0.94. This range can be

TABLE I

ECL AND ΔECL VALUES OF FIVE SERIES OF FATTY ACIDS MEASURED ON THREE DIFFERENT COLUMNS

Total carbon number of fatty acid	*Equivalent chain length (ECL)*								
	Iso series			*Anteiso series*			*ω-Cyclopropyl series*		
	*EGA**	*Silar***	*SE-30***	*EGA*	*Silar*	*SE-30*	*EGA*	*Silar*	*SE-30*
14	13.53	13.53	13.52				14.97		
15	14.54			14.68	14.68	14.70			
16	15.54	15.53	15.60				17.00	17.13	16.35
17	16.57	16.57		16.72	16.72	16.62			
18									
ΔECL	−0.45	−0.46	−0.44	0.30	0.30	−0.34	0.98	1.13	0.35

TABLE II

DIFFERENCE BETWEEN ECL VALUES MEASURED ON EGA AND SE-30 COLUMNS

Carbon number of fatty acid	$ECL_{EGA} - ECL_{SE\text{-}30}$			
	Cyclopropyl	*Cyclobutyl*	*Cyclopentyl*	*Cyclohexyl*
15	—	0.45	—	0.79
16	0.65	—	0.94	—
17	—	0.60	—	0.92
18	—	—	0.91	—

compared with values of 0.75 and 1.48 for oleic and linoleic acid, respectively, on columns of EGA and Apiezon L (Apiezon L is substantially the same as SE-30)[7]. Hence the substitution of an ω-alicyclic ring into a fatty acid appears to be similar in some respects to the introduction of unsaturation. Studies on the biochemical significance of this substitution are continuing.

ACKNOWLEDGEMENTS

The author thanks Drs. H. W. Habgood and S. K. Chakrabartty for useful discussions.

REFERENCES

1 R. G. Ackman, *Prog. Chem. Fats Other Lipids*, 12 (1972) 165.
2 T. Kaneda, *J. Bacteriol.*, 95 (1968) 2210.
3 T. Kaneda, *Can. J. Microbiol.*, 12 (1966) 501.

ω-Cyclobutyl series			*ω-Cyclopentyl series*			*ω-Cyclohexyl series*		
EGA	*Silar*	*SE-30*	*EGA*	*Silar*	*SE-30*	*EGA*	*Silar*	*SE-30*
15.92	15.98 (16.1)***	15.47 (15.6)§		(16.5)***	(15.8)§	16.43	16.42 (16.7)***	14.64 (15.9)§
			17.46	17.40	16.52			
18.03	18.15	17.43				18.54	18.61	17.62
			19.45	19.56	18.54			
0.98	1.07	0.45	1.46	1.48	0.53	1.49	1.52	0.64

* Operated under isothermal conditions at 180° as described under Experimental.
** Operated under temperature-programmed conditions as described under Experimental.
*** Measured on Reoplex column[6].
§ Measured on Apiezon L column[6].

4 R. Dreher, K. Poralla and W. A. König, *J. Bacteriol.*, 127 (1976) 1136.
5 M. Schlenk and J. L. Gellerman, *Anal. Chem.*, 32 (1960) 1412.
6 A. K. Sen Gupta and H. Peters, *Chem. Phys. Lipids*, 3 (1969) 371.
7 H. P. Burchfield and E. E. Storrs, *Biochemical Applications of Gas Chromatography*, Academic Press, New York, London, 1962, p. 549.

Journal of Chromatography, 136 (1977) 328–331

CHROM. 9957

Note

Analysis of low-boiling isomers of phenols by gas chromatography

ASIT BHATTACHARJEE and A. BHAUMIK
Central Fuel Research Institute, P.O. FRI, Dhanbad, Bihar (India)
(First received September 28th, 1976; revised manuscript received January 12th, 1977)

The complete analysis of the low-boiling isomers of phenols by gas chromatography on conventional packed columns has not been achieved so far owing to the excessive peak tailing caused by non-linear adsorption on the solid support on the one hand and the lack of a suitable single stationary phase on the other. Although peak tailing has been eliminated in some instances by converting the phenols into their methyl[1,2] or silyl ethers[3], such procedures are cumbersome and time consuming. In order to overcome the difficulty of using a single stationary phase, Janak and Komers[4] and Kolšek and Matičič[5] suggested the use of two different stationary phases, based on their studies on a number of polar and non-polar phases. The selection of a suitable pair of stationary phases is also important as it is essential that a pair of components that overlap completely on one of the phases should be completely resolved on the other phase, and *vice versa*; partial overlapping or partial separation may lead to erroneous results. Unfortunately, no pair of stationary phases studied by previous workers[4,5] satisfy the above requirements so far as the complete separation of *m*- and *p*-cresol and 2,4- and 2,5-xylenol in particular is concerned.

We have found a pair of stationary phases that overcomes peak tailing appreciably without the need to convert the phenols into their methyl or silyl ethers and facilitates the almost complete separation of all of the peaks. Several 10-component mixtures of phenols containing phenol, three cresols and six xylenols have been analysed on this pair of stationary phases with acceptable accuracy.

EXPERIMENTAL

Equipment

A Perkin-Elmer 810 gas chromatograph fitted with a flame-ionization detector and a Honeywell 1-mV recorder was used. Experiments were carried out on a stainless-steel column (6 ft. × 1/8 in. O.D.) with nitrogen as the carrier gas (flow-rate 30 ml/min). The packing materials for the following two columns were prepared by slurrying the stationary phases with the support in a water–methanol mixture (column I) and in a chloroform–methanol mixture (column II) followed by removal of the solvents by heating: column I, 40% rubidium benzenesulphonate + 2% Carbowax 20M + 2% ascorbic acid on Chromosorb P (60–80 mesh); column II, 12% Apiezon L + 0.5% Carbowax 20M + 1% ascorbic acid on Chromosorb W (60–80 mesh. acid washed). Prior to analysis, column I was activated at 175° for 1 h or longer

until there was a complete separation of *m*- and *p*-cresol at 150°. Column II was pre-conditioned at 160° for 1 h under a flow of nitrogen.

Chemicals

The following pure grade chemicals were used: phenol (BDH, Poole, Great Britain), o-cresol (E. Merck, Darmstadt, G.F.R.), m-cresol (BDH), p-cresol (Naarden, Naarden, The Netherlands), 2,6-xylenol (BDH, recrystallised from cyclohexane), 2,4-, 2,5-, 2,3-, 3,5- and 3,4-xylenol (BDH) and ascorbic acid (BDH).

RESULTS AND DISCUSSION

In a previous study, it was shown that rubidium benzenesulphonate[6] modified with Carbowax 20M and ascorbic acid[7] (column I) was an excellent stationary phase for the separation of cresols and xylenols. All the six xylenols are separated on this phase at a column temperature of 150°. A mixture of 10 phenols containing phenol, three cresols and six xylenols is separated into seven peaks with the order of elution 2,6-xylenol, *o*-cresol, phenol + 2,5-xylenol, *m*-cresol + 2,3-xylenol, *p*-cresol + 2,4-xylenol, 3,5-xylenol, 3,4-xylenol.

Apiezon L grease, one of the most widely used non-polar stationary phases, has the major disadvantage that it causes peak tailing of polar compounds such as phenols. In some instances[1-3], peak-symmetry has been achieved by converting the phenols into their alkyl or silyl ethers. In this study, peak tailing of all compounds except phenol has been successfully eliminated by using Apiezon L modified with 0.5% Carbowax 20M and 1% ascorbic acid (column II) without altering the order of elution obtained on an unmodified Apiezon L column. Carbowax 20M and ascorbic acid when used individually with Apiezon L, however, failed to overcome tailing appreciably. On this column, at a column temperature of 135°, a mixture of 10 phenols was also separated into seven distinct peaks with the following order of elution: phenol, *o*-cresol, *m*-cresol + *p*-cresol, 2,6-xylenol, 2,4-xylenol + 2,5-xylenol, 2,3-xylenol + 3,5-xylenol, 3,4-xylenol.

It can be seen from the orders of elution of the phenols that no two particular

TABLE I

ANALYSIS OF SYNTHETIC MIXTURES OF PHENOLS USING COLUMNS I AND II AT 150° AND 135°, RESPECTIVELY

The figure quoted are percentages by weight.

Compound	*Mixture 1*		*Mixture 2*		*Mixture 3*		*Mixture 4*	
	Found	*Present*	*Found*	*Present*	*Found*	*Present*	*Found*	*Present*
Phenol	6.8	6.8	5.2	5.2	4.8	5.2	5.7	5.7
o-Cresol	15.0	15.8	4.5	4.8	4.4	4.7	8.4	8.4
m-Cresol	8.9	9.6	29.0	29.3	39.7	40.7	39.1	39.7
p-Cresol	18.7	18.7	25.0	23.6	14.0	13.3	6.9	6.8
2,6-Xylenol	5.8	5.7	1.3	1.3	4.4	4.3	6.1	6.2
2,4-Xylenol	5.6	5.5	4.3	4.4	5.0	4.1	6.1	4.8
2,5-Xylenol	16.7	15.7	4.9	5.5	5.6	5.2	9.5	9.4
2,3-Xylenol	3.4	2.8	4.1	4.6	6.7	6.7	4.1	3.6
3,5-Xylenol	7.3	7.2	6.1	5.7	5.4	5.3	8.2	8.5
3,4-Xylenol	12.2	12.2	15.5	15.6	10.4	10.5	6.3	6.9

phenols are eluted together from both columns. The pairs *m*- and *p*-cresol, 2,4- and 2,5-xylenol and 2,3- and 3,5-xylenol, which overlap on column II, are completely resolved on column I. These characteristic elution patterns led us to study the analysis of mixtures of low-boiling isomers of phenols on these two phases. Several synthetic mixtures have been analysed using these two columns and the results were satisfactory (Table I). Figs. 1 and 2 show the chromatograms of synthetic mixture 1 on columns I and column II, respectively. The relative retention times of the phenols on both phases are presented in Table II.

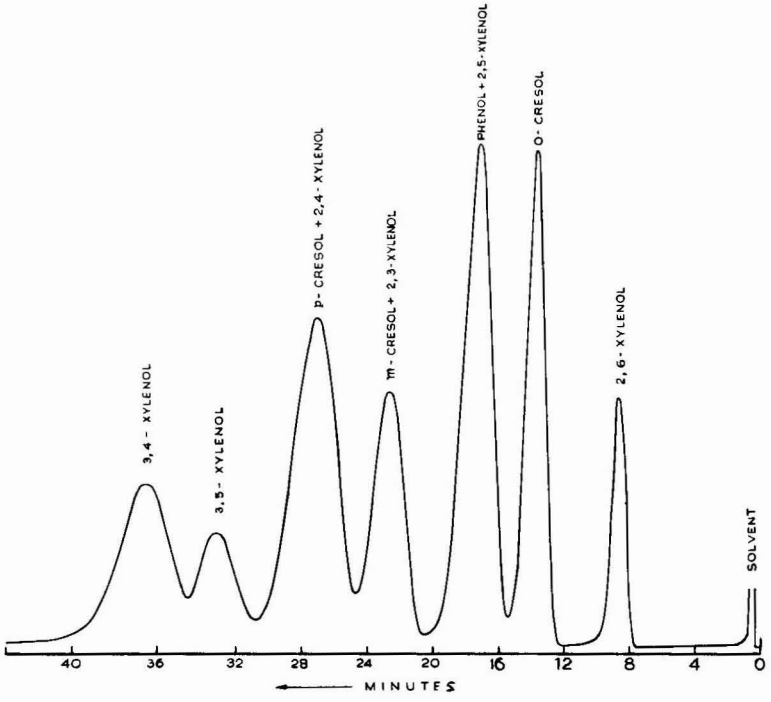


Fig. 1. Chromatogram of synthetic mixture 1 (Table I) on column I at 150°.

TABLE II

RELATIVE RETENTION TIMES OF PHENOLS

Compound	*Boiling point (°C)*	*Relative retention time on column I (150°)*	*Relative retention time on column II (135°)*
Phenol	181.7	1.00	1.00
o-Cresol	190.8	0.82	1.37
m-Cresol	202.1	1.32	1.61
p-Cresol	201.5	1.60	1.58
2,6-Xylenol	200.6	0.51	1.91
2,4-Xylenol	211.3	1.55	2.30
2,5-Xylenol	211.5	1.01	2.30
2,3-Xylenol	217.1	1.31	2.78
3,5-Xylenol	221.0	1.90	2.71
3,4-Xylenol	227.0	2.18	3.14

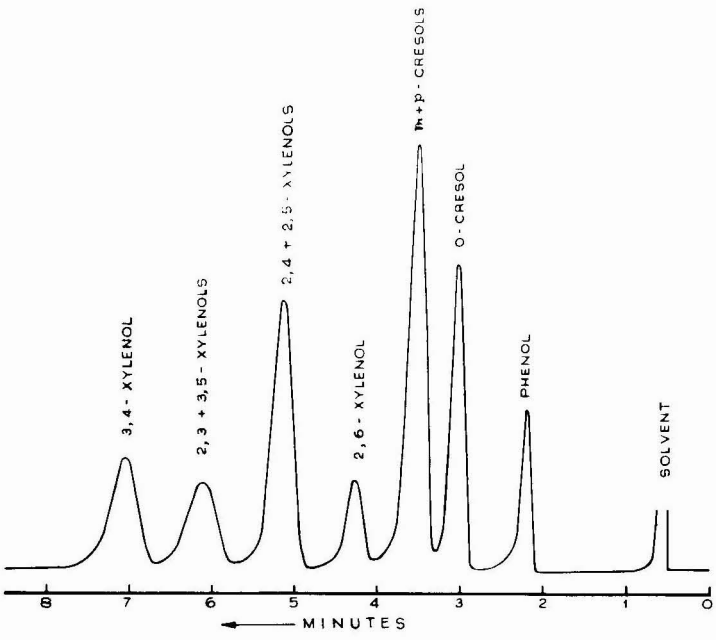


Fig. 2. Chromatogram of synthetic mixture 1 (Table I) on column II at 135°.

ACKNOWLEDGEMENT

The authors are grateful to Dr. C. S. B. Nair for his constant encouragement and helpful suggestions.

REFERENCES

1 L. E. Cook and R. C. Spangelo, *Anal. Chem.*, 46 (1974) 122.
2 A. C. Bhattacharyya, A. Bhattacharjee, O. K. Guha and A. N. Basu, *Anal. Chem.*, 40 (1968) 1873.
3 S. H. Langer, P. Pantages and I. Wender, *Chem. Ind. (London)*, (1958) 1664.
4 J. Janak and R. Komers, in D. H. Desty (Editor), *Gas Chromatography 1958*, Academic Press, New York, 1958, p. 343.
5 J. Kolšek and M. Matičič, *J. Chromatogr.*, 12 (1963) 305.
6 A. Bhattacharjee and A. N. Basu, *J. Chromatogr.*, 71 (1972) 534.
7 A. Bhattacharjee and A. Bhaumik, *J. Chromatogr.*, 115 (1975) 250.

Journal of Chromatography, 136 (1977) 332–336

CHROM. 9933

Note

Simultaneous gas chromatographic analysis for the seven commonly used antiepileptic drugs in serum

C. V. ABRAHAM and DENISE GRESHAM

Lynchburg Training School and Hospital, Lynchburg, Va. (U.S.A.)

(First received September 20th, 1976; revised manuscript received January 3rd, 1977)

Modern antiepileptic therapy frequently involves the simultaneous administration of two or more anticonvulsant drugs[1,2]. The importance of anticonvulsants and the development of methods adds a new dimension to the control of epilepsy[3].

Gas–liquid chromatography appears to be the method of choice for such analyses[1,4–6]. Its sensitivity and specificity allows simultaneous measurement of anticonvulsant drugs. Here, we describe a simple method for simultaneously determining phenobarbital, diphenylhydantoin, primidone, carbamazepine, ethosuccinimide, methsuccinimide and phensuccinimide in 1 ml of plasma or serum in less than one hour.

EXPERIMENTAL

Drugs used were: diphenylhydantoin (Dilantin; Parke-Davis, Detroit, Mich., U.S.A.); carbamazepine (Tegretol; CIBA Pharmaceutical, Summit, N.J., U.S.A.); phenobarbital (Eli Lilly, Indianapolis, Ind., U.S.A.); primidone (Ayerst Laboratory, New York, N.Y., U.S.A.); 5-(*p*-methylphenyl)-5-phenylhydantoin (MPPH; Aldrich, Milwaukee, Wisc., U.S.A.); ethosuccinimide (Zarontin; Parke-Davis); methsuccinimide (Celontin; Parke-Davis); phensuccinimide (Milontin; Parke-Davis); and Fluorene (Eastman-Kodak, Rochester, N.Y., U.S.A.). Trimethylphenylammonium hydroxide (TMPAH, 0.4 moles/l) was prepared as described by Abraham and Joslin[4].

As internal standard, 90 μg of MPPH and 60 μg of fluorene were added to the 5 ml of chloroform used for the extraction. All seven drugs were added to a drug-free pool of serum to obtain five standards with the concentrations listed in Table I. Each serum standard was extracted and chromatographed in duplicate as if it were a patient sample.

The instrumental conditions were the same as explained by Abraham and Joslin[4]. In the chromatographic conditions two temperature modes were used: (a) isothermal heating at 150° for 1.0 min, increased to 300° at a rate of 20°/min; (b) temperature-programmed from 180° to 300° at a rate of 10°/min. Gas flow-rates were: nitrogen, 160 ml/min; hydrogen, 30 ml/min; air, 300 ml/min.

Serum or plasma (1 ml) was combined with 0.5 ml of 0.25 *M* hydrochloric acid in a 150 × 13 mm test tube (PTFE-lined screw cap). Chloroform (5 ml) con-

TABLE I

CONCENTRATIONS OF WORKING STANDARDS (mg/l)

Standard	*Pheno-barbital*	*Diphenyl-hydantoin*	*Primidone*	*Carbama-zepine*	*Etho-succinimide*	*Meth-succinimide*	*Phen-succinimide*
1	5	2.5	2.5	1.0	12.5	3.1	3.1
2	10	5.0	5.0	2.5	25.0	6.2	6.2
3	20	10.0	10.0	5.0	50.0	12.5	12.5
4	40	20.0	15.0	7.5	100.0	25.0	25.0
5	80	40.0	20.0	10.0	200.0	50.0	50.0

taining the internal standards was added to the screw-cap tube and shaken in a vortex-type mixer. The aqueous (upper) phase was aspirated and the organic phase filtered through a Whatman No. 1 filter paper into a conical centrifuge tube and evaporated under a stream of nitrogen.

The dried residue in the centrifuge tube was reconstituted with 50 μl of methanol. This extract (1–2 μl) was chromatographed as explained in chromatographic condition (a). After this first injection, 50 μl of TMPAH were added to the residue and mixed well in a vortex mixer. This extract (1–2 μl) was chromatographed as explained in chromatographic condition (b). The peaks were identified by comparing their relative retention times (relative to internal reference peak) to known standards. Drug concentrations were calculated from the standard curve.

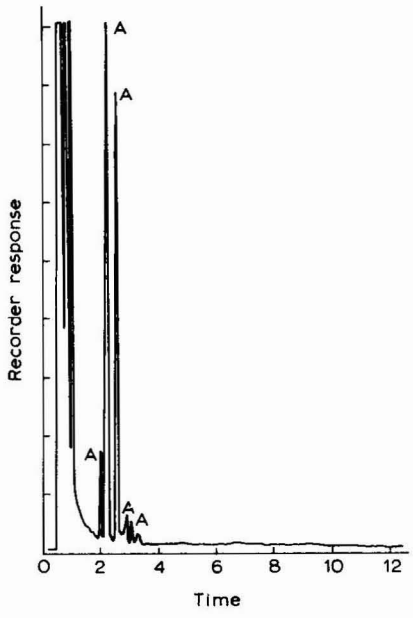
The precision of the proposed method was checked by using aliquots of spiked serum pools which were kept frozen. The serum pool contained all seven drugs.

RESULTS

Chromatograms of four specimens extracted and chromatographed according to our procedure are presented in Figs. 1–5. Fig. 1 is a typical pattern for serum from a normal individual not receiving anticonvulsant drugs when the program was run as explained in chromatographic condition (a). The peaks marked A are attributable to normal serum constituents. Fig. 2 with the internal standard MPPH is a typical pattern for serum from a normal individual not receiving anticonvulsants when the program was run as explained in chromatographic condition (b). The three prominent peaks marked S are attributable to normal serum constituents.

The chromatogram in Fig. 3 is a serum sample spiked with all three of the succinimide drugs. The concentrations calculated from the standard curve were: ethosuccinimide, 64 μg/ml; methsuccinimide, 91 μg/ml; phensuccinimide, 69 μg/ml. The spiked values for the drugs were 60, 90 and 65 μg/ml, respectively. Fig. 4 and 5 are from a patient who is receiving ethosuccinimide, carbamazepine and diphenylhydantoin; 75 μg/ml of primidone and 150 μg/ml of phenobarbital were added to the same sample. The calculated values for this sample were: phenobarbital, 142 μg/ml; carbamazepine, 7 μg/ml; primidone, 70 μg/ml; diphenylhydantoin, 36 μg/ml; ethosuccinimide, 76 μg/ml. All the drug peaks were well separated from one another and there were no interfering peaks.

The between-day precision of the proposed method was evaluated by analyzing the spiked serum sample for 31 consecutive days. The standard deviation varied from 0.4–1.2 mg/l. Analytical recoveries varied from 99–104%.



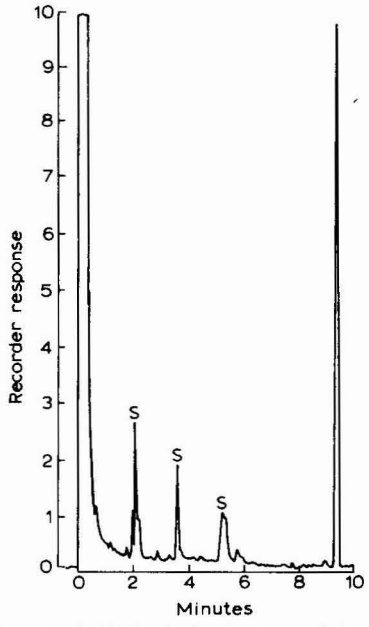
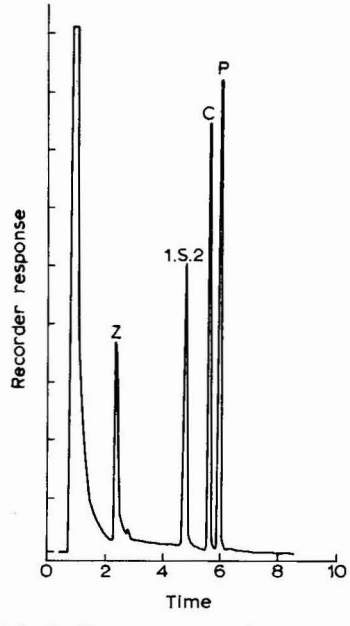


Fig. 1. Chromatogram obtained by injecting a serum extract from an individual who is not receiving any antiepileptic drugs. Chromatographic condition (a) was used. A = unknown constituent.

Fig. 2. Chromatogram obtained by methylation of serum extract from an individual who is not receiving any antiepileptic drugs. Chromatographic condition (b) was used. S = serum constituent; I.S. = internal standard (MPPH).



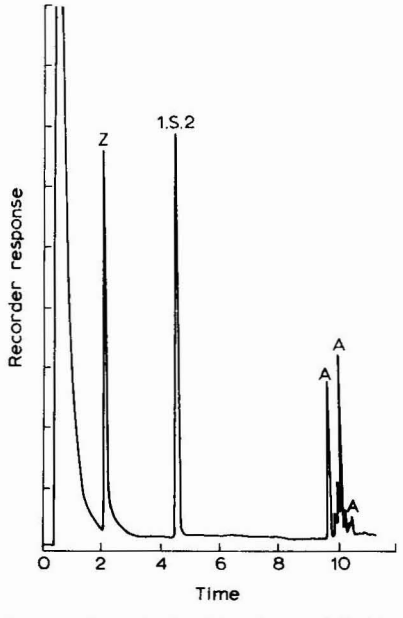


Fig. 3. Chromatogram obtained from a serum extract of a sample spiked with ethosuccinimide, methsuccinimide and phensuccinimide. Chromatographic condition (a) was used. Z = ethosuccinimide; C = methsuccinimide; P = phensuccinimide; I.S.2 = internal standard (fluorene).

Fig. 4. Chromatogram of a serum extract from a patient who is receiving ethosuccinimide, carbamazepine, diphenylhydantoin and the same serum spiked with primidone and phenobarbital. Chromatographic condition (a) was used. Z = ethosuccinimide; A = unknown constituent; I.S.2 = internal standard (fluorene).

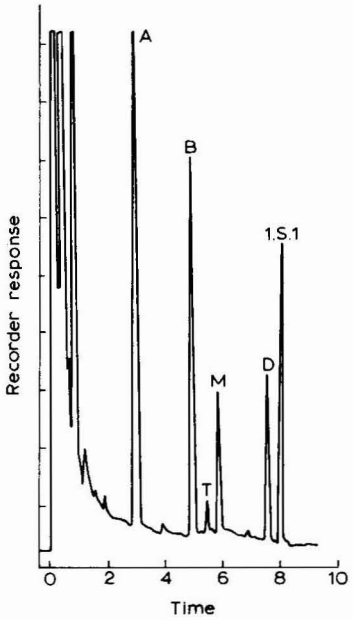


Fig. 5. Chromatogram of a methylated serum extract from a patient who was receiving ethosuccinimide, carbamazepine, diphenylhydantoin and the same serum spiked with primidone and phenobarbital. Chromatographic condition (b) was used. A = unknown constituent; B = phenobarbital; T = carbamazepine; M = primidone; D = diphenylhydantoin; I.S.1 = internal standard (MPPH).

The usual sample volume used was 1 ml of serum or plasma. In pediatric cases, similar results can be obtained by decreasing the amounts of serum, chloroform and reagent proportionately. In some instances we successfully used as little as 0.2 ml of serum.

The proposed method is simple and well suited for the clinical laboratory. A single extraction and chromatographic system is used to analyze all seven of the anticonvulsant drugs. Use of two internal standards in the same extracting solvent for both chromatographic conditions reduces the technical difficulties, the standardization procedure and the analytical time. The non-methylated derivatives of succinimide-type drugs and the methylated derivatives of other drugs were well resolved as were the two internal standards. The use of glass columns and SP 2250 gives good peak characteristics and a linear standard curve.

The five standards that contain all the seven drugs are prepared in pooled serum. In our proposed method the concentration in standard 5 is about twice the toxic concentration, standard 1 is less than a third of the therapeutic concentration.

The most advantageous aspect of the procedure is the capability to determine the most commonly used antiepileptic drugs in a single procedure within one hour.

ACKNOWLEDGEMENT

We would like to thank Mr. Calvin Mason, Lynchburg Training School and Hospital Photographer for taking the photographs.

REFERENCES

1 D. M. Woodbury, J. K. Penry and R. P. Schmidt, *Antiepileptic Drugs*, Raven Press, New York, 1972.
2 S. W. Rose, L. D. Smith and J. K. Penry, *Blood Level Determinations of Antiepileptic Drugs: Clinical Value and Methods*, U.S. Dept. of H.E.W., Bethesda, Md., 1971.
3 E. S. Vesell and G. T. Parsananti, *Clin. Chem.*, 17 (1971) 851.
4 C. V. Abraham and H. D. Joslin, *J. Chromatogr.*, 128 (1976) 281.
5 I. P. Baumel, B. B. Gallagher and R. H. Mattson, *Arch. Neurol.*, 27 (1972) 34.
6 G. Kananen, R. Osiewicz and I. Sunshine, *J. Chromatogr. Sci.*, 10 (1972) 283.

Journal of Chromatography, 136 (1977) 337–341

CHROM. 9924

Note

High-pressure liquid chromatographic separation of pharmaceutical compounds using a mobile phase containing silver nitrate

R. J. TSCHERNE and G. CAPITANO

Quality Control Department, Hoffmann-La Roche, Nutley, N.J. 07110 (U.S.A.)

(Received January 5th, 1977)

Use of impregnated supports for the separation of closely related compounds has been reported for thin-layer chromatographic (TLC) systems[1]. Application of some of these TLC techniques has been extended to high-pressure liquid chromatography (HPLC). An argentated silica gel stationary phase has been used for the separation of *o*-, *m*- and *p*-picoline[2]. Janák *et al.*[3] have reported the use of a porous polyhydrocarbon stationary phase (Porapak Q) and a mobile phase containing silver nitrate. Very recently, Schomburg and Zegarski[4] have reported the use of an argentated mobile phase with a reversed-phase partition system for the separation of geometrical isomers of 2-alkenes and 1,5,9-cyclododecatriene, as well as for the separation of oleic and elaidic acid methyl esters.

Application of this technique for the differentiation of compounds of pharmaceutical importance was realized in this laboratory. Very similar compounds were found to be affected differentially by the presence of silver nitrate in the mobile phase. Vitamins D_2 and D_3, as well as various estrogenic compounds, were examined. Some compounds that were either only partially or totally unresolved from chromatographically similar species, were completely separated when using a mobile phase containing silver nitrate.

EXPERIMENTAL

All mobile phases were prepared using certified A.C.S. grade methanol (Fisher Scientific, Fair Lawn, N.J., U.S.A.) and demineralized water. Reagent A.C.S. grade silver nitrate was purchased from Allied Chemical (Morristown, N.J., U.S.A.). All mobile phases containing silver nitrate were shielded from light using low-actinic glassware.

A Milton Roy Mini-Pump Model 396/2396 (Laboratory Data Control, Riviera Beach, Fla., U.S.A.) was used in conjunction with a 30 cm × 4 mm I.D. μBondapak/C_{18} chromatographic column (Waters Assoc., Milford, Mass., U.S.A.). Sample introduction into a DuPont Instruments Model 830 liquid chromatograph injection port was made using a 5-μl Hamilton HP305 syringe. A DuPont Model 835 filter photometer (DuPont Instruments, Wilmington, Del., U.S.A.) and a Linear Instruments Model 252A recorder (Irvine, Calif., U.S.A.) were used. Because of the high absorbance of mobile phases containing silver nitrate, the recorder pen was electronically offset using the photometer balance and recorder zero adjustments.

Reference standard vitamins D_2 and D_3 were purchased from the United States Pharmacopoeia (Rockville, Md., U.S.A.). Estrogen samples were obtained from a variety of suppliers. All sample solutions were prepared to be approximately 1 mg/ml in methanol.

RESULTS AND DISCUSSION

In the past, chromatographers have shown the partial separation of vitamin D_2 from vitamin D_3 (ref. 5), as well as the separation of various estrogenic compounds[6–8] from each other using HPLC. Structures for some of these compounds are shown in Fig. 1. In many cases, these separations are found to be difficult to reproduce. The use of an argentated reversed-phase partition system is recommended as an alternative method for the separation of these groups of compounds.

Chromatograms showing a vitamin D_2–vitamin D_3 separation using a mobile phase both with and without silver nitrate addition are shown in Fig. 2. An essentially complete separation of vitamin D_2 from vitamin D_3 was accomplished in less than 35 min (Fig. 3). Table I relates the effect of silver nitrate concentration to the retention

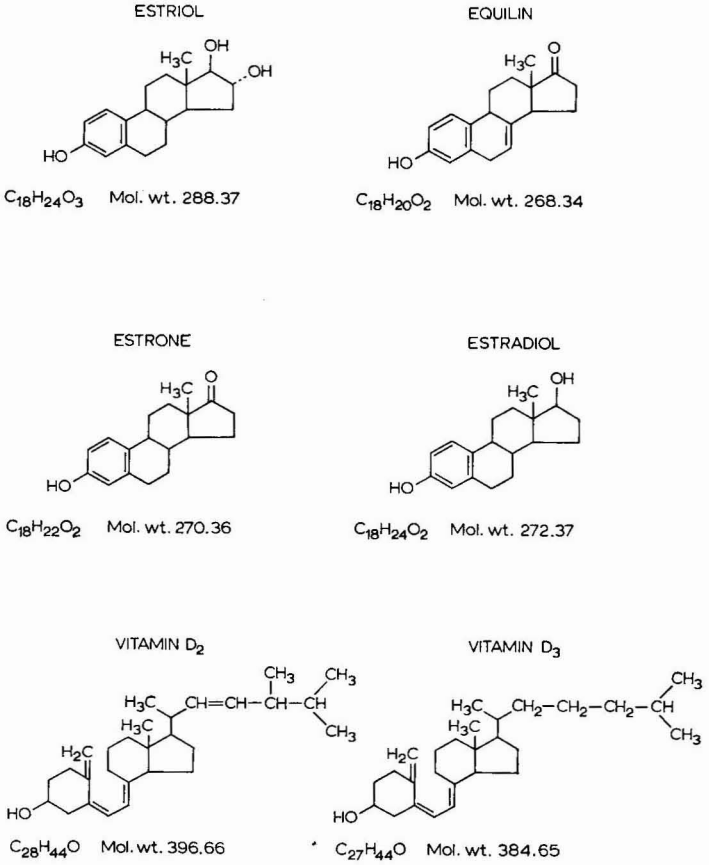


Fig. 1. Structures of various vitamin D and estrogenic compounds.

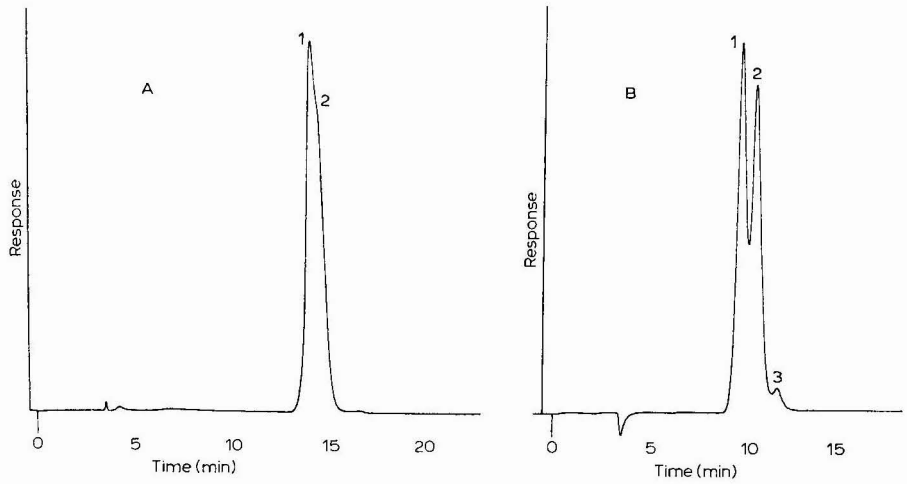


Fig. 2. HPLC separation of vitamin D compounds using a μBondapak/C_{18} column (30 cm × 4 mm I.D.). (A) Mobile phase, 95 ml methanol + 5 ml water; flow-rate, 0.8 ml/min. (B) Mobile phase, 95 ml methanol + 5 ml water + 2 g silver nitrate; flow-rate, 0.8 ml/min. 1 = Vitamin D_2; 2 = vitamin D_3; 3 = impurity.

time of each component. From a study of this set of data, it is evident that the role of the silver nitrate is to allow π-complexation with available sites on these molecules. Both the retention times of vitamin D_2 and vitamin D_3 are noticeably decreased on the addition of silver nitrate to the mobile phase because each of these compounds

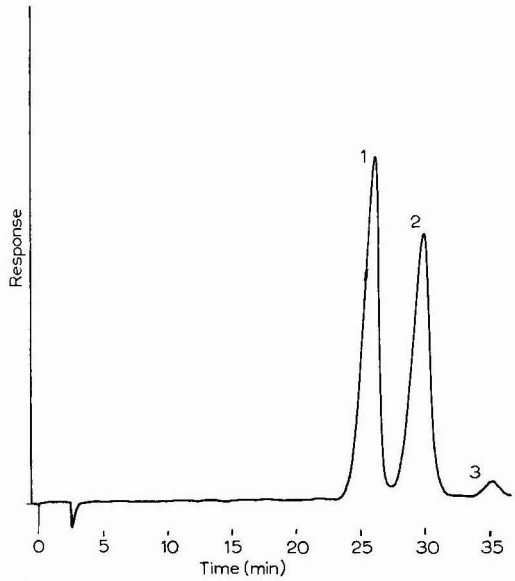


Fig. 3. HPLC separation of vitamin D_2 from vitamin D_3. Column, μBondapak/C_{18}, 30 cm × 4 mm I.D.; mobile phase, 86.5 ml methanol + 13.5 ml water + 2.4 g silver nitrate; flow-rate, 1.1 ml/min. 1 = Vitamin D_2; 2 = vitamin D_3; 3 = impurity.

TABLE I

RETENTION TIMES FOR VITAMIN D_2 AND VITAMIN D_3 IN THE PRESENCE OF VARYING AMOUNTS OF SILVER NITRATE

All runs were made using the following conditions: column, μ-Bondapak/C_{18}; mobile phase, methanol–water (95:5) + varying amounts of silver nitrate; detector, UV at 254 nm; flow-rate, 0.8 ml/min.

Grams of $AgNO_3$/500 ml of mobile phase	*Retention time (min)*	
	Vitamin D_2	*Vitamin D_3*
0	14.3	14.5
2.53	12.5	13.0
5.00	11.6	12.3
7.50	10.3	11.1
10.01	10.0	10.6

has the ability to π-complex with the silver atom. Vitamin D_2, however, has an additional complexation site on the side chain, which provides the main basis for the separation of vitamin D_2 from vitamin D_3.

Chromatograms showing the separation of estriol, equilin, estrone and estradiol both before and after argentation of the mobile phase are shown in Fig. 4. Addition of 20 g/l of silver nitrate resulted in a retention time decrease of 6 min for

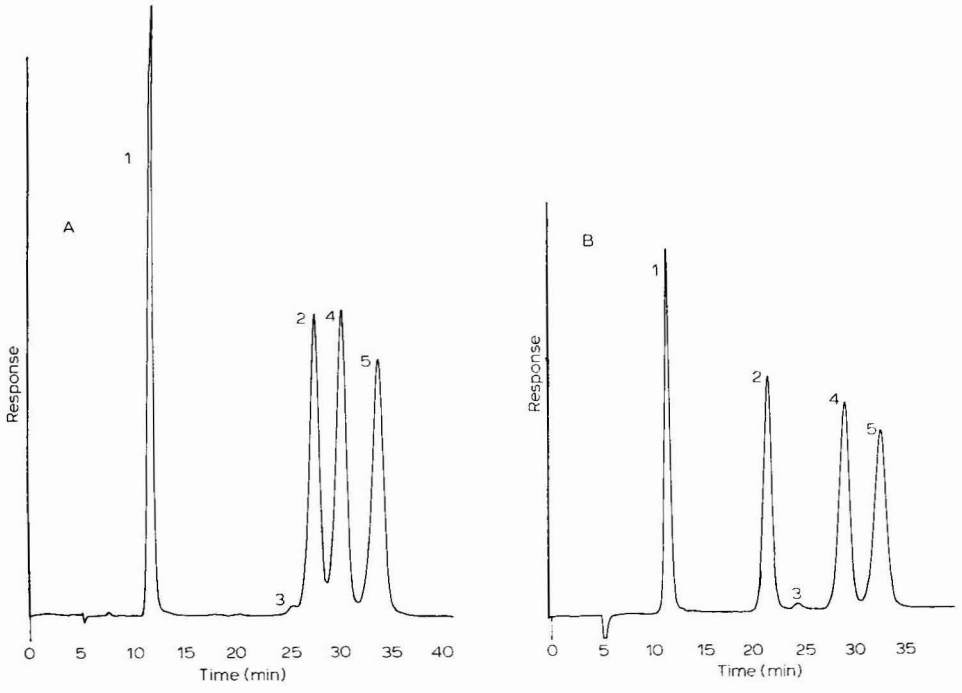


Fig. 4. HPLC separation of estrogenic compounds using a μBondapak/C_{18} column. (A) Mobile phase, 60 ml methanol + 40 ml water; flow-rate, 0.55 ml/min. (B) Mobile phase, 60 ml methanol + 40 ml water + 2 g silver nitrate; flow-rate, 0.55 ml/min. 1 = Estriol; 2 = equilin; 3 = impurity; 4 = estrone; 5 = estradiol.

the equilin compound, while the retention times for estriol, estrone, and estradiol were virtually unchanged. In this case, the double bond between No. 7 and No. 8 carbon atoms in the equilin molecule allows for π-complexation with the silver atom. Because of this phenomenon, the retention time of equilin could be decreased almost independently of the other compounds simply by silver nitrate addition to the mobile phase (Table II).

TABLE II

RETENTION TIMES FOR ESTROGENS IN THE PRESENCE OF VARYING AMOUNTS OF SILVER NITRATE

All runs were made using the following conditions: column, μ-Bondapak/C_{18}; mobile phase, methanol–water (60:40) + varying amounts of silver nitrate; detection, UV at 280 nm; flow-rate, 0.8 ml/min.

Grams of $AgNO_3$/500 ml of mobile phase	*Retention time (min)*			
	Estriol	*Equilin*	*Estrone*	*Estradiol*
0	11.8	27.8	30.7	34.1
2.48	11.7	25.7	30.0	33.7
5.46	11.6	23.9	29.6	33.2
7.60	11.7	22.7	29.3	32.9
10.12	11.7	21.7	29.7	33.3

Complete reproducibility of all systems using silver nitrate was observed. To prevent metallic silver build-up, each evening a water–methanol (50:50) mobile phase was used to thoroughly flush excess silver nitrate from the system. Even so, a small amount of metallic silver build-up was observed on the inner walls of the tubing over a period of time. Rinsing with dilute nitric acid or replacement of the tubing itself was used to remedy this situation.

By no means is the use of this argentation technique expected to be the answer to all separation problems of this type, but it is an alternative. The versatility of the system is shown in the ability to completely regenerate the μBondapak/C_{18} column back to the state in which it was prior to the use of the argentated mobile phase. Applicability of this technique of adding metal ions to the mobile phase should not stop with these two series of compounds, but should be expanded for use on a wide variety of compounds with possibly other metal ions as complexing agents, such as mercury and nickel salts.

REFERENCES

1 E. Stahl, *Thin-Layer Chromatography: A Laboratory Handbook*, Springer, Heidelberg, 2nd Ed., 1969, pp. 48–52.
2 R. Aigner, H. Spitzy and R. W. Frei, *Anal. Chem.*, 48 (1976) 2.
3 J. Janák, Z. Jagarić and M. Dressler, *J. Chromatogr.*, 53 (1970) 525.
4 G. Schomburg and K. Zegarski, *J. Chromatogr.*, 114 (1975) 174.
5 *DuPont Liquid Chromatography Laboratory Report*, No. A-95441 (1974).
6 G. J. Krol, R. P. Masserano, J. F. Carney and B. T. Kho, *J. Pharm. Sci.*, 59 (1970) 1483.
7 A. G. Butterfield, B. A. Lodge and N. J. Pound, *J. Chromatogr. Sci.*, 11 (1973) 401.
8 *Applications Highlights, AG-336*, Waters Associates Inc., Milford, Mass., U.S.A., 1974.

Journal of Chromatography, 136 (1977) 342–347

CHROM. 9967

Note

Use of thin-layer chromatography in the separation of disaccharides resulting from digestion of chondroitin sulphates with chondroitinases*

LINA WASSERMAN, ARTUR BER and DAVID ALLALOUF

Endocrinological Department of the Rogoff-Wellcome Medical Research Institutes, Beilinson Hospital, Petah Tikva (Israel)

(First received September 10th, 1976; revised manuscript received December 14th, 1976)

An approach to the identification of glycosaminoglycans was developed by Saito *et al.*[1], who used specific enzymes from bacterial origin that degrade chondroitin sulphates. Among these enzymes, chondroitinase ABC degrades chondroitin 4-sulphate (C-4S), dermatan sulphate (DS) and chondroitin 6-sulphate (C-6S). Both enzymes also degrade hyaluronic acid and chondroitin. The degradation is carried out by an elimination reaction yielding disaccharides consisting of 4,5-unsaturated uronic acid and hexosamine at the reducing end carrying a sulphate group at the 4- or 6-position, or no sulphate in the case of non-sulphated glycosaminoglycans[2]. These disaccharides exhibit different mobilities in paper chromatography and can be rendered visible by illumination with UV light. They can be quantified either by measuring their absorbances at 232 nm or by the help of colour reactions for their constituent monosaccharides[2,3].

This method has been widely applied to the identification of glycosaminoglycans in various tissues and in fractions obtained by a variety of procedures. In view of the polydispersity of glycosaminoglycans in biological materials, as shown by their different molecular weights and the variability in the degree of sulphation[4], fractionations generally do not yield homogeneous preparations and glycosaminoglycans of a single type. The determination of disaccharides resulting from digestion with chondroitinases was found to be a very useful means for the identification of the components present.

Paper chromatography is applicable to amounts of disaccharides ranging from 25 to 100 μg of each isomer, which might be higher than desirable when dealing with minor components, and the procedure, including a desalting step, requires about 36 h. The present work was aimed at the development of a more rapid and sensitive method for the qualitative and quantitative determination of these disaccharides. Cellulose thin-layer chromatographic (TLC) plates were found to give satisfactory separations and detection of amounts of disaccharides as low as 2.5 μg. The application of colour reactions with the eluted spots made possible their quantification. An advantage of this method is the short period of time (12 h) required for the development of the thin-layer chromatograms.

* Part of a Thesis submitted by Mrs. L. Wasserman to the Tel Aviv University, in partial fulfilment of the requirements for the Ph.D. degree.

MATERIALS AND METHODS

Chondroitin 4- and 6-sulphate were purchased from Miles Labs. (Kankakee, Ill., U.S.A.). Hyaluronic acid and dermatan sulphate were kindly supplied by Dr. Cifonelli, University of Chicago, Chicago, Ill., U.S.A. Commercial unspecified preparations of chondroitin sulphate were obtained from Nutritional Biochemicals (Cleveland, Ohio, U.S.A.; CS-1) and Delta Chemical Works (New York, N.Y., U.S.A.; CS-2). Chondroitinases ABC and AC and unsaturated disaccharides were obtained from Seikagatu Kogyo (Tokyo, Japan). Pre-coated cellulose F TLC plates, 0.1 mm thick, were supplied by Merck, Darmstadt, G.F.R. All chemicals were of analytical-reagent grade.

Digestion with chondroitinases

The digestion mixture consisted of 10 μl of a solution of glycosaminoglycan in water, 10 μl of enriched Tris buffer (pH 8.0)[1] and 20 μl of an aqueous solution of enzyme containing 10 units/ml. After incubation for $2^1/_2$ h at 37°, another 10-μl portion of enzyme solution was added and incubation continued for another $2^1/_2$ h[5]. The control mixture consisted of the same components (except enzyme), brought to the same final volume. Four volumes of 96% ethanol were then added and the mixtures left overnight at 4°. The clear supernatant obtained by centrifugation was dried in a desiccator and the residue, dissolved in a small volume of water, was applied on the TLC plates.

Thin-layer chromatography

Initially, desalting was carried out using 1-butanol–ethanol–water (52:32:16) for 6 h[5]. After drying in air the plates were developed for 6 h using as solvent 1-butanol–acetic acid–1 *N* ammonia solution (2:3:1)[1]. The unsaturated disaccharides were rendered visible under shortwave UV light as brown spots or after immersion in silver nitrate[6,7] and were identified by comparing their mobilities with those of the reference compounds. For quantitative determinations the areas located by UV illumination representing the various materials as well as clear background areas on the plates were scraped off and eluted with 1 ml water using sonication with a MSE ultrasonicator at maximal intensity for 2 min and subsequent heating at 50° for 1 h. After centrifugation and reduction of the volume of the supernatant by evaporation, the disaccharides were determined either by the method of Hascall *et al.*[8], which determines unsaturated uronic acid, or by the Elson-Morgan reaction for hexosamines as modified by Antonopoulos[9], after hydrolysis with 4 *N* hydrochloric acid for 7 h at 100°.

Uronic acid was determined according to the method of Bitter and Muir[10] and protein by the method of Lowry *et al.*[11].

RESULTS AND DISCUSSION

Fig. 1 illustrates the chromatographic separation of a mixture of 20 μg each of the disaccharides ΔDi-4S (3-O-Δ^4-glucuronosyl-N-acetylgalactosamine 4-sulphate), ΔDi-6S (3-O-Δ^4-glucuronosyl-N-acetylgalactosamine 6-sulphate) and ΔDi-0S (3-O-Δ^4-glucuronosyl-N-acetylgalactosamine) and the individual compounds separately. Experiments with smaller amounts showed that spots corresponding to 2.5 μg of material could still be detected. It should be pointed out that parallel experiments

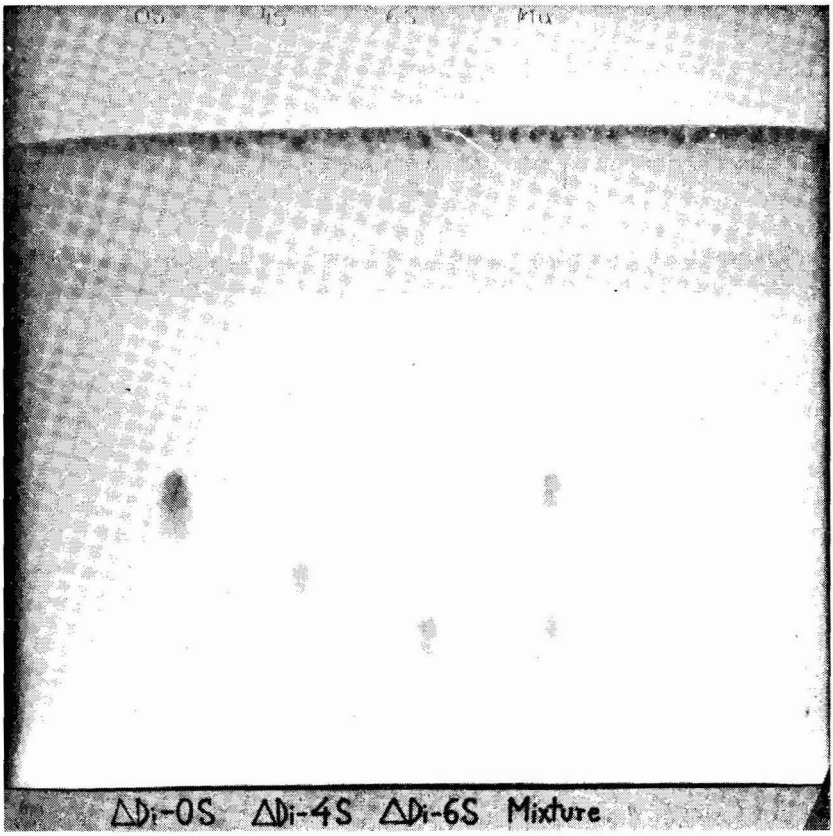


Fig. 1. TLC of standard unsaturated disaccharides. Amounts applied: 20 μg each. Solvents and conditions as described in the text. Spots revealed by UV illumination.

carried out by using paper chromatography indicated that 25 μg was the limit of detection.

Fig. 2 shows the degradation products obtained in the incubation of 50 μg of C-4S and C-6S with chondroitinase ABC and AC. A mixture of the markers developed on the same plate, revealing spots of ΔDi-6S, ΔDi-4S and ΔDi-0S in this order of increasing mobility, permitted the identification of the degradation products. The major spots corresponded to the expected products and the additional minor spots probably represent minor contaminants.

Dermatan sulphate gave, as expected, ΔDi-4S following incubation with chondroitinase ABC and remained undigested by chondroitinase AC (not shown in the figure).

In the experiment illustrated in Fig. 3 we examined the sensitivity of the method by using decreasing amounts of C-6S. The intensity of the spots was proportional to the amount of substrate and the degradation product resulting from 12.5 μg of initial substance is still clearly detectable. In fact, in view of the above-mentioned contamination, the true amount is even lower, indicating the high sensitivity of the method.

The experiment illustrated in Fig. 4 was designed to show the applicability of

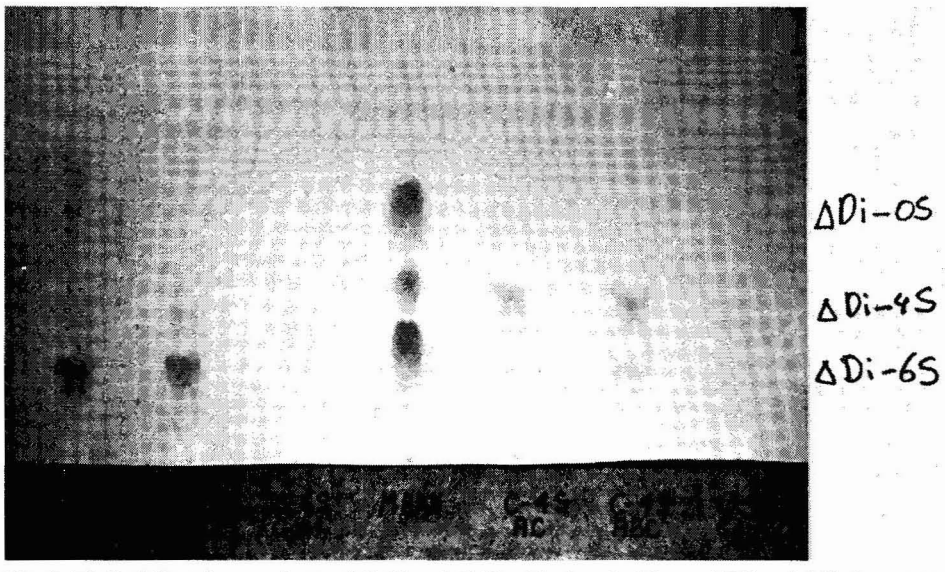


Fig. 2. TLC of digestion products of C-4S and C-6S with chondroitinase ABC and AC. Amounts of substrates used: 50 μg. Controls (cont.) consisted of substrates without enzymes. Mark., reference disaccharide markers.

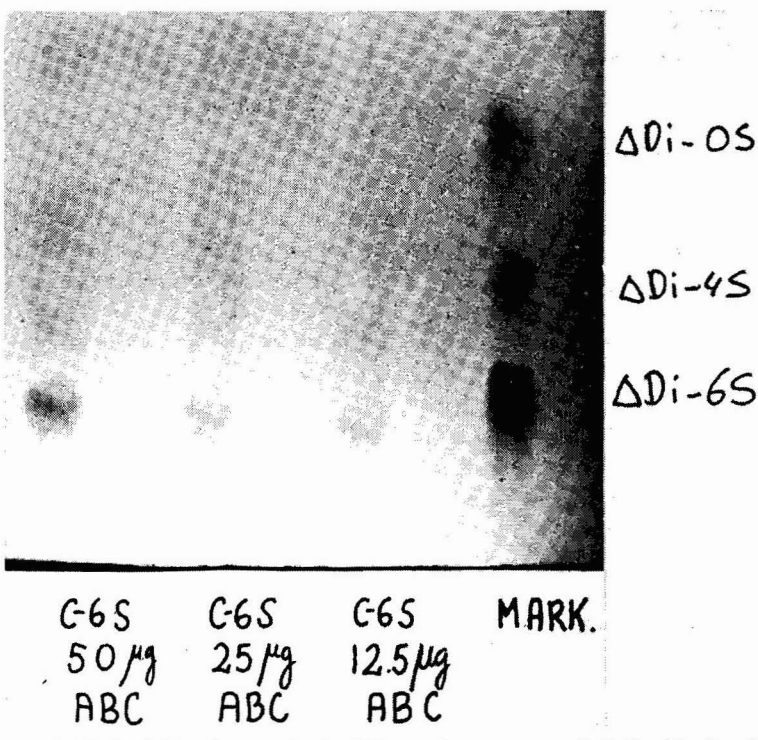


Fig. 3. TLC of digestion products of decreasing amounts of C-6S with chondroitinase ABC.

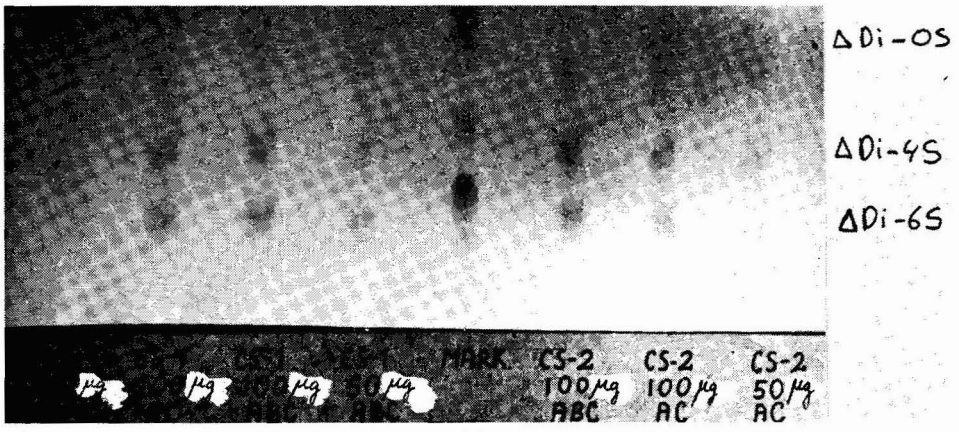


Fig. 4. TLC of digestion products of commercial preparations of chondroitin sulphate with chondroitinase ABC and AC.

TLC in the analysis of impure preparations of mixtures of C-4S and C-6S often encountered in extracts of biological materials. For this purpose, unspecified preparations of chondroitin sulphate from two different commercial sources, found to contain similar concentrations of uronic acid (about 23%) and protein (about 20%), were used. The significant content of protein presumably indicates the presence of proteoglycans. The sensitivity of the method was confirmed by the fact that digestion products from amounts of substrates corresponding to 12 μg of uronic acid were clearly detectable. Other experiments not shown in the figure were carried out with half of this amount and the results were satisfactory.

Experiments not presented in the figures showed that the non-sulphated unsaturated disaccharide derived from the degradation of hyaluronic acid with chondroitinase migrated faster than the reference marker ΔDi-0S supplied by the manufacturer, which is a galactosamine-containing disaccharide, indicating the possibility of resolving mixtures of glucosamine and galactosamine-containing disaccharides. This difference in mobility has also been reported by other workers who used paper chromatography[5].

The experiments reported above included detection of the spots under UV light. It should be mentioned that the spots can also be revealed by the silver nitrate procedure for reducing sugars. The sensitivity of this staining method, however, is lower than that achieved by UV illumination.

For the determination of the various disaccharides separated on the TLC plates two procedures were used. The generally used methods consisting of measurement of absorbance at 232 nm and performance of the carbazole reaction for uronic acid in eluates from paper proved to be inapplicable with the pre-coated TLC plates used in view of the background absorption at this wavelength and the positive carbazole reaction. With two other reactions, however, the results in this respect were satisfactory. Thus, the background absorption with the Elson-Morgan reaction for hexosamines and the periodic acid–thiobarbituric acid reaction for unsaturated uronic acids described by Hascall *et al.*[8] was virtually zero. Experiments with one of the commercial preparations of chondroitin sulphate showed good agreement between

the two colorimetric methods with regard to the relative amounts of the isomeric components. The determination of unsaturated uronic acid has the advantages of being faster and more sensitive than that of hexosamine. It should be noted that with the former method care should be taken to use appropriate standards of disaccharides as the colour intensity of their reaction products is different. Thus, the ratios of the absorbances of equal concentrations were approximately 1:2:3 for ΔDi-4S, ΔDi-0S and ΔDi-6S.

The recovery of the TLC method was examined with the three pure disaccharides. 40 μg of each, individually and in admixture, were applied on the TLC plates. After migration, the recoveries in the eluates of the corresponding spots, using the reaction for unsaturated uronic acid, were 80–105%. Recoveries of this magnitude can be considered satisfactory in view of the possible errors involved in the various steps, and the quantitative procedure can therefore be recommended, especially in experiments in which the relative amounts of isomeric chondroitin sulphates are to be determined in mixtures.

REFERENCES

1 H. Saito, T. Yamagata and S. Suzuki, *J. Biol. Chem.*, 243 (1968) 1536.
2 T. Y. Yamagata, H. Saito, O. Habuchi and S. Suzuki, *J. Biol. Chem.*, 243 (1968) 1523.
3 K. von Figura, W. Kiowski and E. Buddecke, *Hoppe-Seyler's Z. Physiol. Chem.*, 356 (1975) 1517.
4 L. Rodèn, in E. A. Balazs (Editor), *Chemistry and Molecular Biology of the Intercellular Matrix*, Vol. 2, Academic Press, London, 1970, p. 797.
5 T. Y. Lee, A. M. Jamieson and I. A. Schafer, *Pediatr. Res.*, 7 (1973) 965.
6 W. E. Trevelyan, D. P. Procter and J. S. Harrison, *Nature (London)*, 166 (1950) 444.
7 N. Sharon and R. W. Jeanloz, *J. Biol. Chem.*, 235 (1960) 1.
8 V. S. Hascall, R. L. Riolo, J. Hayward, Jr. and C. C. Reynolds, *J. Biol. Chem.*, 247 (1972) 4521.
9 C. A. Antonopoulos, *Ark. Kemi*, 25 (1966) 243.
10 T. B. Bitter and H. M. Muir, *Anal. Biochem.*, 4 (1962) 330.
11 O. H. Lowry, N. J. Rosenbrough, L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.

Journal of Chromatography, 136 (1977) 348–352

CHROM. 10,005

Note

Detection of bile salts with Komarowsky's reagent and group specific dehydrogenases

IAN A. MACDONALD

Department of Medicine, Dalhousie University, Halifax, Nova Scotia (Canada)

(Received January 5th, 1977)

A number of spray reagents for the detection of bile salts and neutral steroids on thin-layer chromatography (TLC) are in common use. Some of the more widely used reagents include various sulfuric acid solutions[1], molybophosphoric acid[1] antimony(III) chloride[2] and anisaldehyde-sulfuric acid solution[3]. Kellogg[4] has documented the use of 8-hydroxy-1(3,6-pyrene)trisulfonic acid for bile salt and neutral steroid detection. More recently, Goswami and Frey[5] have introduced a novel copper–ammonium molybdate–sulphuric acid reagent to detect a variety of bile salts, lecithin and cholesterol on TLC plates.

Komarowsky's reagent (*p*-hydroxybenzaldehyde–sulfuric acid solution) has been used to detect a limited number of keto-steroids and sapogenins[1,6]. It is the purpose of this communication to describe the general usefulness of this reagent in the detection of hydroxy- and keto- bile salts and the application of group specific dehydrogenases against eluates from sprayed thin-layer plates.

MATERIALS

Preparation of Komarowsky's reagent was modified from Stevens[6]: 5 ml of concentrated sulfuric acid were added to 100 ml of a 2% solution of *p*-hydroxybenzaldehyde (Baker, Phillipsburg, N.J., U.S.A.) in methanol. All bile salts and neutral steroids were dissolved (5 mg/ml) in methanol–water (4:1). TLC plates (20 × 20 cm) were made as described earlier[7].

Solvent systems consisted of chloroform–methanol–acetic acid: (A), 40:4:2; (B), 40:2:1; (C), 40:1:1.

3α-Hydroxysteroid dehydrogenase (3α-HSDH) was purchased from Worthington (Freehold, N.J., U.S.A.) enzymes; 7α-hydroxysteroid dehydrogenase (7α-HSDH) was made as previously described[7].

METHODS

Standard steroids (5–20 μl) were spotted approximately 2 cm from the edge of a TLC-plate (pre-washed by leaving in a tank containing chloroform–methanol–water (85:35:5) and pre-activated at 100° for 1 h). Prior to spotting, plates were divided

into 10 lanes and the appropriate solvent system allowed to equilibrate in a glass tank for 2 h. On completion of spotting, the plates were dried by blowing cold air onto the surface and quickly placed in a solvent tank. Plates were developed for 2 h, dried (in a fumehood), sprayed liberally with Komarowsky's reagent and left at 80° for 5–8 min. Alternately, plates were gently warmed by use of a heat gun (1–2 min). Colors and R_F values were noted, when the plate was cooled (approximately 15 min).

The colors were allowed to partially fade (1 or 2 days); individual spots corresponding to selected compounds were scraped from the plate and the scrapings were placed in pasteur pipettes, pre-packed with cotton-wool (approximately 1 cm high). Scrapings were eluted directly into a cuvette with 0.5 ml methanol (or methanol–diethyl ether, 50:50, for the more non-polar compounds; *e.g.* di- or tri-keto bile salts). The solvent was blown down to dryness and 3.0 ml reaction mixture, consisting of 0.17 *M* glycine–NaOH buffer pH 9.5, $0.8 \cdot 10^{-3}$ *M* NAD, was added. A baseline was obtained by reading the cuvette at 340 nm in a Beckman DBGT spectrophotometer and recorder at 0.1 in./min. A 50-μl volume of purified 3α-HSDH (2 mg/ml) or 7α-HSDH (4 mg/ml) were added and the change in absorbance at 340 nm observed.

RESULTS AND DISCUSSION

Results are summarized in Table I. The reagent reacted with both hydroxylated and ketonic bile salts and gave a wide range of colors. It appears to be able to differentiate isomers such as 3α,7α-dihydroxy- and 3α-,12α-dihydroxy-cholanoates, and 3,7 diketo- and 3,12 diketo-cholanoates which are not generally differentiated by their R_F values. It can be noted however that there was no color difference between a free bile salt and the methylester or glycine or taurine conjugates (not shown) or between 5α-H bile salts and 5β-H bile salts. Thus the color formed with *p*-hydroxybenzaldehyde appears to be dependent on the number and positions of the hydroxyl- and keto-substituents. The range and type of compounds detected by this reagent is far greater than the original paper suggests[6]. All compounds detected under visible light, fluoresced when viewed under the long wave or short wave ultraviolet light although the former gave a more intense fluorescence.

The spectrum of colors seen with *p*-hydroxybenzaldehyde is similar (but not identical) to that seen with anisaldehyde, which has been extensively investigated by Kritchevsky *et al.*[3] and Lisboa[8]. Similar to their finding, the limit of sensitivity was found to be approximately 1 μg of steroid in day light. The use of a long wave UV-lamp will detect less than 0.5 μg cholic acid not visible in day light. Colors were stable for a period of approximately 6 h although some fading of the more "delicate" shades was evident after about 2 h. A plate which had been allowed to fade nearly completely (over a weekend) could be re-stored by simply re-heating at 80° although the restored color was not necessarily identical to the original.

The solvent systems A, B, and C in our hands gave excellent results. Behaviour of most bile salts in solvent B was somewhat similar to their behaviour in solvent S_1 of Eneroth[9] but with less diffusion and improved separations (Benzene–dioxane–acetic acid, 75:20:2). An increase or decrease in the chloroform–methanol ratio (solvents C and A respectively) permits the separation of less polar or more polar compounds without essentially changing the order on the TLC plate. However,

TABLE I

THIN-LAYER CHROMATOGRAPHY OF TRI- DI- AND MONO-FUNCTIONAL BILE SALTS AND COLORS WITH KOMAROWSKY'S REAGENT

Cholanoate	*Source**	*Color*	*Solvent system*	R_F**
3α,7α,12α-Trihydroxy-5β-	b	deep purple	A	0.21
			B	0.06
3α,7α,12α-Trihydroxy-5β-methyl	a	deep purple	A	0.39
			B	0.12
3α,7α,12α-Trihydroxy-5α-methyl	d	deep purple	A	0.28
			B	0.11
7α,12α-Dihydroxy-3-keto-5β-methyl	a	rose wine	A	0.70
			B	0.42
3α,12α-Dihydroxy-7-keto-5β-	a	khaki green	A	0.40
			B	0.15
3α,12α-Dihydroxy-7-keto-5β-methyl	a	khaki green	A	0.65
			B	0.37
3α,7α-Dihydroxy-12-keto-5β-	a	green-gray	A	0.40
			B	0.15
3α,7α-Dihydroxy-12-keto-5β-methyl	a	green-gray	A	0.65
			B	0.37
3α-Hydroxy-7,12-diketo-5β-	c	lime	B	0.37
7α-Hydroxy-3,12-diketo-5β-	e	bright red	B	0.39
12α-Hydroxy-3,7-diketo-5β-	e	brown	B	0.39
3,7,12-Triketo-5β-	a	orange-crimson	B	0.52
			C	0.36
3α,7α-Dihydroxy-5β-	b	violet	B	0.25
3α,7α-Dihydroxy-5β-methyl	a	violet	B	0.49
3α,7α-Dihydroxy-5α-methyl	d	violet	B	0.41
3α,7β-Dihydroxy-5β-	b	blue-purple	B	0.32
3α-Hydroxy-7-keto-5β-	a	violet-red	B	0.51
			C	0.44
7α-Hydroxy-3-keto-5β-	a	oxblood	C	0.48
3,7-Diketo-5β-	a	rust-yellow	B	0.57
			C	0.48
3α,12α-Dihydroxy-5β-	b	mauve-gray	B	0.25
3α,12α-Dihydroxy-5β-methyl	a	mauve-gray	B	0.49
3α-Hydroxy-12-keto-5β-	a	green	B	0.40
			C	0.27
12α-Hydroxy-3-keto-5β-	a	mahogany	C	0.42
3,12-Diketo-5β-	c	brick red	C	0.47
3α,6α-Dihydroxy-5β-	b	medium blue	B	0.22
3α,12α-Dihydroxy-5β-	a	blood red	B	0.45
7α-Hydroxy-5β-	c	navyblue-purple	B	0.66
			C	0.48
3-Keto-5β-	e	brick red	B	0.74

* (a) Steraloids (Wilton, N.H., U.S.A.); (b) Calbiochem. (Los Angeles, Calif., U.S.A.); (c) Applied Science Labs. (State College, Pa., U.S.A.); (d) kindly donated by Dr. W. H. Elliot (St. Louis University, Mo., U.S.A.); (e) Synthesized enzymatically (refs. 7 and 13).

** Average of two determinations.

similar to those results of Eneroth[9], certain isomeric pairs were not separated (*e.g.* 3α, 7α- and 3α,12α-dihydroxy-5β-cholanoates).

Low concentrations of *p*-hydroxybenzaldehyde (10^{-5} *M*) in the cuvette did not

inhibit either *P. testosteroni* 3α-HSDH or *E. coli* 7α-HSDH, however higher concentrations absorbed excessively at 340 nm and thereby interfered. Sulfuric acid in correspondingly small amounts was readily buffered in the glycine–NaOH reaction system. Thin-layer eluates of 3α,7α,12α-trihydroxy-5β-cholanoate, 3α,7α-dihydroxy-12-keto-5β-cholanoate and 3α,12α-dihydroxy-7-keto-5β-cholanoate from a sprayed plate were tested as substrates. In each case, the presence of the appropriate OH-group, a measurable evolution of NADH occurred on addition of 3α-HSDH or 7α-HSDH or 7α-HSDH. Approximately 1.5 μg of bile salt was required for enzymatic detection. The color complex when eluted into a cuvette, caused a somewhat variable increase in background, but did not interfere in the enzymatic reactions. Although enzymatic reactions could be observed to go to "completion", no attempt was made to obtain quantitative results.

Because of the fairly mild conditions required for detection of bile salts and sterols with Komarowsky's reagent[6], this spray reagent is essentially non-destructive; its use with appropriate solvent systems enables the investigator to obtain rapid preliminary structural evidence on the basis of 3 criteria: mobility, color and reactivity with enzymes of established position and stereospecificity. Preliminary evidence suggests that this reagent can also be used with two independent sources of 12α-HSDH and can serve as a valuable aid in the identification of microbial degradation products[10].

It must be emphasized that the last criterion depends strongly on substrate specificity pattern of the enzyme involved. For example, Haslewood[11] has demonstrated that sulfation of the 3 position of cholate (or side chain elongation) will abolish reactivity of 7α-HSDH at the 7α-OH position. Similarly we have demonstrated[10] that oxidation of the 3α-OH group to the ketone will drastically reduce reactivity of the molecule with 7α-HSDH. Additionally, since the visualization of bile salts is more sensitive than the enzymatic detection, insufficient amounts may be present in the eluate for the latter. Thus a positive enzyme reaction with an unknown compound may be used for evidence that a given OH-group is present but a negative test in the absence of other evidence may not permit an investigator to claim the absence of this OH-group in the molecule. The importance of the investigation of substrate specificity patterns of recently discovered enzymes[11–13] cannot be understated.

ACKNOWLEDGEMENT

This work was supported by Grant MA 5075 from the Medical Research Council of Canada and by the National Cancer Institute.

REFERENCES

1 R. Neher, in E. Stahl (Editor), *Thin-Layer Chromatography: A Laboratory Handbook*, Springer, Heidelberg, 1969, p. 311.
2 W. L. Anthony and W. T. Beher, *J. Chromatogr.*, 13 (1964) 567.
3 D. Kritchevsky, D. S. Martak and G. H. Rothblat, *Anal. Biochem.*, 5 (1963) 388.
4 T. F. Kellogg, *J. Lipid Res.*, 11 (1970) 498.
5 S. K. Goswami and C. F. Frey, *J. Chromatogr.*, 89 (1974) 87.
6 P. J. Stevens, *J. Chromatogr.*, 14 (1964) 269.
7 I. A. Macdonald, C. N. Williams and D. E. Mahony, *Anal. Biochem.*, 57 (1974) 127.

8 B. P. Lisboa, *J. Chromatogr.*, 16 (1964) 136.
9 P. Eneroth, *J. Lipid Res.*, 4 (1963) 11.
10 I. A. Macdonald, T. P. Forrest, G. A. Costain and B. G. Rao, unpublished results.
11 E. S. Haslewood and G. A. D. Haslewood, *Biochem. J.*, 157 (1976) 207.
12 I. A. Macdonald, C. N. Williams, D. E. Mahony and W. H. M. Christie, *Biochim. Biophys. Acta*, 384 (1975) 12.
13 I. A. Macdonald, C. N. Williams and D. E. Mahony, *Proc. Can. Fed. Biol. Soc.*, Abstract 18 (1975) 130.

Journal of Chromatography, 136 (1977) 353–357

CHROM. 9981

Note

Thin-layer chromatography of Sudan dyes

P. N. MARSHALL

Department of Haematology, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Rd., London W.12 OHS (Great Britain)

(First received October 26th, 1976; revised manuscript received January 31st, 1977)

The Sudan dyes are widely used for the histological demonstration of fats[1]. However, few rapid and effective methods for the quality control of these dyes appear to have been published. Most analytical methods have been paper chromatographic ones[2–12] which are extremely slow and sometimes of low resolution. By and large, rapid analytical thin-layer chromatographic (TLC) methods have been devised primarily to distinguish between the various Sudan dyes and not to separate the impurities of individual dyes[5,13–17]. The majority of these methods are therefore unsuitable for the quality control of individual dyes.

In this paper a rapid, highly effective TLC system for the quality control of Sudan dyes is described. Analytical data on commercial samples of various dyes are also presented.

MATERIALS AND METHODS

Reagents and equipment

Acetone, benzene and chloroform of AnalaR grade were used. The various Sudan dye samples studied are listed in Tables I–V.

Aluminium-backed, silica gel TLC sheets were obtained from Merck (type 5553, 200 × 200 mm without fluorescent indicator, adsorbent thickness 0.25 mm). Disposable micropipettes (Microcaps®, Drummond, Broomall, Pa., U.S.A.) or a special applicator (serum applicator 51225, Gelman, Ann Arbor, Mich., U.S.A.) was used for sample application. Chromatograms were developed in a sandwich tank (Eastman Chromagram developing apparatus 6071).

Chromatography

Two microlitres of dye solutions (about 4 mg/ml in acetone) were applied to unactivated TLC sheets as bands 20 mm in length. The developing solvent consisted of benzene–chloroform (10:1, v/v). Development was performed in darkness.

RESULTS AND DISCUSSION

Several reports, *e.g.* those of Schweppe[16], Walker and Beroza[17] and Jordan[18,19], indicate that good separations of Sudan dyes may be obtained on thin layers of silica

TABLE I

TLC DATA ON COMMERCIAL SAMPLES OF OIL BLUE N (CI 61555), SUDAN BLUE (NO CI NO.), SUDAN BLUE GA (CI 61525) AND SUDAN GREEN (CI 62545)

m = Major component; t = trace; i = intermediate amount. Data on components: (no., mean R_F value, colour): 1 = immobile, grey; 2 = immobile, brown; 3 = 0.05, mauve; 4 = 0.09, purple; 5 = 0.10, blue; 6 = 0.13, mauve; 7 = 0.17, blue; 8 = 0.26, blue; 9 = 0.31, blue; 10 = 0.32, pink; 11 = 0.39, blue; 12 = 0.44, mauve; 13 = 0.46, mauve; 14 = 0.46, yellow; 15 = 0.51, orange; 16 = 0.62, mauve; 17 = 0.64, blue; 18 = 0.68, yellow.

Dye, supplier and batch no.	*Component*																
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*
Oil Blue N																		
Difco, 1358								t			m							
Gurr, 23619	t		t	i		t	m	m			m		t			t		
R. A. Lamb, 0572	t			t		t	m	m	t		m							
Sudan Blue																		
Difco, 1040		i						m				i			t			
Gurr, 0886		t								i	m							
Sudan Blue GA																		
R. A. Lamb, 0486	t				t			m				t						
Sudan Green																		
Difco, 1041	t							m							t		t	m
Gurr, 1372	t							m							t			m
R. A. Lamb, 0488	i				t			m				t		t	t			m

TABLE II

TLC DATA ON COMMERCIAL SAMPLES OF OIL RED O (CI 26125), SUDAN II (CI 12140), SUDAN III (CI 26100) AND SCARLET R (MICHAELIS) (CI 26105)

m = Major component; t = trace; i = intermediate amount. Data on components (no., mean R_F value, colour): 1 = immobile, orange; 2 = immobile, brown; 3 = 0.06, brown; 4 = 0.08, brown; 5 = 0.12, brown; 6 = 0.19, brown; 7 = 0.31, yellow; 8 = 0.40, maroon; 9 = 0.41, yellow; 10 = 0.51, maroon; 11 = 0.56, orange; 12 = 0.59, pink; 13 = 0.62, amber; 14 = 0.65, yellow; 15 = 0.68, orange; 16 = 0.73, pink.

Dye, supplier and batch no.	*Component*															
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*
Oil Red O																
Difco, 0210		t			t	t		i		m	i	i				
Gurr, 0770		t						t		m						
R.A. Lamb, 3682		t			t	t	t	i		m		i				
Sudan II																
Gurr, no batch no.											m		i		m	
R. A. Lamb, no batch no.	i		m													
Sudan III																
Difco, 2374									t		m			t		t
Gurr, 71427		t						i		m	m		i			
R. A. Lamb, 4308									t		m					t
Scarlet R (Michaelis)																
Difco, 0686		i		t		t		m		m	m	m				
Gurr, no batch no.		i		t		t		m		m	m	m				
R. A. Lamb, 3626		i		t		t		m		m	m	m				

TABLE III

TLC DATA ON COMMERCIAL SAMPLES OF SUDAN BLACK B (CI 26150) AND ACETYLATED SUDAN BLACK B (NO CI NO.)

m = Major component; t = trace; i = intermediate amount. Data on components (no., mean R_F value, colour): 1 = immobile, brown; 2 = 0.02, brown; 3 = 0.04, brown; 4 = 0.08, brown; 5 = 0.11, black; 6 = 0.29, brown; 7 = 0.37, brown; 8 = 0.45, brown; 9 = 0.49, blue-black; 10 = 0.52, grey; 11 = 0.57, grey; 12 = 0.90, grey-green; 13 = 0.96, pink.

Dye, supplier and batch no.	*Component*												
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*
Sudan Black B													
Difco, 1870	i	i	i	t	m				m	t	t	t	t
Gurr, 80167	i	i	i	t	m				m	t	t	t	t
R. A. Lamb, 2715	i	i	i	t	m	t	i	i	m	t	t	t	t
Acetylated Sudan Black B													
Difco, 1549	i	i	i	t	m	t	i	i	m	t	t	t	t
Gurr, MN 1058	i	i	i	t	m	t	i	i	m	t	t	t	t
R. A. Lamb, 1994	i	i	i	t	m	t	i	i	m	t	t	t	t

TABLE IV

TLC DATA ON COMMERCIAL SAMPLES OF SUDAN BROWN (CI 12020)

m = Major component; t = trace; i = intermediate amount. Data on components (no., mean R_F value, colour): 1 = immobile, brown; 2 = 0.04, brown-orange; 3 = 0.15, brown; 4 = 0.25, brown-red; 5 = 0.31, brown-red; 6 = 0.50, orange; 7 = 0.62, red; 8 = 0.74, brown; 9 = 0.86, pink; 10 = 0.92, grey; 11 = 0.97, green-yellow.

Supplier and batch no.	*Component*										
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*
Difco, 0206	m	i	m	t	t	m	m	t	i	i	t
Gurr, 14517	m	i	m	t	t	m	m	t	i	i	t
R. A. Lamb, 0487	m	i	m	t	t		m		i	i	t

TABLE V

TLC DATA ON COMMERCIAL SAMPLES OF SUDAN YELLOW (CI 12055)

m = Major component; t = trace; i = intermediate amount. Data on components (no., mean R_F value, colour): 1 = immobile, cerise; 2 = 0.46, orange; 3 = 0.55, mauve; 4 = 0.60, orange; 5 = 0.65, yellow; 6 = 0.77, orange.

Supplier and batch no.	*Component*					
	1	*2*	*3*	*4*	*5*	*6*
Difco, 0267	i	m	t	t	t	t
Gurr, 23813	t	m				t
R. A. Lamb, 3660		m				

using benzene as the developing solvent. In the author's hands a mixture of benzene and chloroform proved much more effective.

Chromatographic data on commercial Sudan dyes are shown in Tables I–V, where "m" indicates a major component, "t" a trace one and "i" one present in an intermediate amount. A developed chromatogram is shown in Fig. 1.

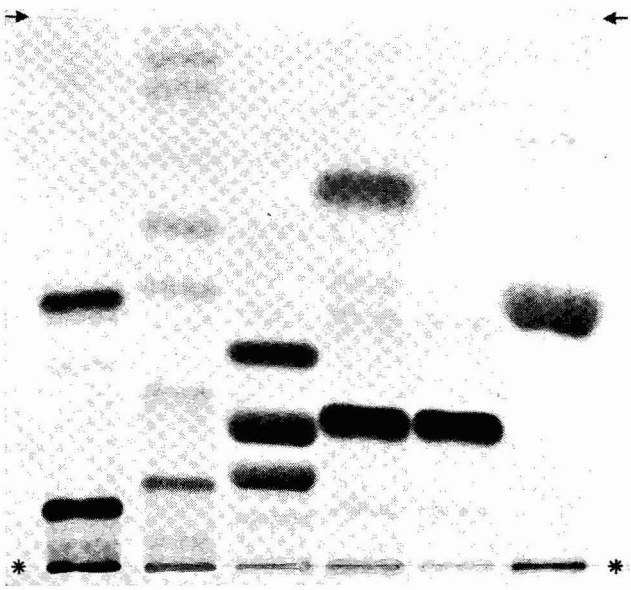


Fig. 1. Thin-layer chromatogram of commercial samples of Sudan dyes. Samples are from left to right: Sudan Black B (R. A. Lamb, 2715); Sudan Brown (Gurr, 14517); Oil Blue N (Gurr, 23619); Sudan Green (R. A. Lamb, 0488); Sudan Blue GA (R. A. Lamb, 0486) and Sudan Yellow (Difco 0267). Asterisks indicate the origin, arrows the solvent front. The chromatogram has been somewhat overloaded in order to demonstrate as many components as possible. In spite of this, certain trace components may not be clearly visible.

Oil Blue N, Sudan Blue, Sudan Blue GA and Sudan Green (Table I)

Most of these dyes were extremely heterogeneous. With the samples of Oil Blue N, Sudan Blue and Sudan Blue GA it is impossible to decide which, if any, of the major components correspond to the nominal ones. The samples of Sudan Green do not correspond to the Colour Index[20] formulation since they contain no green dye. They are, in fact, merely mixtures of a blue Sudan dye with an unidentified yellow component.

Oil Red O, Sudan II, Sudan III and Scarlet R (Michaelis) (Table II)

The majority of the samples of these dyes were complex mixtures. Those of Oil Red O contained a single major component, which was also identified in samples of Scarlet R (Michaelis). Coloured impurities in Oil Red O have previously been detected using paper chromatography[5,8] and TLC[5,21]. The two samples of Sudan II examined were completely dissimilar; one contained a large proportion of an orange component identified in the samples of Sudan III and Scarlet R (Michaelis). Previous authors have detected coloured impurities in Sudan II by paper chromatography[6] and TLC[16,22]. Sudan III and Scarlet R (Michaelis) contained several common components. Indeed, it is alleged that some commercial samples of Sudan III are mixtures of the nominal dye and Scarlet R (Michaelis)[20]. Impurities in these dyes have been identified previously by both paper chromatography[2,3,5,6,11] and TLC[5,16].

Sudan Black B and Acetylated Sudan Black B (Table III)

Sudan Black B is an extremely heterogeneous product. The three samples contained the same pair of major components. Ten components were common to all samples and these may correspond to the fractions isolated by column chromatography[23]. Side reactions occuring during the synthesis of Sudan Black B may produce as many as seven contaminants[24]. The number of components in fact detected may perhaps indicate the presence of *cis*- and *trans*-isomers.

Coloured impurities in this dye have been detected by many authors using gel-filtration chromatography[25], paper chromatography[7,10] and TLC[5,16,18,19].

Surprisingly, the samples of Acetylated Sudan Black B contained no component that was not present in the parent dye.

Sudan Brown (Table IV)

Samples of this dye contained up to eleven coloured components.

Sudan Yellow (Table V)

All samples of this dye contained the same major component, possibly the nominal one. The sample from R. A. Lamb was exceptional amongst those of the Sudan dyes examined in that it was free from detectable coloured contaminants.

REFERENCES

1 R. D. Lillie, *Histopathologic Technic and Practical Histochemistry*, McGraw-Hill, New York, 3rd ed., 1965, p. 454.
2 J. F. Christman and R. H. Trubey, *Stain Technol.*, 27 (1952) 53.
3 J. F. Christman and H. J. Werner, *Stain Technol.*, 28 (1953) 259.
4 J. Gasparič and M. Matrka, *Collect. Czech. Chem. Commun.*, 25 (1960) 1969.
5 R. W. Horobin and L. B. Murgatroyd, *Histochemie*, 11 (1967) 141.
6 H. Kutt, D. Lockwood and F. McDowell, *Stain Technol.*, 34 (1959) 197.
7 H. Kutt, D. Lockwood and F. McDowell, *Stain Technol.*, 34 (1959) 203.
8 H. Kutt and T. T. Tsaltas, *Clin. Chem.*, 5 (1959) 149.
9 W. Lindberg, *Z. Lebensm.-Unters.-Forsch.*, 103 (1956) 1.
10 S. I. Rosenthal, H. Puchtler and F. Sweat, *Arch. Pathol.*, 80 (1965) 190.
11 R. H. Trubey and J. F. Christman, *Stain Technol.*, 27 (1952) 87.
12 M. R. Verma and R. Dass, *J. Sci. Res. (Hardwar, India)*, 16B (1957) 131.
13 J. W. Copius-Peereboom and H. W. Beekes, *J. Chromatogr.*, 20 (1965) 43.
14 J. Davídek and G. Janíček, *J. Chromatogr.*, 15 (1964) 542.
15 R. A. Hoodless, J. Thomson and J. E. Arnold, *J. Chromatogr.*, 56 (1971) 332.
16 H. Schweppe, in E. Stahl (Editor), *Thin-layer Chromatography, A Laboratory Handbook*, Springer, Berlin, Heidelberg, New York, 2nd ed., 1969, p. 612.
17 K. C. Walker and M. Beroza, *J. Ass. Offic. Agr. Chem.*, 46 (1963) 250.
18 D. M. Jordan, *J. Chromatogr.*, 57 (1971) 427.
19 D. M. Jordan, *J. Chromatogr.*, 63 (1971) 442.
20 *The Colour Index*, Society of Dyers and Colourists, Bradford, 2nd ed., 1956.
21 E. V. Truter, *J. Chromatogr.*, 14 (1964) 57.
22 J. G. Kirchner, *J. Chromatogr.*, 63 (1971) 45.
23 E. W. Bermes and H. J. McDonald, *Arch. Biochem. Biophys.*, 70 (1957) 49.
24 J. Y. Terner, J. Schnur and J. Gurland, *Lab. Invest.*, 12 (1963) 405.
25 R. W. Horobin, *Stain Technol.*, 46 (1971) 297.

Journal of Chromatography, 136 (1977) 358–359

CHROM. 10,064

Book Review

Advances in chromatography, Vol. 14, edited by J. C. Giddings, E. Grushka, J. Cazes and P. R. Brown, Marcel Dekker, New York, Basel, 1976, xv + 459 pp., price SFr. 114.00, ISBN 0-8247-6436-6.

In a rapidly advancing field like chromatography, even advances become rapidly outdated. Most chapters in the last volume of the "Advances" had been written before 1975 and when the book finally finds its place on the library shelf the reader's excitement will be as yesteryears' snow. It is hoped that fresh blood will be infused and the series rejuvenated by the enlarged team of editors, so that it will present up to date accounts of new developments in the various branches of chromatography.

The first chapter by A. J. Clifford is an invitation to apply high-performance liquid chromatography in the field of nutrition. It is not very clear whether nutritionists and food technologists are invited to use liquid chromatography or chromatographers should turn to nutritional samples for analysis. Nevertheless a nice, if somewhat outdated, account is given of the chromatography of various purines and folate coenzyme forms. It is quite stimulating that the various techniques mainly developed in the realm of physiological chemistry find important applications in the nutritional field which becomes increasingly sophisticated. B. Stenlund's chapter on "Polyelectrolyte effects in gel chromatography" goes back to P. Flodin's dissertation and elaborates on the role of the fixed charges on the Sephadex matrix in gel filtration. The treatment is mainly concerned with the behaviour of lignosulfonates on Sephadex G-50 columns and the last reference is from 1970.

The minichapter by I. Sebestian and I. Halász describes chemically bonded phases in both gas and liquid chromatography on ten pages. The authors made significant contributions to the development of bonded phases, which are the workhorses of today's liquid chromatography, and their treatment written in 1974 makes an interesting reading. D. C. Locke wrote a delightful essay on "Physicochemical measurements using chromatography" with a minimum of equations so that the less mathematically inclined reader can follow what has been done in this field. The addendum to this chapter at the end of the book should not be missed. "Gas–liquid chromatography in drug analysis" is the title of the chapter by W. J. A. VandenHeuvel and A. G. Zacchei, who write exactly that what they promise and present a solid treatment of the subject by covering the pertinent literature of the early seventies. C. L. de Ligny's chapter is on "The investigation of complex association by gas chromatography and related chromatographic and electrophoretic methods". The field of complexation in chromatography is in a state of convulsion at present and we hope in the parturience of a clearer understanding of what is going on. Liquid chromatography is just about to move into this field strongly. This chapter mainly covers the literature until 1974 with some recent references postfixed. "Gas–liquid–solid chromatography" is the title of the chapter by A. di Corcia and A. Liberti. Whereas the

technique is widely practiced, mostly unintentionally, the authors restrict themselves to present a well written account of their own work on liquid coated graphited carbon black as the stationary phase in gas chromatography. J. K. Haken's chapter, "Retention indices in gas chromatography" considers "the abscissa response on the usual differential chromatographic record" and the "abscissa relationship and its expression principally as retention indices for the specification of retention". By and large the topics of the chapters are balanced and the book is well produced. It presents useful information and offers stimulating reading in spite of the delay in its publication.

New Haven, Conn. (U.S.A.) CSABA HORVATH

Journal of Chromatography, 136 (1977) 359–360

CHROM. 10,065

Book Review

Gas chromatography of polymers, (Journal of Chromatography Library, Vol. 10), by V. G. Berezkin, V. R. Alishoyev and I. B. Nemirovskaya, Elsevier, Amsterdam, Oxford, New York, 1977, XIII + 225 pp., price Dfl. 103.00, US$ 41.95, ISBN 0-444-41514-9.

The English edition of this book which originally appeared in Russian in 1972 is said to incorporate many additions which reflect the extent of the increasingly important analytical role played by gas chromatography (GC) in the area of polymer chemistry. The title is therefore perhaps misleadingly restrictive for a large part of the text is concerned with the scrutiny of monomers, solvents etc. and also of the many polymerisation processes themselves.

A brief survey of the theory and practice of GC with suitable emphasis upon quantitative procedures is followed by a comprehensive survey of raw material quality control. The chapter "The study of polymer formation reactions" rightly describes the advantages accruing from the use of GC as opposed to, for example, dilatometry but disappointingly cites little or nothing exemplary of the many high-volume polymerisation processes which are of major industrial importance; indeed emulsion polymerisation is almost completely ignored.

Detail is restored in consideration of the determination of volatiles in polymeric systems which is treated at length. The alternative procedure of cold extraction as a means of combatting problems peculiar to thermolabile samples appears to have been overlooked.

Polymer reactions are examined in depth and sensible import accorded techniques of, for example, oxidative degradation. Regrettably there are no observa-

tions on weathering and artificial weathering studies wherein GC methods have been widely used.

Both reaction GC and pyrolysis GC enjoy the most comprehensive of summaries and it may be fairly said that this is as good a text as any presently available and should be of considerable value to workers in these fields.

The concluding chapter deals effectively with applications of inverse phase chromatography to polymer identification, the determination of molecular weight, interaction and phase transition studies and the investigation of the kinetics and equilibria of polymer reactions.

Throughout one is impressed by the thoroughness and accuracy of referencing; how the authors overlooked the S.C.I. Symposium on High Temperature Resistance and Thermal Degradation of Polymers (S.C.I. Monograph 13, 1961) must remain a mystery as so much that could be described as obscure has been located. Pertinent references are occasionally divorced from the actual statements which could be a little confusing and it is a pity that diagrams/figures frequently fail to bear indication of origin.

The text is singularly free from typographical error and although the language is at times quaint the book is eminently readable, this latter point is particularly important in that the authors have produced a useful source-book for polymer technology students. A broader horizon and more detail would have resulted in an acceptable laboratory manual appealing to a far wider readership.

Even though the lay-out of the contents is systematic and definitive, the lack of an index is to be deprecated; it is a considerable annoyance to search for something only to discover it be not there.

Redhill (Great Britain) C. E. ROLAND JONES

Vol. 136, No. 2

journal of
chromatography news section

MEETING

TWELFTH INTERNATIONAL SYMPOSIUM ON ADVANCES IN CHROMATOGRAPHY
"CHROMATOGRAPHY '77"

The Twelfth International Symposium on Advances in Chromatography will be held November 7–10, 1977 at the International Congress Centre RAI, in Amsterdam, The Netherlands.

A total of 84 papers will be presented at the Symposium representing contributions from 20 countries. A special feature of the meeting will be an exposition of the latest instrumentation and books. There will also be informal discussion groups held during the course of the Symposium.

Registration should be made in advance. The programs, registration forms and hotel reservation cards can be obtained from:

Organisatie Bureau Amsterdam B.V.,
International Congress Centre RAI,
P.O. Box 7205,
Europaplein 14,
Amsterdam,
The Netherlands,

or

Prof. A. Zlatkis,
Chemistry Department,
University of Houston,
Houston, Texas 77004,
U.S.A.

The detailed program of the Symposium is given below.

Monday, November 7, 1977

NEW HORIZONS

L.S. Ettre, presiding

9.00	G. Dijkstra, Chairman, Royal Dutch Chemical Society (K.N.C.V.): Welcome to Symposium
9.15	Presentation of the M.S. Tswett Chromatography Medals
9.30	J.E. Lovelock and A.J. Watson (Bowerchalke, Great Britain) – Electron capture: theory and practice, 2
10.00	Egon Stahl (Universität des Saarlandes, Saarbrücken, G.F.R.) – Extraction with supercritical gases in coupling with TLC
10.30	I. Halász (Universität des Saarlandes, Saarbrücken, G.F.R.) – New horizons and problems in HPLC
11.00	Intermission

G. Guiochon, presiding

11.10 J.C. Giddings and M.N. Myers (University of Utah, Salt Lake City, Utah, U.S.A.) – Biological applications of field-flow fractionation (one-phase chromatography)

11.30 W. Al-Thamir, R.J. Laub and J.H. Purnell (University College of Swansea, Swansea, Great Britain) – Separation of all C_1–C_4 hydrocarbons by multi-substrate gas–solid–liquid chromatography

11.50 A.A. Zhukovitskii and S.M. Yanovskii (Institute of Steel and Alloys, Moscow, U.S.S.R.) – Different variants of chromadistillation

12.10 D.H. Desty and A. Douglas (British Petroleum Research Centre, Sunbury-on-Thames, Great Britain) – Non-round capillary columns for gas chromatography

GAS CHROMATOGRAPHY

C.A. Cramers, presiding

14.00 C. Vidal-Madjar and G. Guiochon (École Polytechnique, Palaiseau, France) – Experimental characterization of elution profiles using the central statistical moments and their relation with the mass transfer kinetics of the column

14.20 G. Schomburg (Max Planck Institut für Kohlenforschung, Mülheim-Ruhr, G.F.R.) – Sampling techniques in capillary gas chromatography

14.40 R.D. Schwartz, R.G. Mathews and N.A. Pedro (Pennzoil Company, Shreveport, La., U.S.A.) – High-temperature gas–liquid chromatography with a dentritic salt support

15.00 S. Nygren (The National Food Administration, Uppsala, Sweden) – Summary of exponential flow programming in gas chromatography

15.20 F.I. Onuska, P.D. Goulden, M.E. Comba and R.J. Wilkinson (Canada Centre for Inland Waters, Burlington, Ontario, Canada) – Surface-modified wide-bore wall-coated open tubular columns, their preparation and application in trace analysis

15.40 Intermission

ENVIRONMENTAL PROBLEMS

R.E. Kaiser, presiding

15.50 R.E. Sievers, R.H. Shapiro, H.F. Walton, R.M. Barkley, G.A. Eiceman, L.R. Field and J.H. Duncan (University of Colorado, Boulder, Colo., U.S.A.) – Chromatographic analysis of environmental pollutants

16.10 G. Holzer, H. Shanfield and A. Zlatkis (University of Houston, Houston, Texas, U.S.A.), W. Bertsch, P. Juarez and H. Mayfield (University of Alabama, University, Ala., U.S.A.) and H.M. Liebich (Medizinische Universitätsklinik Labor, Tübingen, G.F.R.) – Collection and analysis of trace organic emissions from natural sources

16.30 J.F.K. Huber and R. Becker (Institut für Analytische Chemie der Universität, Vienna, Austria) Enrichment of trace compounds by displacement column liquid chromatography

16.50 B.S. Middleditch, B. Basile and E.S. Chang (University of Houston, Houston, Texas, U.S.A.) – Environmental effects of offshore oil production: Alkanes.

17.10 B.V. Ioffe, V.A. Isodorov and I.G. Zenkevich (University of Leningrad, Leningrad, U.S.S.R.) – Gas chromatographic–mass spectrometric determination of organic volatiles in an urban atmosphere

Tuesday, November 8, 1977

BIOCHEMICAL GAS CHROMATOGRAPHY

H. Poppe, presiding

9.00 A. Karmen and N.S. Longo (Albert Einstein College of Medicine, Bronx, N.Y., U.S.A.) – High-sensitivity radioassay by gas chromatography

9.20 C.M. Wels (Wellcome Research Laboratories, Berkhamsted, Great Britain) – A high-sensitivity radio gas chromatography detector for single- or dual-labelled compounds

9.40 S.J. Gaskell and C.J.W. Brooks (The University, Glasgow, Great Britain) – GC–MS of phospholipids after enzymic hydrolysis

10.00 P. Hartvig, N.O. Anfelt and K.E. Karlsson (University of Uppsala, Uppsala, Sweden) – Electron-capture gas chromatography of tertiary amines as trichloroethyl or pentafluorobenzyl carbamates

10.20 Intermission

G. Dijkstra, presiding

10.30 E.C. Horning, D.I. Carroll, I. Dzidic, S.-N. Lin, R.N. Stillwell and J.-P. Thenot (Baylor College of Medicine, Houston, Texas, U.S.A.) – Atmospheric pressure ionization mass spectrometry. Studies of negative ion formation for detection and quantification purposes

10.50 S. Goodman, P. Helland, A. Flatmark, O. Stokke and E. Jellum (University of Oslo, Oslo, Norway) – Organic acid profiles of human tissues determined by capillary GC MS

11.10 H.M. Liebich and J. Wöll (Medizinische Universitätsklinik Labor, Tübingen, G.F.R.) – Volatile substances in blood serum – Profile analysis and quantitative determination

11.30 B. Davis, D. Durden, P. Pun-Li and A. Boulton (University Hospital, Saskatoon, Saskatchewan, Canada) – The detection and quantitation of the acid metabolites of some aryl alkyl amines

11.50 E. Reiner and F.L. Bayer (Emory University, Atlanta, Ga., U.S.A.) – Differentiation of medically important substances by means of pyrochromatography

12.10 H.-Ch. Curtius, M. Zagalak, W. Leimbacher and E. Redweik (University Childrens Clinic, Zurich, Switzerland) – Quantitation of phenylalanine and tyrosine in plasma using multiple-ion detection method

THIN-LAYER CHROMATOGRAPHY

M. Lederer, presiding

14.00 V. Pretorius and B.J. Hopkins (University of Pretoria, Pretoria, Republic of South Africa) – A TLC detector employing a photo-diode array

14.20 H. Shanfield and A.J.P. Martin (University of Houston, Houston, Texas, U.S.A.) – The use of activated nitrogen as a general visualizing agent in thin-layer chromatography

14.40 D.C. Fenimore, C.J. Meyer and C.M. Davis (Texas Research Institute of Mental Sciences, Houston, Texas, U.S.A.) and F. Hsu and A. Zlatkis (University of Houston, Houston, Texas, U.S.A.) – Determination of psychotropic drugs in blood serum by high-performance TLC

15.00 J.A. Vinson, J.A. Nebzydoski and L. Shebby (University of Scranton, Scranton, Pa., U.S.A.) - Preadsorbent thin-layer chromatography: Detection of lipid abnormalities in serum without extraction

15.20 R.E. Kaiser (Institut für Chromatographie, Bad Dürkheim, G.F.R.) – C_8 and C_{18} reversed-phase high-performance TLC on chemically bonded layers for environmental trace analysis and for high-performance liquid chromatography optimization

15.40 Intermission

R. Segura, presiding

15.50 D.B. Faber (Vrije Universiteit, Amsterdam, The Netherlands) – Quantitation with high-performance TLC and PMD with HP-micro thin-layer material for drug analyses in biological fluids

16.10 H. Jork and B. Roth (Universität des Saarlandes, Saarbrücken, G.F.R.) – A comparison between HPTLC, HPLC and LC in triazine investigation

16.30 W. Ritter (Bayer AG, Wuppertal, G.F.R.) – Thin-layer densitometric assay in the nanogram range of BAY g 2821, a structurally new diuretic drug, in biological fluids

16.50 H. Halpaap and K.F. Krebs (E. Merck, Darmstadt, G.F.R.) – TLC- and HPTLC-ready-for-use preparations with concentrating zones

Wednesday, November 9, 1977

INFORMAL DISCUSSION GROUPS

9.00 High-performance thin-layer chromatography. Chairman: V. Pretorius; starter: R.E. Kaiser

10.00 Environment. Chairman: W. Bertsch; starter: R.E. Sievers

11.00 Selective detectors. Chairman: E. Bayer; starter: W. Aue

14.00 Liquid chromatography. Chairman: I. Halász; starter: R.P.W. Scott

15.00 Biomedical gas chromatography. Chairman: M.G. Horning; starter: A. Karmen

16.00 High-resolution columns. Chairman: G. Schomburg; starter: D.H. Desty

POSTER SESSIONS (15.30–19.00 h)

GAS CHROMATOGRAPHY

1 R. Annino and R. Voyksner (Canisius College, Buffalo, N.Y., U.S.A.) – Operating characteristics of a pneumatic detector suitable for gas chromatography

2 W.A. Aue and C.G. Flinn (Dalhousie University, Halifax, Nova Scotia, Canada) – A photometric tin detector for gas chromatography

3 A.G. Smith and C.J.W. Brooks (The University, Glasgow, Great Britain) – GC–MS of thromboxane B_2 derivatives

4 P.H. Degen (Ciba–Geigy, Basel, Switzerland) – The quantitative determination of drugs in biological material by means of extractive alkylation and GLC

5 W. Engewald, G. Mann and M. Sieler (Karl-Marx-Universität, Leipzig, G.D.R.) – Retention behavior of methylcyclohexanes in gas–liquid and gas–solid chromatography

6 C. Sunol and E. Gelpi (Instituto de Biofisica y Neurobiologia, Barcelona, Spain) – Direct GLC–MS connection of glass capillary columns for the analysis of serotonine and metabolites by selected ion monitoring

7 J.F.K. Huber and E. Kenndler (Institut für Analytische Chemie der Universität, Vienna, Austria) – Quantitation of the information attainable from identification data in multi-dimensional gas chromatography and mass spectrometry and application in doping analysis

8 G. Eklund, B. Josefsson and C. Roos (University of Göteborg, Göteborg, Sweden) – Gas chromatography of monosaccharides in the picogram range using glass capillary columns, trifluoroacetyl derivatization and electron capture detection

9 A. Need, N.S. Longo and A. Karmen (Albert Einstein College of Medicine, Bronx, N.Y., U.S.A.) – Specific nitrogen detection for gas chromatography by wet chemical methods

10 M.M. Kopečni, Z.E. Ilić and S.K. Milonjić (Boris Kidrič Institute of Nuclear Sciences, Belgrado, Yugoslavia) – Investigation of tri-*n*-butyl phosphate–organic diluents system by gas-liquid chromatography

11 W.J.J. Leunissen and J.H.H. Thijssen (Eindhoven University of Technology, Eindhoven, The Netherlands) – Gas chromatographic determination of steroid profiles from urine; a quantitative study

12 E. Matisová and J. Krupčík (Slovak Technical University, Bratislava, Czechoslovakia) – Capillary gas chromatography of *s*-triazines

13 J.A. Moulijn, R. Spijker and J.F.M. Kolk (Institute for Chemical Technology, Amsterdam, The Netherlands) – Axial dispersion of gases flowing through coiled columns

14 S. Wičar, J. Novák, J. Drozd and J. Janák (Institute of Analytical Chemistry, Czechoslovak Academy of Sciences, Brno, Czechoslovakia) – Retention volume in high-pressure gas chromatography. Systems squalane–carbon tetrachloride, isooctane, toluene–nitrogen, hydrogen, carbon dioxide

15 B. Plazonnet (Merck, Sharp and Dohme, Clermont-Ferrand, France) and W.J.A. Vanden Heuvel (Merck, Sharp and Dohme, Rahway, N.J., U.S.A.) – Preparation, gas chromatography and mass spectrometry of methyl and trimethylsilyl esters of indomethacin

16 L. Soják (Chemical Institute of Comenius University, Bratislava, Czechoslovakia), J. Janák (Institute of Analytical Chemistry, Czechoslovak Academy of Sciences, Brno, Czechoslovakia) and J.A. Rijks (Eindhoven University of Technology, Eindhoven, The Netherlands) – Capillary gas chromatography of alkylbenzenes. IV. Correlations on two and/or three stationary phases

17 J. Ševčík (Packard-Becker B.V., Delft, The Netherlands) – The information content of multi-dimensional switching systems

18 W. Bruening and F.G.M. Concha (Petroleo Brasileiro S.A., Rio de Janeiro, Brazil) – Improved ozone chemiluminescence detector

LIQUID CHROMATOGRAPHY

19 S.R. Bakalyar and R. McIlwrick (Spectra-Physics, Santa Clara, Calif., U.S.A.) – Improving resolution in reversed-phase high-performance liquid chromatography by using secondary solvent effects: Ternary programming, trace modifiers, dissolved gases

20 J.J. de Kok, U.A.Th. Brinkman and A. de Kok (Free Reformed University, Amsterdam, The Netherlands) – The analysis of polybrominated biphenyls

21 J.E. Greving, J.H.G. Jonkman, F. Fiks and R.A. de Zeeuw (State University, Groningen, The Netherlands) – Determination of oxyphenonium bromide in plasma and urine by means of ion-pair extraction, derivatization and electron capture gas chromatography

22 S. Hara and S. Hayashi (Tokyo College of Pharmacy, Tokyo, Japan) – The correlation of retention behaviors of the steroidal pharmaceuticals in polar and bonded reversed-phase liquid column chromatography

23 J. Krupčík (Slovak Technical University, Bratislava, Czechoslovakia), J. Kříž and D. Prušová (Institute of Chemical Technology, Prague, Czechoslovakia) and Z. Červenka and P. Suchánek (Chemko, Strazske, Czechoslovakia) – Analysis of PCBs by capillary gas chromatography and high-performance liquid chromatography

24 C. Merritt, Jr. and S.G. Kayser (U.S. Army Natick Development Center, Natick, Mass., U.S.A.) and B.A. Bidlingmeyer (Waters Associates, Milford, Mass., U.S.A.) – Application of a porous polymer bead column for liquid chromatographic separation of polyol polyesters

25 R.A.A. Muzzarelli (Università Degli Studi di Ancona, Ancona, Italy) – Advances in the chromatography of chitosan

26 F.E. Rickett (Wellcome Research Laboratories, Berkhamsted, Great Britain) – Use of HPLC to separate very small quantities of radio-labelled metabolites from biological tissue

27 F.P.B. van der Maeden, P.T. van Rens and F.A. Buytenhuys (Akzo Research Laboratories, Arnhem, The Netherlands) – Quantitative analysis of *d*-tubocurarine chloride in curare by liquid column chromatography

28 H.G.M. Westenberg, B.F.H. Drenth and R.A. de Zeeuw (State University, Groningen, The Netherlands) – Analysis of clomipramine and desmethylclomipramine in plasma by means of liquid chromatography

29 W.S.M. Geurts van Kessel (State University of Utrecht, Utrecht, The Netherlands) and W.M.A. Hax (Philips Research Laboratories, Eindhoven, The Netherlands) – High-performance liquid chromatography separation and direct UV detection of phospholipids

THIN-LAYER CHROMATOGRAPHY

30 K. Kreuzig (Forschung und Entwicklung der Biochemie G.m.b.H., Kundl, Austria) – Application of quantitative HPTLC in the antibiotic industry

31 E. Kučan and M. Prošek (L.E.K. Pharmaceutical and Chemical Works, Ljubljana, Yugoslavia) – Quantitative fluorodensitometric determination of ergot alkaloids

Thursday, November 10, 1977

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

I. Halász, presiding

9.00 R.P.W. Scott and P. Kucera (Hoffmann-La Roche, Nutley, N.J., U.S.A.) – Solute–solvent interactions at the surface of non-polar bonded phases

9.20 E. Bayer, E. Grom, B. Kaltenegger, W. Voelter and K. Zech (Institut für Organische Chemie, Universität Tübingen, Tübingen, G.F.R.) – HPLC of amino acids and biogenic amines in the attomole range

9.40 I. Molnar and C. Horvath (Yale University, New Haven, Conn., U.S.A.) – High-performance liquid chromatography of amino acids and peptides on non-polar stationary phases

10.00 R. Tijssen (Koninklijke/Shell Laboratorium, Amsterdam, The Netherlands) – Liquid chromatography in helically coiled open tubular columns

10.20 Intermission

J.F.K. Huber, presiding

10.30 J.W. Jorgenson, S.L. Smith and M. Novotný (Indiana University, Bloomington, Ind., U.S.A.) – Light-scattering detection in liquid chromatography

10.50 R.W. Frei, L. Michel and W. Santi (Sandoz, Basel, Switzerland) – New aspects of post-column derivatization in high-performance liquid chromatography

11.10 J.C. Gfeller, G. Frey and R.W. Frei (Sandoz, Basel, Switzerland) – Post-column derivatization in HPLC using the air segmentation principle; application to digitalis glycosides

11.30 J. Miksic and P.R. Brown (University of Rhode Island, Kingston, R.I., U.S.A.) – Use of high-pressure reversed-phase and ion-exchange chromatography to study reactions of reduced nicotinamide adenine dinucleotide

R.S. Deelder, presiding

14.00 J.H. Knox and J. Jurand (University of Edinburgh, Edinburgh, Great Britain) – Determination of paracetamol and its metabolites in urine by HPLC using reversed-phase bonded support

14.20 J. Crommen, B. Fransson and G. Schill (University of Uppsala, Uppsala, Sweden) – Ion-pair chromatography in the low-concentration range by use of highly absorbing counter ions

14.40 W.K. Fong and E. Grushka (State University of New York at Buffalo, Buffalo, N.Y., U.S.A.) – The properties of peptides as bonded stationary phases in liquid chromatography

15.00 H. Engelhardt and D. Mathes (Universität des Saarlandes, Saarbrücken, G.F.R.) Chemically bonded phases for aqueous high-performance exclusion chromatography

15.20 C.L. Guillemin, J.P. Thomas, S. Thiault and J.P. Bounine (Rhone-Poulenc Industries, Aubervilliers, France) – Spherosil for high-performance, moderate-pressure liquid chromatography

15.40 Intermission

E. van Kreveld, presiding

15.50 D.W. Grant and R.B. Meiris (The British Carbonization Research Association, Derbyshire, Great Britain) – The application of HPLC and TLC techniques to the separation of polycyclic aromatic hydrocarbons in bituminous materials

16.10 W. Voelter (Universität Tübingen, Tübingen, G.F.R.) – Preparative high-performance liquid chromatographic separations of isomeric peptides with TSH-releasing activity

16.30 R.P. Chaplin, J.K. Haken and J.J. Paddon (C.S.R. Chemicals Ltd., Rhodes, New South Wales, Australia) – Use of narrow and broad molecular weight distribution polymers as calibration standards in gel permeation chromatography

16.50 J.C. Kraak, K.M. Jonker and J.F.K. Huber (University of Amsterdam, Amsterdam, The Netherlands) – Rapid separation of amino acids by means of solvent-generated ion-exchange chromatography

17.10 Closing of Symposium.

CONTACT CATALYSIS

edited by Z.G. SZABÓ and D. KALLÓ

1976. Vol. 1: 540 pages. Vol. 2: 480 pages
Price: US $101.95/Dfl. 250.00 (per two volume set)
ISBN 0-444-99852-7

This monograph provides detailed practical instructions for the application of catalysis, based on a rigorous scientific background. The subject of contact catalysis ranges so widely from solid-state physics to reaction kinetics and reactor design that multi-author treatment was essential. Each chapter has been written by an authority in the field concerned.

REACTION KINETICS IN HETEROGENEOUS CHEMICAL SYSTEMS

Proceedings of the 25th International Meeting of the Société de Chimie Physique, Dijon, 8-12 July, 1974.

edited by P. BARRET

1975. xvi+788 pages
Price: US $65.50/Dfl. 160.00
ISBN 0-444-41351-0

This volume contains the complete text of 16 plenary and special lectures, and 42 communications together with discussions. The papers describe the current state of knowledge concerning corrosion of metals and alloys, and solid-solid and gas-solid reactions involving inorganic compounds. Fundamental ideas of chemical kinetics and thermodynamics of irreversible processes are covered in relation to these subjects.

ELSEVIER SCIENTIFIC PUBLISHING COMPANY

P.O. Box 211, Amsterdam,
The Netherlands.

Distributor in the U.S.A. and Canada:
ELSEVIER NORTH-HOLLAND, INC.,
52 Vanderbilt Ave., New York, N.Y. 10017

1903 E

The Dutch guilder price is definitive. US $ prices are subject to exchange rate fluctuations.

PREPARATION OF CATALYSTS

Scientific Bases for the Preparation of Heterogeneous Catalysts

Proceedings of the International Symposium held at the Solvay Research Centre, Brussels, October 14-17, 1975.

edited by B. DELMON, P.A. JACOBS *and* G. PONCELET.

1976. xvi+706 pages
Price: US $65.50/Dfl. 160.00
ISBN 0-444-41428-2

This book contains the proceedings of the first international symposium devoted to the processes involved in the preparation of industrial heterogeneous catalysts. The subjects covered include the preparation of phases associating two or more metallic elements in the form of alloys or mixed oxides: the preparation of "complex catalysis" by impregnation, coprecipitation, mixing or other novel methods; the activation stage, with particular reference to decomposition, reduction, influence of the carrier, promotion or inhibition of crystallite growth and of compound formation with the carrier. This book is practically oriented and will be of particular value to those involved in the fabrication of catalysts in manufacturing plants and industrial research laboratories. It will also interest academic workers in the field.

CATALYSIS: HETEROGENEOUS AND HOMOGENEOUS

Proceedings of the International Symposium on the Relations between Heterogeneous and Homogeneous Catalytic Phenomena, Brussels, 23-25 October, 1974.

edited by B. DELMON *and* G. JANNES

1975. xxvi+550 pages
Price: US $44.95/Dfl. 110.00
ISBN 0-444-41346-4

These proceedings comprise twenty-eight contributed papers and ten invited lectures or reviews. The main topics are: similitude of active species or elementary steps: heterogenization of homogeneous catalysts; interpretation of heterogeneous phenomena through approaches pertaining to homogeneous catalysis; metallic clusters in solution.

GENERAL INFORMATION

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

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (full-length papers), short communications and notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as full-length papers, but should preferably not exceed four printed pages. For reviews, see page 2 of cover under Submission of Papers.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (*e.g.*, Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc. should be on separate sheets.

Title. The title of the paper should be concise and informative. Since titles are widely used in information retrieval systems, care should be taken to include the key words. The title should be followed by the authors' full names, academic or professional affiliations, and the address of the laboratory where the work was carried out. If the present address of an author is different from that mentioned, it should be given in a footnote. Acknowledgements of financial support are *not* to be made in a footnote to the title or name of the author, but should be included in the Acknowledgements at the end of the paper.

Summary. Full-length papers and review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes are published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. One original and two photocopies are required. Attention should be given to any lettering (which should be kept to a minimum) and to spacing on axes of graphs in order to ensure that numbers etc. remain legible after reduction. Axes of a graph should be clearly labelled. The figures should preferably be of such a size that the same degree of reduction can be applied to all of them. Photographs should have good contrast and intensity. Sharp, glossy photographs are required to obtain good halftones. References to the illustrations should be included in appropriate places in the text using arabic numerals. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet*. If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the authors' expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

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 in square brackets. In the reference list, periodicals [1], books [2], multi-author books [3] and proceedings [4] 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 H. C. S. Wood and R. Wrigglesworth, in S. Coffey (Editor), *Rodd's Chemistry of Carbon Compounds, Vol. IV, Heterocyclic Compounds*, Part B, Elsevier, Amsterdam, Oxford, New York, 2nd ed., 1977, Ch. 11, p. 201.
4 E. C. Horning, J.-P. Thenot and M. G. Horning, in A. P. De Leenheer and R. R. Roncucci (Editors), *Proc. 1st Int. Symp. Quantitative Mass Spectrometry in Life Sciences, Ghent, June 16–18, 1976*, Elsevier, Amsterdam, Oxford, New York, 1977, p. 1.

Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts*. Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication". The *Journal of Chromatography; Journal of Chromatography, Biomedical Applications* and *Chromatographic Reviews* should be cited as *J. Chromatogr.*

Proofs. One set 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.

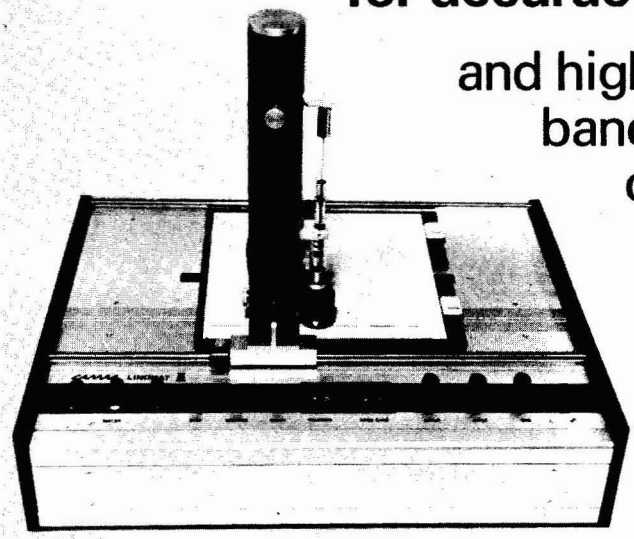
News. News releases of new products and developments, and information leaflets of meetings should be addressed to: The Editor of the News Section, Journal of Chromatography/Journal of Chromatography, Biomedical Applications, Elsevier Scientific Publishing Company, P.O. Box 330, Amsterdam, The Netherlands.

Advertisements. Advertisement rates are available from the publisher on request. The Editors of the journal accept no responsibility for the contents of the advertisements.

Quantitative Sample Application in Thin-Layer Chromatography

for accuracy in volume

and high precision band application of samples



CAMAG LINOMAT III

The spray on technique, with nitrogen as carrier gas, prevents damage to the layer and allows any sample volume to be applied in the form of narrow bands of desired length.

- Digital preselection of sample volume between 1 and 99 μl and automatic control by stepping motor at every 0.05 μl.
- Band-length adjustable between 0 (= spot) and 199 mm.
- The sample solution is only in contact with the precision syringe. Changing of sample requires only seconds by simply inserting a preloaded syringe.
- An additional feature of the instrument: It meets the criteria of preparative TLC when operated with a 500 μl syringe.

Please ask for detailed information on CAMAG LINOMAT III.

CAMAG

4132 Muttenz/Switzerland
Homburgerstrasse 24
Tel. (061) 61 34 34

United States Affiliate, Main Offices and Warehouse:
CAMAG Inc. 16229 West Ryerson, New Berlin, Wisconsin 53151

20171-E

ref.no.142