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*edited by* A. J. BARNES *and* W. J. ORVILLE-THOMAS, *Department of Chemistry and Applied Chemistry, University of Salford, Great Britain.*

This book gathers together leading exponents of recent advances in the field of vibrational spectroscopy to explain the experimental and theoretical developments, and their application to problems in molecular structure. It will not only be of value to practising spectroscopists as a reference source for modern methods and current developments in vibrational spectroscopy, but will also be suitable as a basic text for graduate students and those entering the field.

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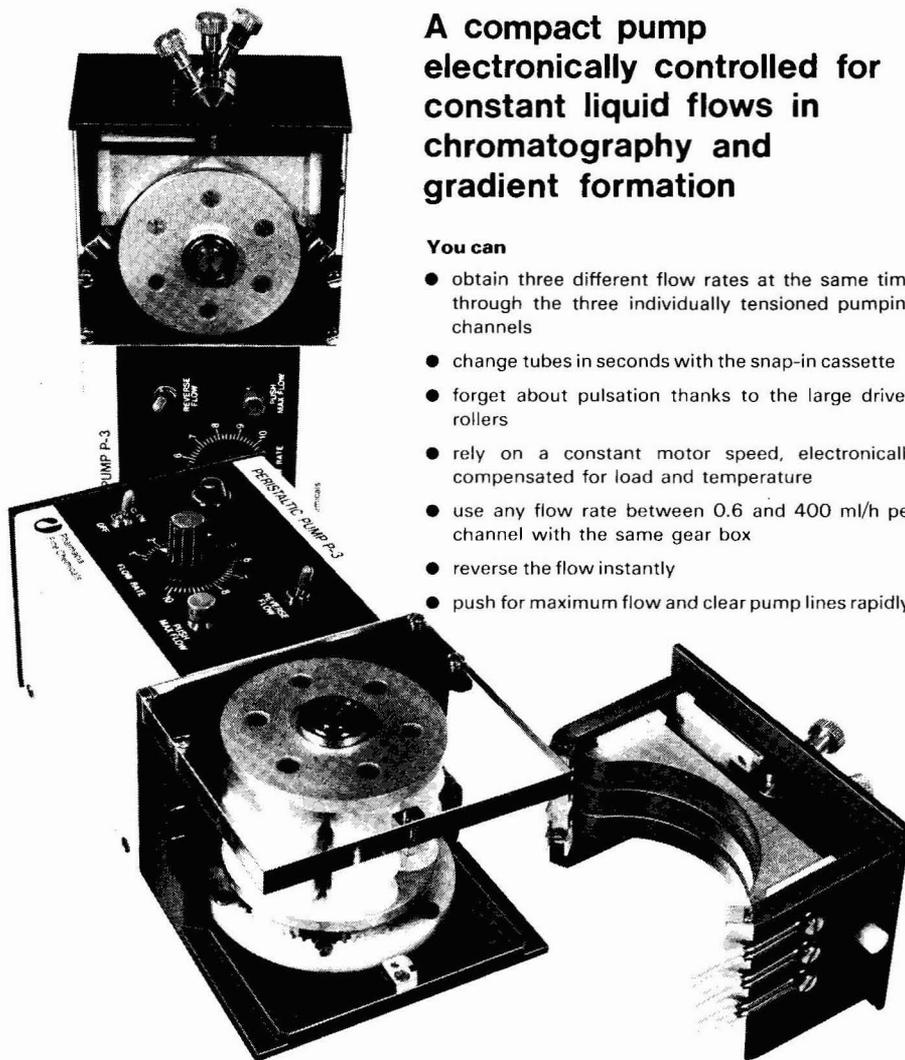
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*edited by* MICHAEL A. WEST, *The Royal Institution, London.*

As lasers and associated electro-optics have been developed in the past few years, chemists have rapidly adapted and used these new light sources in many diverse ways. This conference was held in order to review and discuss the present state-of-the-art in this fast-growing field and the proceedings contain 79 papers organized into seven sections. Each section, except one, contains a review paper by an invited speaker and a set of contributed papers which, taken together, indicate the overall scope of current research and point to likely future advances in a particular area. This volume will be of value to academic, government and industrial scientists as well as to technologists in chemistry, physics and electro-optics.

*In the contents listed below, the main topics of the conference are given with the total number of papers in each section noted in parentheses. Limitation of space allows titles of only a few randomly selected papers to be mentioned.*

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# A SILICON DETECTOR FOR GAS CHROMATOGRAPHY[*]

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(Received April 25th, 1977)

SUMMARY

Silicon compounds can be detected by a hydrogen-atmosphere flame ionisation detector with moderate sensitivity (nanogram range) and selectivity (two to three orders of magnitude *vs.* hydrocarbons). The detector can be operated with or without ferrocene vapor present in its hydrogen atmosphere: The former mode is more sensitive, the latter more practical.

INTRODUCTION

Volatile compounds of silicon are frequently encountered in chemistry, both academic and industrial. For many of them, gas chromatography (GC) is the preferred method of analysis. Conversely, the particular demands of GC have stimulated considerable activity in silicon chemistry.

Foremost among these activities are the silylation reactions which have in recent years been used in a multitude of cases to prepare more volatile and less polar derivatives of alcohols, acids, etc., for GC analysis. On occasion, these silylations have also served to introduce halogen into the analyte molecule for increased response in the electron capture detector, although the more common detector for silicon compounds is, no doubt, the flame ionization detector (FID).

The FID responds, of course, to the carbon portion of the molecule, but the matter is more complicated than that: the response of organosilicon compounds can be negative; *i.e.*, inverted peaks may be observed. The inversion depends on the presence of carbon in the flame and a critical silicon mass flow-rate[1].

It is obvious that a GC detector selective for silicon would be an advantage to many types of analysis. No such detector is commercially available and, to our knowledge, only one system designed for selective determination of silicon has been described[2]. It uses the 2516-Å line of silicon and is based on either atomic emission in an oxygen–acetylene flame or atomic absorption in a nitrous oxide–acetylene flame.

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[*] Presented at the 58th CIC Conference, Toronto, Ont., Canada, May 1975.

In some recent studies on the detection of organometallics containing Fe, Al, Sn, Pb, Cr, and other elements, we noted an unexpected phenomenon: the response of a detector named hydrogen-atmosphere flame ionization detector (HAFID) was influenced by the presence of silane[3]. In fact, silane (or other silicon compounds) had to be introduced continuously to keep the detector at its optimum performance level, a level characterized by sensitivities in the lower picogram range and selectivities (against C, H, N, O compounds) of five orders of magnitude.

Compared to an undoped detector, the addition of silicon-containing compounds brought about an improvement in performance, *i.e.* the response towards organometallics, of two to three orders of magnitude. It appeared likely, therefore, that the changes in the response of organometallics caused by the introduction of organosilanes could be used to determine the latter rather than the former. Furthermore, it was interesting to investigate whether the HAFID would also respond to silicon-containing species in the absence of other organometallics.

A study along these lines is contained in this paper. Its subject is treated in a strictly exploratory manner, designed to indicate the feasibility and potential of the chosen approach rather than to optimize the technique for a particular analytical usage or to elucidate the flame mechanism(s) responsible for the observed effects.

EXPERIMENTAL

The general construction of the HAFID was described earlier (*e.g.* ref. 3). The particular model used is shown in Fig. 1; it was situated under appropriate exhaust facilities to vent safely the large amounts of unburned hydrogen as well as any other toxic gases. Unless stated otherwise, the detector flow-rates (ml/min) were hydrogen 1600, oxygen 150, nitrogen 25, nitrogen (column) 40; and the collector electrode, polarized at $-90$ or $-240$ V, was situated 50 mm above the quartz jet tip.

For experiments involving the addition of ferrocene, the organometallic was coated onto, and mixed with, Chromosorb W (80–100 mesh). This material was filled into a 12 $\times$ 1/4 in. O.D. copper tube mounted in by-pass configuration on the hydrogen supply line. A variable and easily measured flow of hydrogen could thus be passed through the finely dispersed ferrocene at ambient temperature, and an estimation of the ferrocene introduction rate could be obtained from vapor pressure data and the assumption of equilibrium. The total hydrogen flow-rate entering the detector remained approximately constant at 1600 ml/min.

The electrode height was varied by using appropriate "spacers". The only other parameter investigated was the oxygen flow-rate.

Several silicon-containing compounds were injected onto a 5 ft. $\times$ 4 mm I.D. U-tube borosilicate glass column packed with Chromosorb W modified by a non-extractable layer of Carbowax 20 M (ref. 4), except where noted. No efforts were made to optimize chromatographic conditions.

RESULTS AND DISCUSSION

*Ferrocene present in the hydrogen atmosphere*
The presence of ferrocene in the hydrogen atmosphere of the detector definitely affects the response of organosilanes coming from the chromatographic column and
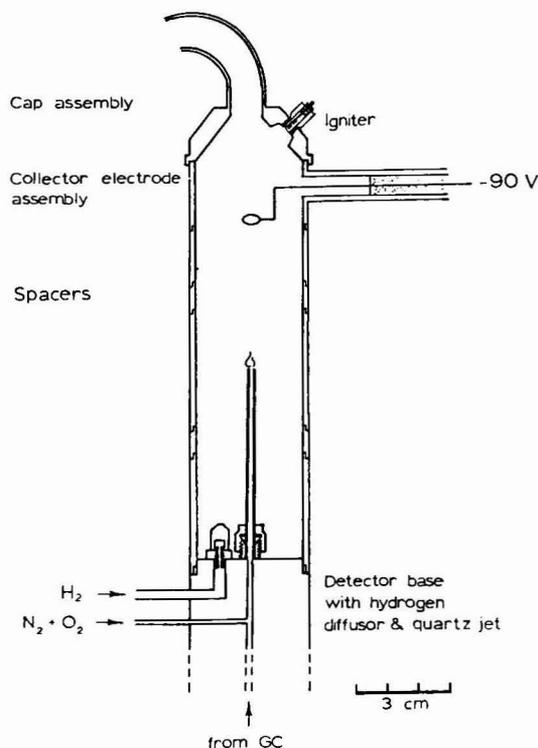
Fig. 1. Hydrogen-atmosphere FID as used for silicon compounds.

burning in the flame (Fig. 2). At low concentrations of ferrocene, positive peaks (increases in detector current) are obtained as expected. At high concentrations of ferrocene, however, the peaks are negative (inverted).

The HAFID has a very large baseline current, and peak inversion is not an uncommon phenomenon. It shares the latter, albeit only formally, with such detectors as the alkali flame, the electron capture, or the flame photometric detector. Here as there, a precise correlation of detector processes with peak inversion can be difficult to establish. The response of the HAFID reflects, most likely, a rather complex system of reactions that is difficult to investigate. A general, speculative picture of these reactions has been developed[5]; however, no experimental data are at present available that would unequivocally establish the detector mechanism(s).

Fig. 3 shows two chromatograms obtained with different ferrocene levels but under otherwise identical conditions; tetramethylsilane is the compound chosen to demonstrate peak inversion.

Fig. 4 presents the variation of organosilane response with oxygen flow, the results being close to expectations based on earlier studies of organometallics[6]: increased oxygen flow increased response of the silanes and decreased response of hydrocarbons.

Determination of organosilanes in the presence of ferrocene is fairly sensitive (down to nanogram amounts of injected compounds) and selective (up to four orders
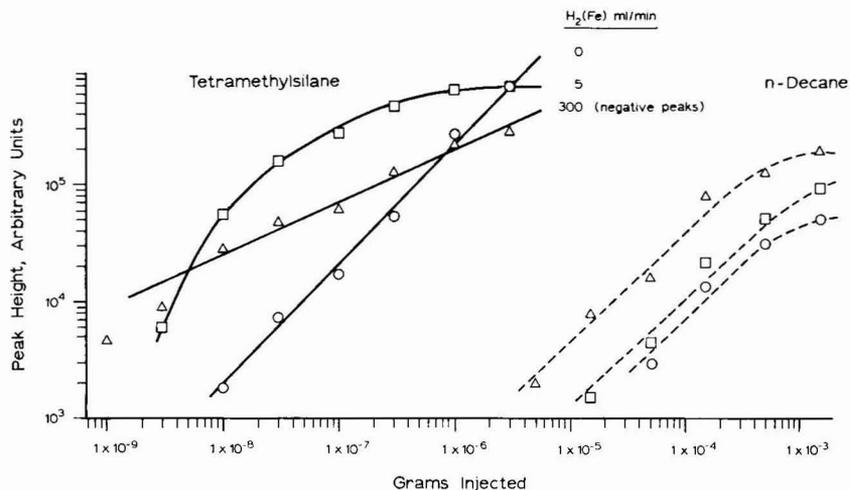
Fig. 2. Calibration curves for tetramethylsilane (TMS) and decane with different amounts of ferrocene present in the detector atmosphere. Fraction of hydrogen saturated with ferrocene at room temperature: $\triangle$ = 300 ml/min; $\square$ = 5 ml/min; $\bigcirc$ = 0 ml/min (blank). Response of TMS for 300 ml/min: negative (inverted peak); for 5 and 0 ml/min: positive. Response of decane: all positive. General conditions: column 5 ft. $\times$ 4 mm I.D. borosilicate U-tube packed with Chromosorb W AW (80–100 mesh) modified by *ca.* 0.2% Carbowax 20M. Electrode height, 50 mm; potential $-240$ V. Temperatures, detector 240°, injection port 220°, column 70°. Flow-rates (ml/min): $H_2$, 1600 (total), $N_2$ (detector), 25; $N_2$ (column), 40; $O_2$, 150.
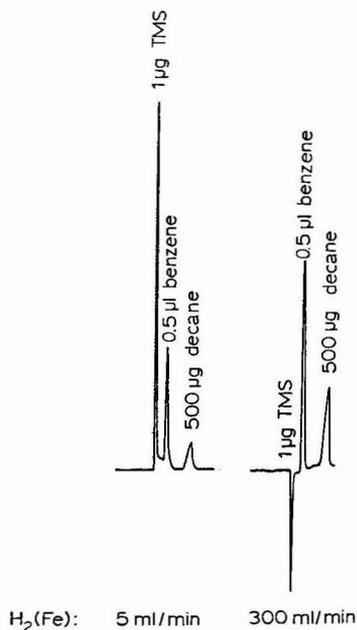


Fig. 3. Inversion of TMS peak caused by larger amounts of ferrocene in the detector atmosphere. Fraction of hydrogen saturated with ferrocene at room temperature: left, 5 ml/min; right, 300 ml/min; out of a total 1600 ml/min. Other conditions as in Fig. 2.
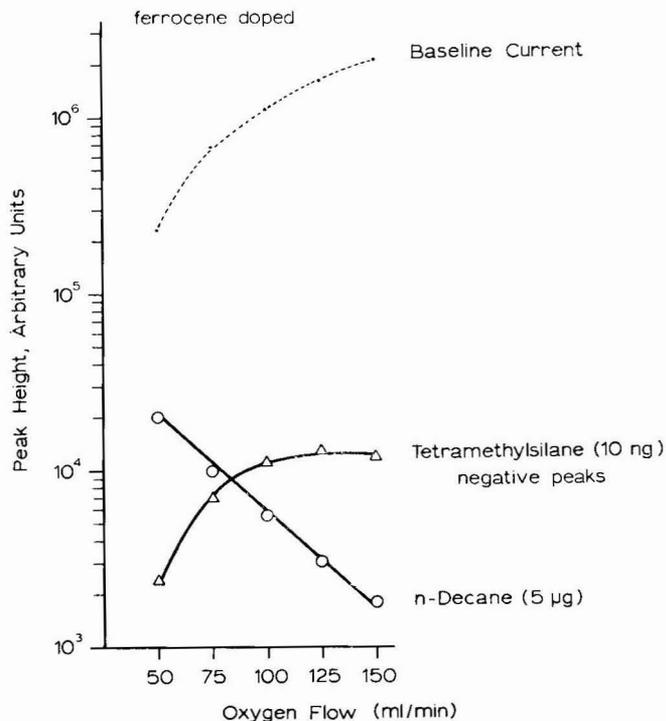
Fig. 4. Variation in response of TMS with different oxygen flows in a ferrocene-doped HAFID. Fraction of hydrogen saturated with ferrocene: 300 ml/min; out of a total 1600 ml/min. Response: TMS negative, decane positive. Other conditions as in Fig. 2.

of magnitude depending on concentrations). On the other hand, the calibration curves (Fig. 2) are non-linear and the amount of ferrocene entering the detector would obviously have to be carefully controlled. Ferrocene was the only doping agent investigated; but it is reasonable to assume that other organometallics could also bring about increased response. If small amounts of their vapors were added to the bottled hydrogen supply, analytical reliability and ease of operation may also be improved.

In its present form, though, the HAFID is less cumbersome to operate without ferrocene, and some noteworthy responses can still be obtained.

*Ferrocene absent from the hydrogen atmosphere*

Fig. 5 shows the expected variation of response with a change in electrode height. The baseline current and the response to hydrocarbons decrease as the electrode is moved upwards, a characteristic of the HAFID and presumably the FID as well. The response to silicon compounds stays constant up to over 60 mm in contrast to the organometallics determined earlier in a silane-doped detector[3], whose response increased. This behavior was expected from a working hypothesis developed to explain the response of organometallics[5], which speculates that $SiH_3^+$ (or a similar ion) has a lower rate of neutralization than other ions, *e.g.* $H_3O^+$, under HAFID conditions and can therefore serve as the principal charge carrier. When silane compounds are
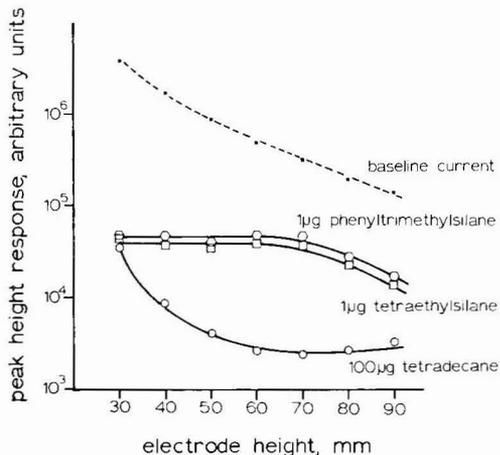
Fig. 5. Variation in response of two silicon compounds and a hydrocarbon with height of the collector electrode above the jet tip. No ferrocene doping. Electrode potential, − 90 V. Temperatures: column, 60° (phenyltrimethylsilane), 30° (tetraethylsilane), 120° (tetradecane); detector, 250°; injection port, 225°. Other conditions as in Fig. 2.

chromatographed, the $SiH_3^+$ (or similar) ion would presumably form in the upper parts of, or right above the flame, explaining the unusual phenomenon of an ion current which remains approximately constant over more than 60 mm above the flame. While the still speculative nature of this explanation needs to be stressed, it is obvious that a judicious choice of electrode height, together with a suitable adjustment of oxygen flow-rate, can lead to an effective suppression of the sample matrix for analytical purposes.

Fig. 6 gives calibration curves for three organosilanes, in the absence of any deliberately added doping agent. These are linear, though only over a restricted range. Minimum detectable amounts are around 10 ng and selectivities are between two and
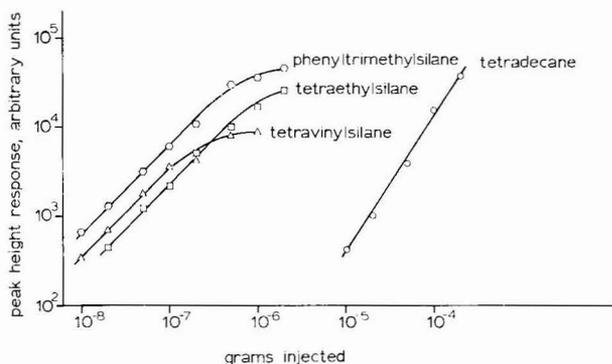


Fig. 6. Calibration curves for three silicon compounds and a hydrocarbon. No ferrocene doping. Electrode height, 50 mm; potential, −90 V. Column: 5 ft. × 4 mm I.D. borosilicate U-tube packed with 5 % Carbowax 20M on Chromosorb W AW (80–100 mesh). Column temperatures: 30° (tetraethylsilane), 60° (tetravinylsilane), 80° (phenyltrimethylsilane), 125° (tetradecane). Other conditions as in Fig. 2.

three orders of magnitude. Fig. 7 shows a chromatogram of a typical standard mixture.

This performance should make the detector of interest for several types of GC analysis. While it would not be considered suitable at present for trace analysis (*e.q.* the sub-nanogram range), typical applications could provide information on whether or not a particular GC peak represents a silane, and could facilitate the analysis of samples with interfering carbonaceous backgrounds.
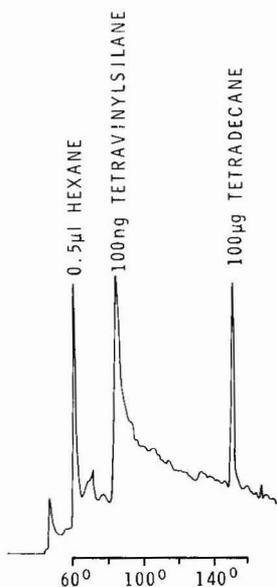


Fig. 7. Temperature-programmed chromatography of tetravinylsilane and a hydrocarbon standard. Program 60° to 150° at 40°/min. Other conditions as in Fig. 6.

There is little doubt that a more thorough study, perhaps involving varied doping agents, improved chromatographic conditions, optimized construction and operation parameters, etc., could lead to future improvements in detector performance.

ACKNOWLEDGEMENT

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CHROM. 10,141

# NICKEL GAS CHROMATOGRAPHIC COLUMNS: AN ALTERNATIVE TO GLASS FOR BIOLOGICAL SAMPLES

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(Received March 22nd, 1977)

## SUMMARY

Nickel tubing may be substituted for glass in the fabrication of gas chromatographic columns for use with samples of biological interest. Comparisons of separations of mixtures of steroids, narcotic alkaloids, phenothiazines, and amphetamines on stainless steel, glass, and nickel packed columns showed little or no observable sample decomposition on glass or nickel as contrasted to complete loss of certain compounds on stainless steel. The nickel columns are easily prepared, durable, economical, and not subject to breakage.

## INTRODUCTION

Nearly all gas chromatographic (GC) packed columns currently in use are constructed of either stainless steel or glass tubing. That the choice is shared by these materials indicates that neither possesses all the qualities desired in a chromatographic tubing. Stainless steel is certainly the more easily handled and is quite satisfactory for many analytical applications. Glass, on the other hand, has a less reactive surface which permits separations of labile compounds at high column temperatures; and for this reason, glass is the preferred tubing material for biomedical applications. The surface inertness of glass is perhaps the only property that recommends it in the face of numerous disadvantages. Glass columns are often difficult to connect into the chromatographic system, they lack flexibility and must be formed with precision in order to fit a specific instrument, and they usually require the services of a skilled glass-blower for fabrication which adds to their expense. But these detractions are minor compared to the dismaying fragility of glass which makes changing columns a test of skill for the most experienced chromatographer.

The usual alternatives to glass and stainless steel are, however, even less attractive: copper oxidizes rapidly at elevated temperatures, and decomposition is often severe on copper columns[1]; aluminum has an oxidized surface that is quite active and is difficult to passivate[2]; noble metals such as gold and platinum are far too expen-

sive for general use; and plastics are usually limited to low temperatures. Another possibility was suggested by the introduction of nickel capillary tubing for preparation of high resolution open tubular columns[3]. At least one application of nickel capillary tubing showed that this material could be used in high temperature separation of certain compounds where decomposition on stainless steel had been a problem[4]. Analyses of compounds of biomedical interest, however, are often performed more conveniently on packed columns. Consequently, the extension of nickel tubing to this application was the next logical step.

A comparison of separations of several classes of compounds which experience had shown were subject to decomposition was undertaken using closely matched columns made of stainless steel, glass, and nickel. In these studies nickel columns proved to be surprisingly inert, and yielded chromatographic separations comparable to glass columns.

EXPERIMENTAL

*Columns*

Nickel columns were prepared from 1/8 in. O.D., 0.020 in. wall, nickel-200 tubing obtained from Handy and Harman (Norristown, Pa., U.S.A.). The tubing was cleaned sequentially with ethyl acetate, methanol and water. The interior surface of the tubing was then etched by filling the tubing with 50% nitric acid and after about 10 min rinsing with water until neutral. This treatment was followed by an acetone rinse, and the tubing was then dried with a stream of air.

The stainless-steel column of tubing of identical dimensions (Handy and Harman) was cleaned in the same manner as the nickel column but with omission of the nitric acid treatment. The glass column was 1/8 in. O.D., 0.08 in. I.D. and was cleaned with ethyl acetate, and toluene. This was followed with a silylation treatment which consisted of filling the column with Tri-Sil (Pierce, Rockford, Ill., U.S.A.), diluted about 1:1 with toluene followed by rinsing with toluene and methanol. All three columns were 6 ft. long and coiled.

The columns were packed with 5% SE-30 on 80–100 mesh Gas-Chrom Q obtained from Supelco (Bellefonte, Pa., U.S.A.). In each case packing was accomplished with light tapping with aspirator vacuum drawn on the column.

*Gas chromatograph*

A Varian Model 1200 instrument was utilized in this study and was selected because the columns could be installed for on-column injection, and the outlet of the column could be terminated at the jet of the flame ionization detector. Thus any effect of injector, transfer lines, and connectors on the experimental compounds was avoided.

*Samples*

All sample mixtures were prepared from compounds which had been examined for purity on other chromatographic systems. In each case the compounds were dissolved in redistilled ethyl acetate at 1 mg/ml concentration and 1 $\mu$l was injected on the chromatographic columns.

RESULTS AND DISCUSSION

A variety of classes of compounds known to present difficulties in GC analysis were examined on each of the three columns. The stationary phase, SE-30, was not necessarily the optimum for all of the experimental samples. Experience had shown, however, that if decomposition were to occur on a column, the loss of sample, distortion of peak shape, or appearance of extraneous peaks would be most noticeable with this non-polar, methyl silicone polymer.

Exact replication of retention characteristics of the three columns was not attainable even though some of the columns were repacked several times under vary-



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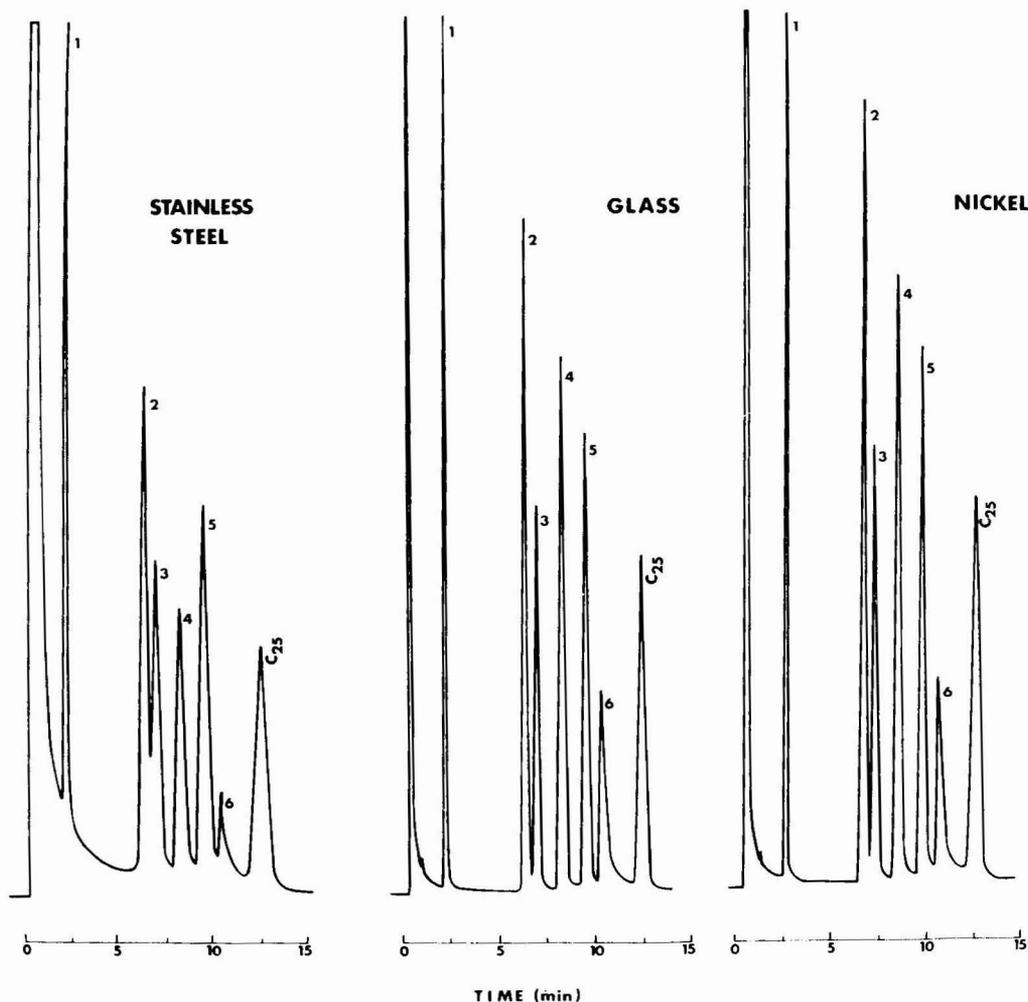
Fig. 1. Separation of a mixture of alkaloids and narcotic analgesics on stainless steel, glass, and nickel packed chromatographic columns. Peaks: 1, meperidine; 2, methadone; 3, cocaine; 4, pentazocine; 5, codeine; 6, morphine; and 7, n-pentacosane added as an internal standard. Columns were temperature programmed at 4"/min from 180" to 220".

ing conditions in an effort to achieve this. Consequently, adjustment of carrier gas flow-rate was used to bring the retention times of test compounds into close agreement on the columns. Column temperature was the same for each column for a given test mixture so that this operating parameter would not be an influence on observed differences.

Fig. 1 shows a comparison of the separation of a mixture of narcotic analgesics on the three columns. The nickel and glass columns are very similar in performance, but some loss of sample has occurred on the stainless-steel column. The peak for morphine is noticeably attenuated, and tailing of the solvent peak is more pronounced on stainless steel which often indicates the presence of breakdown products having lower molecular weights than the parent compounds.
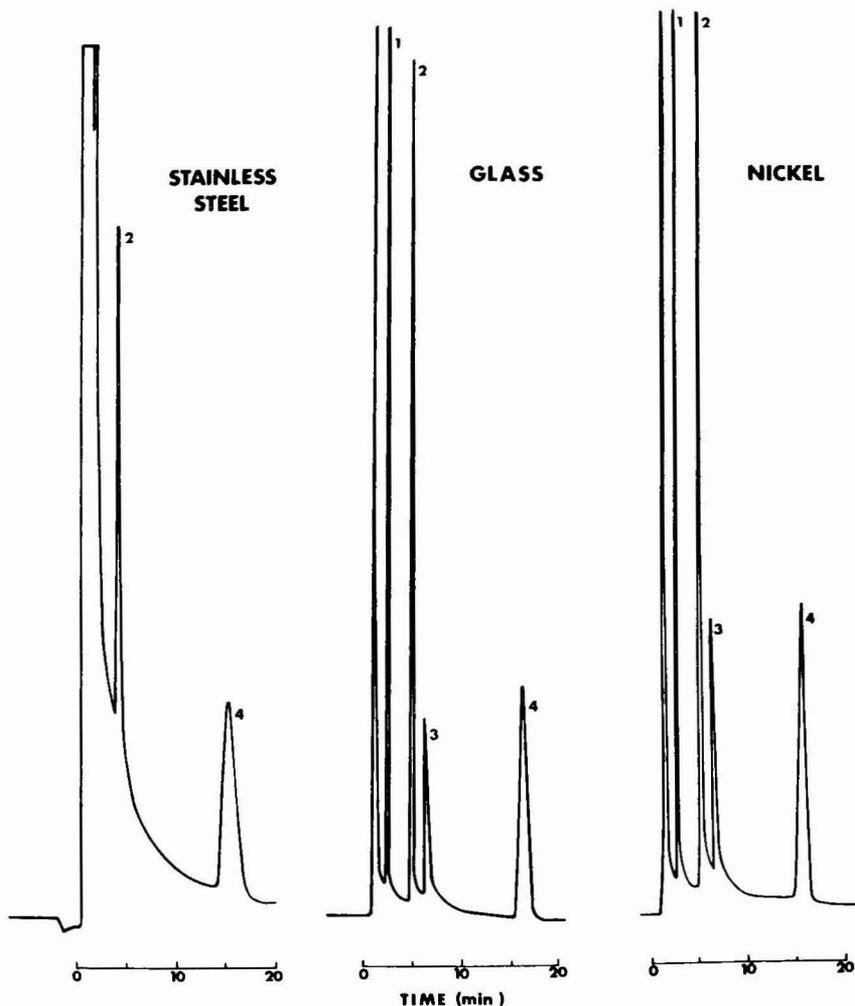


Fig. 2. Comparative chromatograms of a mixture of: 1, methamphetamine; 2, p-chloro-N-metham-phetamine; 3, p-hydroxyamphetamine, and 4, n-hexadecane as internal standard. Column temperatures were 120°.

Somewhat more dramatic differences are observed in the analysis of amphetamines as seen in Fig. 2. Pronounced tailing of the solvent peak has all but obscured the methamphetamine peak, and the hydroxyamphetamine peak has disappeared on the stainless-steel column. The chromatograms obtained on glass and nickel are again quite similar, although some peak tailing has occurred. This tailing is not unexpected considering the SE-30 stationary phase employed in these comparisons is not the usual choice for analysis of amines[5].

The phenothiazine drugs constitute a particularly difficult class of compounds for successful GC analysis, because these substances are not only labile but also
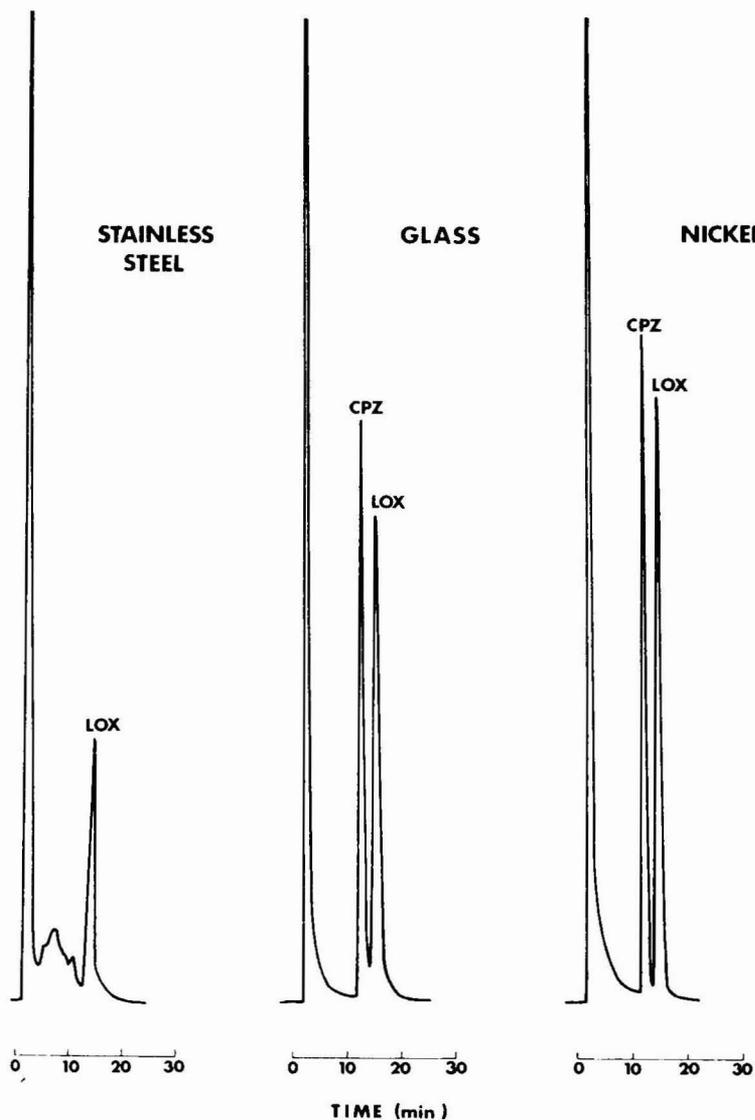


Fig. 3. Separation of chlorpromazine and loxapine at 230° on stainless-steel, glass, and nickel columns.

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require high column temperatures for elution in any reasonable time. As can be seen in Fig. 3, chlorpromazine is entirely decomposed on the stainless-steel column and for this reason glass columns have been a necessity in the analysis of chlorpromazine as well as most other psychotropic drugs having similar structures[6]. The nickel column, however, yielded a very acceptable chromatogram, and similar columns have been employed in our laboratories for many months in routine determinations of drug blood levels[7].

While the effect of column material is not as apparent on the separation of the
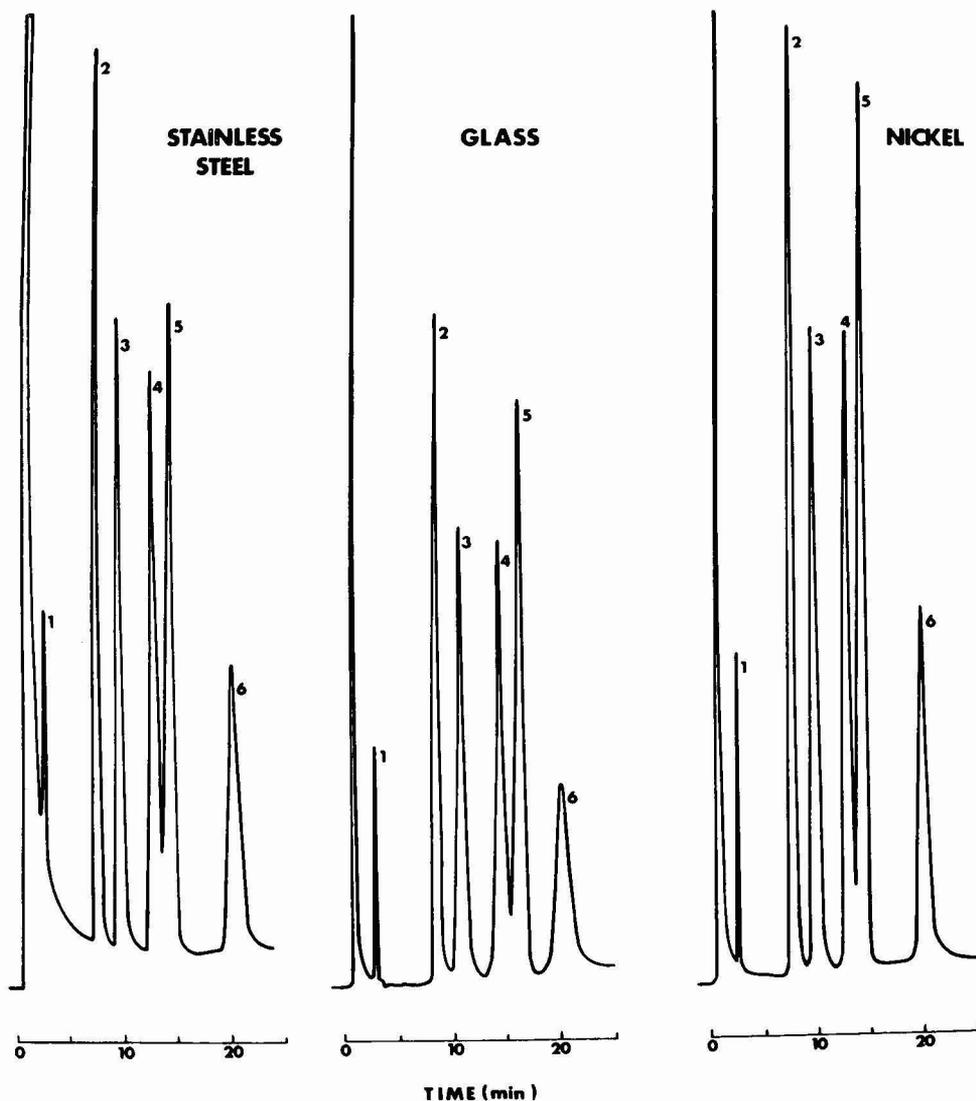


Fig. 4. Comparative separation of a steroid mixture containing: 1, eicosane as internal standard; 2, dehydroepiandrosterone; 3, testosterone; 4, progesterone; 5, 5α-cholestane; and 6, cholesterol. The columns were programmed at 2°/min from 210° to 250°.

steroid mixture shown in Fig. 4, some differences do exist. Again tailing of the solvent front is more noticeable on stainless steel which suggests some decomposition of the sample mixture.

The performance of packed nickel columns may sometimes be improved by silylation which is usually performed by injection of silylating reagents such as Silyl-8 (Pierce) at elevated temperatures, assuming, of course, that the stationary phase is compatible with this treatment. Whether silylation acts on the packing, tubing walls, or both remains to be determined, but the improvement is comparable to that observed in glass columns through similar treatment.

Other classes of compounds which we have chromatographed on nickel column are barbiturates, cannabinoids, catecholamines and catecholamine metabolites, and various perfluorinated ester derivatives used in electron capture detection. Without exception compounds which could be chromatographed on glass columns yielded comparable chromatograms on columns constructed of nickel tubing.

An additional advantage to be gained through the use of these columns is purely economic. Apart from the total lack of breakage that accompanies use of metal columns, the cost of nickel tubing at the time of this report is, measure for measure, about one-tenth that of glass columns obtained from instrument manufacturers or chromatographic supply houses.

Although nickel would appear to serve quite satisfactorily in most applications now requiring glass columns, there is the possibility that only glass will provide the requisite inertness at temperatures above those employed in this study. Studies of metal columns for GC at elevated temperatures are, however, far from being exhausted. Our own experience with metal *versus* glass is such that where performance is comparable, the glass column is relegated to the storage cabinet rather than being used in a working instrument.

## ACKNOWLEDGEMENTS

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# METHOD FOR THE SEPARATION OF ORGANOCHLORINE RESIDUES BEFORE GAS–LIQUID CHROMATOGRAPHIC ANALYSIS

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## SUMMARY

The adsorption characteristics of alumina and silica for column chromatography have been assessed to develop a method, using a single solvent, for the separation of seventeen organochlorine residues into four eluates prior to gas–liquid chromatographic analysis. The effects on the adsorbents of thermal activation and subsequent deactivation with water, variation of column size and choice of eluents have been critically examined. The lipid capacity of the alumina and the effect of co-extracted materials from animal tissue upon the elution profile of the organochlorines have been determined.

## INTRODUCTION

A number of methods have been described[1–5] using adsorption chromatography for the preparation of extracts of chlorinated hydrocarbon residues from environmental samples for gas–liquid chromatographic (GLC) analysis with electron capture detection (ECD). Morley[6] reviewed a number of high surface energy adsorbents used for the clean-up of pesticide residues and Moats[1] compared silicic acid, alumina, decolourizing carbon and Florisil for the clean-up of butter fats. He found little success with the first three and required a 5% deactivation with water on Florisil to elute dieldrin with methylene chloride. Florisil has been widely used, but due to the variability observed between batches[7] some workers have preferred to use alumina, which has a higher adsorption coefficient, particularly for lipids. Law and Goerlitz[3] found that Florisil did not clean-up river water extracts as well as alumina and Leoni[4] used a silicic acid column, deactivated to 5% with water to clean-up similar types of aqueous extracts.

The method used in this laboratory, to date, has been based on the twin column clean-up and separation techniques developed by Holden and Marsden[2] and has been extended to analyse organochlorine residues in rain, river and sea water, plankton and algae, as well as fish tissue. The method employs 2 g alumina columns (deactivated with 5% water) and *n*-hexane as the eluent; the alumina retaining the co-extracted lipid material and many of the unwanted contaminants. The constituent organochlorines are then separated into two groups using a 2-g silica column (deactivated

with 5 % water). The first group, which includes polychlorinated biphenyls (PCBs) and
$p,p'$-DDE, is eluted with $n$-hexane, followed by 10 % diethyl ether in $n$-hexane to
remove $p,p'$-DDT and the more polar organochlorines.

Although this technique has been satisfactory for much of the routine analysis
of organochlorine residues in fish tissue, a number of disadvantages have become
apparent during the analysis of other types of sample. Many samples (*e.g.*, rain, river
waters and sediments), even after concentration of the solvent extract, contain concen-
trations of organochlorines at or near the minimum detection limit of the instrumenta-
tion. The analysis, therefore, demands a stable baseline, free from background inter-
ference derived from the solvents, adsorbents and contaminated glassware. To a large
extent this was obtained by scrupulous cleaning, but it was often difficult to remove
the background interference from the 10 % diethyl ether–hexane eluate. Solvents were
only used when their GLC chromatograms were free of interfering peaks after a one
hundred-fold concentration by evaporation, but impurities were often extracted from
the silica using this solvent mixture, even after the silicic acid had been fired at 600°
prior to deactivation. These impurities were particularly obtrusive in the determina-
tion of hexachlorocyclohexane (HCH) isomers, which appear early in the GLC
chromatogram, and their elimination was necessary to avoid misinterpretation of the
results.

Some commercial DDT formulations contain up to 20–30 % of the $o,p'$ isomer
which may therefore subsequently appear in natural samples. To quantify the DDT
isomers in these samples a complete separation from PCBs into another fraction was
required. The technique of Holden and Marsden[2] divides the $o,p'$-DDT approximately
1:1 between the two silica eluates, part being included with the PCB fraction, while
the remainder is included in the second eluate with $p,p'$-DDT, $p,p$-DDD and dieldrin.
Ideally the $o,p'$-DDT also requires isolation from $p,p'$-DDD and dieldrin, as this
separation would not only simplify the total DDT analysis but also avoid the possibil-
ity of confusion with other residues, *e.g.*, endrin, chlordane.

The technique of using alumina and silica micro-columns has been developed
to enable seventeen organochlorine residues to be separated into four eluates using a
single solvent, $n$-hexane. The compounds have been separated in such a manner that
all residues in each eluate (with the exception of $p,p'$-DDE and the PCBs) can be
completely resolved on a single GLC column. The reliability and reproducibility of
the adsorbents chosen have been critically examined for use with standard solutions
and extracts from natural samples of varying origins and organic content.

EXPERIMENTAL

Borosilicate glass micro-columns 450 × 6 mm I.D. were used, with a taper at
the lower end and a solvent reservoir at the top. Hexane-washed cotton wool balls
were used to plug the tapered end and support the column packing. Alumina powder,
initially BDH (Poole, Great Britain) No. 27076 and subsequently Reeve Angel
(Clifton, N.J., U.S.A.) type A11-O-col, was activated at 800° for 4 h, cooled, and deacti-
vated to the desired level with hexane-washed distilled water. The Reeve Angel A11-
O-col alumina (Reeve Angel, Clifton, N.J., U.S.A.) was sieved to remove the fines
and agglomerates and the particle size band 64–125 $\mu$m was retained (85 % of the total
material). The silica Merck No. 7734 70-325 (ASTM) (Merck, Darmstadt, G.F.R.)

was activated at 600° for 4 h, cooled to 150°, placed in a vacuum desiccator and cooled to room temperature under reduced pressure. Each complete batch of the activated material (400 g for alumina and 200 g for silica) was deactivated to the required level immediately by shaking with distilled water. Both deactivated materials were stored in stoppered flasks. Sodium sulphate (AR), used as a drying agent, was heated to 200° for 4 h, cooled and stored in a glass-stoppered bottle. High purity glass-distilled *n*-hexane and acetone (Rathburn, Walkerburn, Great Britain) were used for rinsing the glassware, and as solvents. A 100-ml aliquot of *n*-hexane from each batch was concentrated to 1 ml and examined for any background interference.

The adsorption columns were freshly prepared for each sample. Each empty column was rinsed with acetone followed by hexane and allowed to drain and air-dry. The column packing was measured out by volume using a calibrated tube, poured into the column and packed by tapping the sides of the column. A small charge (200 mg) of anhydrous sodium sulphate was placed at the column head to ensure complete dryness of the sample before passing to the adsorbent. The sample or standard, contained in 1 ml of *n*-hexane, was pipetted on column and allowed to drain into the column before the sample rinsings and the hexane charge were added to the reservoir. The appropriate eluate volumes were collected in graduated tubes and evaporated to 1 ml, either for analysis by GLC or for further separation. The final eluates were injected into a Varian 1400 single column chromatograph fitted with a tritium ECD instrument. The GLC glass column was 1525 × 2 mm I.D., containing 4% SE-30 + 6% OV-210 on Chromosorb W HP (80–100 mesh). Nitrogen was used as a carrier gas at a flow-rate of 30–35 ml/min. The column and injector temperatures were 200° and the detector temperature was 220°. Identification was based on relative retention times using dieldrin as a reference, and samples were quantified by comparing the peak heights with those from standard pesticide solutions. These standards were injected at similar times to the sample to minimise errors in sensitivity caused by fluctuations of the instrument.

RESULTS

*Adsorbent activation*

An investigation of the effect of thermal treatment on the purity and the final activity of both alumina and silica used as adsorption column packing confirmed that a high temperature firing at 600–800°, as outlined previously, was necessary to minimise the trace impurities. The high purity obtained by thermal treatment outweighed the inherent loss of activity, particularly of the alumina, due to firing.

*Column size*

The diameter and length of the micro-columns were varied to improve the chromatographic efficiency of the packing. However the wider columns (8 and 10 mm) gave much poorer resolution while longer columns of narrower bore (4–5 mm) increased the residence time by restricting the solvent flow, without any significant gain in separation. The 6 mm I.D. column was therefore chosen for the remaining experiments.

*Silica columns*

The original 2-g columns of silica, deactivated with 5% water did not completely separate *o,p'*-DDT from the PCBs and *p,p'*-DDE, but this was improved by increasing the column size from 2 to 2.5 g and by continuing to elute with *n*-hexane until all the *o,p'*-DDT and *p,p'*-DDT were removed. However, it was still necessary to remove the more polar species from the column with 10% diethyl ether in *n*-hexane.

The optimum activity of the silica column for the *o,p'*-DDT–*p,p'*-DDE separation was found by varying the water content from 0 to 7% and monitoring the effect upon the elution pattern (Table I). The results show that the best separation was obtained when 3% water was added to the silica. Further additions of water to 5% and 7% speeded up the elution, but the resolution of the column tended to decrease. The active silica, being strongly hygroscopic, readily altered its activity upon exposure to moist air and was found unsuitable for use, particularly after long-term storage. The silica deactivated with 3% water offered the best resolution and this was chosen for inclusion in the present scheme. Although there is an overlap of *p,p'*-DDE (62%) and *o,p'*-DDT (17%) at the 6-ml elution volume, the separation was completed by changing the collection tube at a more appropriate point druing the elution, *e.g.* at 5.5 ml, giving less than 8% of *o,p'*-DDT in the first eluate.

TABLE I

EFFECT OF WATER CONTENT ON THE SEPARATION CHARACTERISTICS OF SILICA

Silica (2.5 g; Merck No. 7734) activated at 600° for 4 h; eluent, *n*-hexane; column, 6 mm I.D. glass.

| Elution (% water) | Pesticide | Recovery (%) | | | | |
|---|---|---|---|---|---|---|
| | | Volume (ml) | | | | |
| | | 1–4 | 5 | 6 | 7 | 8–13 |
| 0 | *p,p'*-DDE | — | 3 | 14 | 45 | 38 |
| | *o,p'*-DDT | — | — | — | 2 | 98 |
| | *p,p'*-DDT | — | — | — | — | 100 |
| 2 | *p,p'*-DDE | — | 52 | 41 | 7 | — |
| | *o,p'*-DDT | — | — | 30 | 26 | 44 |
| | *p,p'*-DDT | — | — | — | 5 | 95 |
| 3 | *p,p'*-DDE | — | 38 | 62 | — | — |
| | *o,p'*-DDT | — | — | 17 | 25 | 58 |
| | *p,p'*-DDT | — | — | — | 1 | 99 |
| 5 | *p,p'*-DDE | 50 | 50 | — | — | — |
| | *o,p'*-DDT | — | 43 | 34 | 20 | 3 |
| | *p,p'*-DDT | — | — | 10 | 51 | 39 |
| 7 | *p,p'*-DDE | 55 | 43 | 2 | — | — |
| | *o,p'*-DDT | — | 21 | 57 | 22 | — |
| | *p,p'*-DDT | — | — | 8 | 48 | 44 |

The reproducibility of this separation was strongly dependent upon the standardization of the activation and subsequent deactivation of the adsorbent. Each batch, initially 100 g, remained stable throughout its shelf-life (4–5 weeks) giving reproducible chromatographic results. However, it was found that the elution volume

and the degree of separation varied from batch to batch and occasionally, using the previous method of Holden and Marsden[2], a 2-g silica column, deactivated to 5% with water, did not successfully separate $p,p'$-DDE and $p,p'$-DDT. Prior to this study the silica (500 g) was activated and stored in a vacuum desiccator, and working quantities (100 g) were subsequently deactivated and tested when required. Consequently the moisture content of the active adsorbent was dependent upon the number of times it was removed from the desiccator and exposed to the atmosphere, and the effectiveness of the vacuum seal on the desiccator. It was found that the failure of the deactivated material in separating the pesticide residues was related to the age of the active silica. A comparison of the separation obtained from freshly activated silica and active silica, three months old, both deactivated with 3% water, is given in Table II. When this older active silica had been freshly made and tested it had given acceptable results for the separation of $p,p'$-DDE and $o,p'$-DDT. However after three months had elapsed it gave poor resolution, indicating the limited shelf-life of the active material. Reliable and reproducible results were obtained from silica if the material was deactivated immediately after cooling from 600°. Each batch once deactivated was tested and the chromatographic characteristics were found to remain constant throughout the lifetime of the batch.

TABLE II

EFFECT OF AGE OF ACTIVE SILICA ON THE CHROMATOGRAPHIC PROPERTIES AFTER DEACTIVATION

Silica (2.5 g; Merck No. 7734) deactivated with 3% water; eluent, $n$-hexane; column, 6 mm I.D. glass. A, column prepared from activated silica, 3 months old; B, column prepared from activated silica fired the same day.

| Elution volume (ml) | Recovery (%) | | | | | |
|---|---|---|---|---|---|---|
| | A | | | B | | |
| | $p,p'$-DDE | $o,p'$-DDT | $p,p'$-DDT | $p,p'$-DDE | $o,p'$-DDT | $p,p'$-DDT |
| 1–5 | 82 | 3 | – | 73 | 4 | – |
| 5–13 | 18 | 97 | 100 | 27 | 96 | 100 |
| 1–5.5 | 83 | 25 | ... | 96 | 6 | – |
| 5.5–13 | 17 | 75 | 100 | 4 | 94 | 100 |
| 1–6 | 86 | 36 | – | 97 | 35 | – |
| 6–13 | 14 | 64 | 100 | 3 | 65 | 100 |

Although the silica columns were able to separate the early eluting compounds by using $n$-hexane, it was still necessary to add a more polar solvent to remove $p,p'$-DDD, dieldrin and the HCH isomers. The purity of the mixed solvent after eluting from the silica column was frequently checked and found unacceptable due to extracted impurities from the silica. Prewashing the packed columns had previously been investigated, but the process was both time-consuming and tended to give erratic results. Two other alternatives were considered, namely a different solvent of similar polarity (dielectric constant) or removal of the more polar compounds before the silica

column stage. The latter appeared to be a more promising alternative, utilizing the alumina column to effect the separation.

*Alumina columns*

Most extracts from environmental samples require the removal of unwanted co-extracted materials before being introduced into a GLC or gas chromatography–mass spectrometry (GC–MS) system, and alumina has been successfully used for this purpose. Although it is more absorbent than silica, the alumina is less polar at similar water-content levels, and only requires *n*-hexane as a solvent to elute the organo-chlorine residues of current interest from the column. By careful manipulation of the column parameters a division at a suitable point during the elution was considered possible, to separate the more polar compounds which have a larger retention volume on the silica column. The most useful division of the alumina column eluate falls between *p,p'*-DDT and *p,p'*-DDD. The PCBs and *p,p'*-DDE are eluted prior to the *p,p'*-DDT and dieldrin is removed long after *p,p'*-DDD.

The original 2 g alumina, deactivated to 5% with water, used for clean-up did not achieve this, but the results from work by Holden and Marsen[2] gave every indication that it could be done by changing the column conditions. The resolution was much improved by decreasing the water content of the alumina from 5 to 3% and by increasing the column loading from 2 to 3 g. However, using the BDH aluminium hydroxide as a starting material, the elution time to remove dieldrin from the column was increased from 3 to 5 h. This extension, although not an analytical problem, did offset the advantages gained in column performance for a routine method.

Another grade of alumina (Reeve Angel alumina A11-O-col) gave a superior performance with a reduced elution time. This material was sieved and the particle size band 64–125 µm retained. The elution time for dieldrin using a 3-g column was reduced from 5 to 1.2 h, which was a 50% reduction in time over the original columns.

TABLE III

SEPARATION OF ORGANOCHLORINES ON ALUMINA

3 g Al₂O₃ (A11-O-col) 64–125 µm activated at 800° for 6 h and deactivated with 3% water.

| Elution volume (ml) | Recovery (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | p,p'-DDE | o,p'-DDT | p,p'-DDT | p,p'-DDD | α-HCH | γ-HCH | Dieldrin | β-HCH |
| 1 | | | | | | | | |
| 2 | 74 | 44 | | | | | | |
| 3 | 26 | 56 | 46 | | | | | |
| 4 | | | 54 | | | | | |
| 5 | | | | | 9 | | | |
| 6 | | | | | 54 | | | |
| 7–12 | | | | 100 | 37 | 100 | | |
| 13–17 | | | | | | | | |
| 18–22 | | | | | | | 82 | |
| 23–27 | | | | | | | 18 | |
| 28–32 | | | | | | | | |
| 33–37 | | | | | | | | |
| 38–42 | | | | | | | | 66 |
| 43–47 | | | | | | | | 34 |

TABLE IV

ELUTION PATTERN OF SEVENTEEN ORGANOCHLORINE RESIDUES ON ALUMINA AND SILICA COLUMNS

3 g Al$_2$O$_3$ (A11-O-col) containing 4% water; 2.5 g SiO$_2$ (Merck No. 7734) containing 3% water. Eluent, n-hexane; columns, 6 mm I.D. glass.

| Elution volume (ml) | HCB | PCB (1254) | Aldrin | Hepta-chlor | p,p'-DDE | o,p'-DDT | p,p'-DDT | α-HCH | γ-HCH | γ-Chlor-dane | p,p'-DDD | Endo-sulfan I | Hepta-chlor epoxide | Endrin | Dieldrin | p,p'-DCBP | β-HCH |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Silica** | | | | | | | | | | | | | | | | | |
| 1–3 | 100 | | | | | | | | | | | | | | | | |
| 4 | | 30 | 9 | | | | | | | | | | | | | | |
| 5 | | 54 | 48 | | 32 | | | | | | | | | | | | |
| 6 | | 15 | 37 | 14 | 57 | | | | | | | | | | | | |
| 7 | | | 6 | 60 | 12 | 59 | | | | | | | | | | | |
| 8–14 | | | | 26 | | 41 | 100 | | | | | | | | | | |
| *Alumina* | | | | | | | | | | | | | | | | | |
| 5 | | | | | | | | 9 | | 18 | | | | | | | |
| 6 | | | | | | | | 56 | | 57 | | | | | | | |
| 7 | | | | | | | | 30 | 4 | 23 | 26 | | | | | | |
| 8 | | | | | | | | 5 | 28 | 2 | 49 | | | | | | |
| 9 | | | | | | | | | 56 | | 23 | 3 | | | | | |
| 10 | | | | | | | | | 12 | | 2 | 33 | | | | | |
| 11 | | | | | | | | | | | | 38 | | | | | |
| 12 | | | | | | | | | | | | 22 | 2 | 4 | | | |
| 13 | | | | | | | | | | | | 4 | 16 | 21 | | | |
| 14 | | | | | | | | | | | | | 32 | 34 | | | |
| 15 | | | | | | | | | | | | | 33 | 29 | | | |
| 16–17 | | | | | | | | | | | | | 17 | 12 | 3 | | |
| 18–19 | | | | | | | | | | | | | | | 39 | | |
| 20–21 | | | | | | | | | | | | | | | 52 | | |
| 22–23 | | | | | | | | | | | | | | | 6 | | |
| 24–25 | | | | | | | | | | | | | | | | | |
| 26–30 | | | | | | | | | | | | | | | | 3 | 53 |
| 31–35 | | | | | | | | | | | | | | | | 92 | 47 |
| 36–40 | | | | | | | | | | | | | | | | 5 | |
| 41–45 | | | | | | | | | | | | | | | | | |

* First 4 ml from alumina column chromatographed on silica (see Fig. 1).

The separation between p,p'-DDT and p,p'-DDD was increased from 0.5 to 3–4 ml in elution volume and α- and β-HCH were also isolated from the early eluting peaks. However a considerable increase in the elution volume (over 50 ml) was necessary to remove β-HCH from the column (Table III). Reduction of the activity of the Reeve Angel alumina by increasing the water content from 3 to 4% decreased the total elution volume without a loss in resolution between p,p'-DDT and p,p'-DDD. This gave an extremely useful separation of a number of organochlorine residues when used in conjunction with the silica columns, as described earlier (Table IV).

With the modified alumina column the collection tubes were exchanged after the elution of p,p'-DDD. This fulfilled a dual role by isolating α- and γ-HCH from β-HCH in particular, and by separating the later eluting compounds into two groups to assist in the quantitative analysis.

## Lipid content of extracts

The elution profile and recovery of organochlorine residues on alumina has been observed to alter with increasing lipid content of the sample[5], and it was necessary to determine the effect of other co-extracted materials upon the performance of the columns which had been developed using standard solutions. A series of solutions were made containing a fixed concentration of organochlorines with a range of concentrations of cod liver oil. One milliliter of each soluiton was chromatographed on a 3-g Reeve Angel alumina column, deactivated to 4% with water, and separated using

TABLE V

EFFECT OF LIPID CONTENT ON THE RECOVERY OF ORGANOCHLORINES FROM ALUMINA

3 g Al$_2$O$_3$ (A11-O-col) 64–125 μm activated at 800° for 6 h and deactivated with 4% water. Eluent, n-hexane; column, 6 mm I.D. glass.

| Lipid content (mg) | Elution volume (ml) | Recovery (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | p,p'-DDE | o,p'-DDT | p,p'-DDT | p,p'-DDD | α-HCH | γ-HCH | Dieldrin | β-HCH |
| 0 | 1–4 | 112 | 100 | 108 | | | | | |
| | 5–12 | | | | 110 | 104 | 90 | | |
| | 13–35 | | | | | | | 98 | 86 |
| 21 | 1–4 | 100 | 94 | 101 | | | | | |
| | 5–12 | | | | 109 | 104 | 99 | | |
| | 13–35 | | | | | | | 104 | 87 |
| 38 | 1–4 | 104 | 110 | 112 | | | | | |
| | 5–12 | | | | 109 | 106 | 105 | 28 | |
| | 13–35 | | | | | | | 82 | 87 |
| 61 | 1–4 | 104 | 98 | 104 | | | | | |
| | 5–12 | | | | 104 | 96 | 94 | 87 | |
| | 13–35 | | | | | | | 24 | 103 |
| 89 | 1–4 | 92 | – | 112 | * | 21 | – | | |
| | 5–12 | | – | | 77 | ** | –– | 73 | ** |
| | 13–35 | | | | | | | | 38 |
| 178 | 1–4 | 84 | – | 103 | * | 81 | | | |
| | 5–12 | | | | | ** | | 37 | ** |
| | 13–35 | | | | | | | | 16 |

* p,p'-DDD found in 1–4 ml masked by PCBs present in the oil.
** γ-HCH and β-HCH overlapped in the same eluate.

the elution columes found most suitable for the lipid-free standards. The background values obtained for the organochlorines in the original oil were subtracted to obtain the percentage recovery values of the added compounds (Table V). At no or low lipid content the separations and recoveries are quantitative and reproducible, except for $\beta$-HCH which tends to be retained on the column. As the lipid content is increased the elution profile is gradually compressed, decreasing the elution volume of the more polar materials. This compression did not initially affect the early sections of the profile, but with further additions of fat (*ca.* 90 mg) *p,p'*-DDD and $\gamma$-HCH began to emerge along with *p,p'*-DDE and *p,p'*-DDT. At this loading and at higher lipid values the recoveries of the residues became variable and unreliable. These variations in column performance with lipid content do not preclude the use of the method for the analysis of animal tissue extracts provided that the weight of lipid transferred to the column is controlled, the maximum acceptable lipid loading being 60 mg for a 3-g column. This lipid limit was easily maintained by analysing for the extractable residues prior to the organochlorine residue analysis. For an extract of high lipid, but suspected low organochlorine content a small 1-g alumina column was used prior to the main 3-g separation column. The Reeve Angel alumina has a lipid-holding capacity of approximately 50 mg/g. This was determined by loading a series of 1-g alumina columns with a range of weights of lipid and measuring the amount eluted by 40 ml of hexane (Table VI).

TABLE VI

LIPID HOLDING CAPACITY OF ALUMINA

1 g Al$_2$O$_3$ (A11-O-col) 64–125 $\mu$m activated at 800° for 6 h and deactivated with 4% water. Eluent, 40 ml *n*-hexane.

| Lipid | Weight of lipid (mg) | | | |
|---|---|---|---|---|
| On column | 52.5 | 101.6 | 145.1 | 213.0 |
| Recovered | 3.7 | 51.6 | 102.7 | 165.8 |
| Retained | 48.8 | 50.0 | 42.7 | 47.2 |

This 1-g column could therefore be used to reduce the fat content in the sample prior to separation on the main 3-g alumina column. A volume of 15 ml of *n*-hexane was required to elute a sample of $\beta$-HCH from a 1-g alumina column, deactivated to 4% with water.

The partial elution of dieldrin in the second eluate caused by the presence of more than 20 mg lipid was avoided by dividing the eluting *n*-hexane at 10 ml. This was found suitable for non-lipid samples as well as tissue extracts. The standards used for testing each batch of deactivated alumina were in hexane solution, but standardization can be improved by the addition of 50 mg/ml lipid.

*The separation procedure*

The final form of the separation procedure is given in Fig. 1. This flow diagram outlines the complete method and lists the constituent compounds in each eluate. The chromatogram obtained from the separation of the listed organochlorines (Table IV) is given in Fig. 2a–d and can be compared to the traces obtained by the separation with silica alone, Fig. 2e. The compounds which are currently of particular interest in

　　　　　　　　　　　　　　　　　　　　　D. E. WELLS, S. J. JOHNSTONE

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                                                                    HCB
                                                                    Aldrin
              ┌─1-4ml evaporate to 1ml        ┌─1-6ml─E1a─ p,p'DDE
              │                               │            PCB
              │                               │            Heptachlor(part)
              │                               │
              │        2·5g SiO₂ 3% H₂O ──────┤
              │                               │
              │                               │            Heptachlor(part)
              │                               └─7-13ml-E1b─ o,p'DDT
              │                                            p,p'DDT
              │
Sample in 1ml │   3g Al₂O₃ 4% H₂O
n·hexane ─────┤   64µ-125µ
              │
              │                                            α-HCH
              │                                            γ-Chlordane
              ├── 5-12 ml ──────────────────────── E2 ──  γ·HCH
              │                                            p,p'DDD
              │                                            Endosulfan I
              │
              │                                            Heptachlor epoxide
              │                                            Dieldrin
              └── 13-40 ml ─────────────────────── E3 ──  Endrin
                                                           β-HCH
                                                           p,p'DCBP
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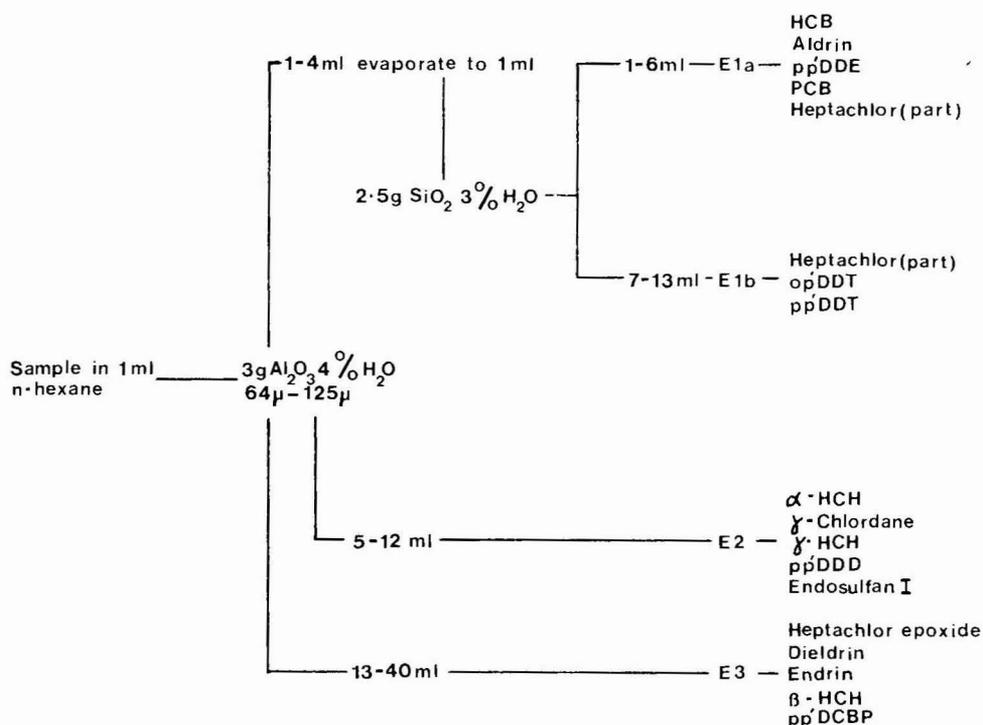
Fig. 1. Flow diagram for the separation of seventeen organochlorine residues using alumina and silica microcolumns and *n*-hexane as the eluent.

environmental samples are clearly separated into four eluates. Heptachlor which is rarely detected in United Kingdom samples, is split between two eluates, but should it occur its presence would not be masked in GLC analysis by other organochlorines included here.

DISCUSSION

This new technique (Fig. 1), incorporating the dry packed alumina and silica columns, has significantly shortened the time required for analysis and improved the identification and quantification of organochlorine residues in natural samples. The time spent on clean-up and separation has been decreased by 50%, giving an overall processing time of approximately 4 h prior to GLC analysis. While the later eluates (5–40 ml) are developing on the alumina column the first alumina eluate can be separated on silica. Both alumina and silica can be reliably deactivated from freshly activated materials in quantities of up to 500 g and will retain their activity for at least three months.

The preparation of the columns and the measurement of the elution volumes were found to be quite critical, particularly at the silica column stage. Although the silica performance was acceptably reproducible, the amount of $p,p'$-DDE which was found in the second (E1b) eluate varied from 2–10%, and the level of $o,p'$-DDT found in the first elutae was occasionally as much as 8% but generally less than 5%. This
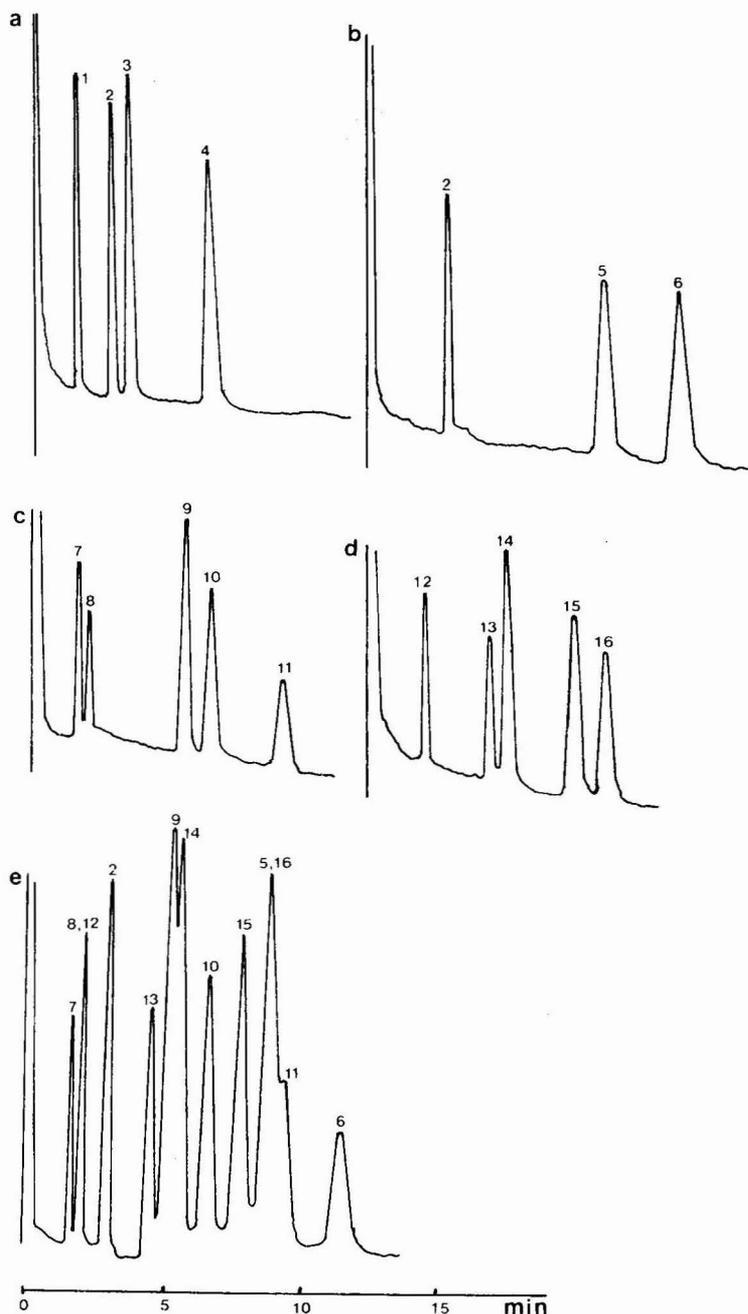
Fig. 2. Chromatograms obtained from (a–d) the eluates using the current method, and (e) the method of Holden and Marsden[2]. Column, 4% SE-30 + 6% OV-210 on Chromosorb W HP; temperature, 200°, nitrogen carrier gas, 35 ml/min. (a) Eluate 1a; 1 = HCB, 2 = heptachlor, 3 = aldrin, 4 = $p,p'$-DDE. (b) Eluate 1b; 2 = heptachlor, 5 = $o,p'$-DDT, 6 = $p,p'$-DDT. (c) Eluate 2; 7 = $\alpha$-HCH, 8 = $\gamma$-HCH, 9 = $\gamma$-chlordane, 10 = endosulfan I, 11 = $p,p'$-DDD. (d) Eluate 3; 12 = $\beta$-HCH, 13 = $p,p'$-DCBP, 14 = heptachlor epoxide, 15 = dieldrin, 16 = endrin. (e) Eluate 2 (Holden and Marsden[2]) which incorporates eluates 1b, 2 and 3 of the new method.

variation reflected the difficulty in packing a column with identical chromatographic characteristics and in measuring small volumes of volatile solvent accurately.

The identification and quantification of $o,p'$-DDT and HCH isomers have been simplified by altering the characteristics of the silica, and by removing the more polar compounds at the alumina stage. This avoids the use of 10% diethyl ether in $n$-hexane on the silica columns. The separation of the HCH isomers into two eluates (E2 for $\alpha$- and $\gamma$-HCH, E3 for $\beta$-HCH) on alumina also assists in their identification.

The four eluates obtained in the preparation of each sample increase the overall chromatographic time, but this is not without justification. As well as improving the separation of the more common organochloriens present it reduces the possibility of confusion with unknown substances. The method has been successfully applied to the analysis of a considerable variety of environmental samples. The analysis of animal tissue extracts is only limited by the amount of lipid which can be applied to the alumina columns, but reproducible results can be obtained if this does not exceed 60 mg.

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# GASCHROMATOGRAPHISCHE BESTIMMUNG DER FLÜCHTIGEN FETTSÄUREN VON C₁ BIS C₅ EINSCHLIESSLICH DER MILCHSÄURE ALS BENZYLESTER UNTER VERWENDUNG VON PHENYLDIAZOMETHAN ALS BENZYLIERUNGSMITTEL

## BESTIMMUNG DER SÄUREN IN SILAGEN

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SUMMARY

*Gas chromatographic determination of the volatile fatty acids ($C_1$-$C_5$) including lactic acid after conversion into their benzyl esters by phenyldiazomethane. Determination of the acids in silages.*

A gas chromatographic method for the determination of the volatile fatty acids ($C_1$-$C_5$) including lactic acid is described. The acids are converted into their benzyl esters by means of phenyldiazomethane without previous purification of the reagent by distillation. The benzyl esters are well separated from the solvent as well as from one another. Silicone SE-30 is used as the stationary phase. An application of the method on silage acids is demonstrated.

EINLEITUNG

Im allgemeinen werden die flüchtigen Fettsäuren mit Ausnahme der Ameisensäure gaschromatographisch (GC) als freie Säuren bestimmt, wobei zur Unterdrückung des sog. Memory-Effektes Ameisensäure verwendet wird. Bezüglich der zahlreichen Veröffentlichungen hierüber sei auf das ausführliche Literaturverzeichnis in der Arbeit von van Huyssteen[1] verwiesen. Eine Einbeziehung der Ameisensäure in diese Bestimmung ist wegen der Unempfindlichkeit des Flammenionisationsdetektors (FID) dieser Säure gegenüber nicht möglich[2]. Zugleich anwesende Milchsäure wurde bisher gesondert bestimmt, meist nach der Methode von Barker–Summerson[3,4]. Die GC-Bestimmung dieser schwerflüchtigen und bei stärkerem Erhitzen zur Kondensation und schliesslich Zersetzung neigenden Säure erfolgt überwiegend in Form ihres Methylesters[5]. Es ergibt sich, dass für eine gemeinsame GC-Bestimmung, wie sie u.a. für die Untersuchung der Gärsäuren in Silagen erwünscht wäre, alle diese Säuren derivatisiert werden müssen. Wegen ihrer beträchtlichen Flüchtigkeit (vgl. Tabelle I), welche zu Substanzverlusten während der Analyse führt und auch für die schlechte Trennung ihrer Peaks vom Lösungsmittelpeak im Chromatogramm verantwortlich ist, kommen die Methylester als Derivate für die niederen Fettsäuren nicht in Betracht, so sehr sie sich auch in der GC der höheren Fettsäuren bewährt haben. Eine Reihe höher

TABELLE I

SIEDEPUNKTE (°C) EINIGER ESTER DER FLÜCHTIGEN FETTSÄUREN $C_1$ BIS $C_5$

| Säure | Methylester | Propylester | Butylester | Benzylester |
|---|---|---|---|---|
| Ameisensäure | 32.0 | 81.3 | 106.9 | 202/747 Torr |
| Essigsäure | 57.1 | 101.5 | 126.5 | 213.5 |
| Propionsäure | 79.7 | 122.5 | 146.0 | 221.0 |
| Isobuttersäure | 92.5 | 135.0 | 155.0 | 229.5 |
| n-Buttersäure | 102.3 | 142.5 | 165.7 | 239.0 |
| Isovaleriansäure | 116.5 | 155.5 | 176.0 | 245.0 |
| n-Valeriansäure | 127.3 | 167.5 | 186.5 | 250/730 Torr |

siedender Derivate wie Propylester[6], Butylester[7-12], Benzylester[13-16], p-Bromphenyl-
und p-Phenylphenacylester[17], Anilide und Toluidide[18,19] sowie Trimethylsilyl(TMS)-
Verbindungen[20] wurde Gegenstand von Untersuchungen. Nach Tabelle I zeichnen
sich die Benzylester gegenüber den anderen dort aufgeführten Estern durch besondere
Schwerflüchtigkeit aus. Sie lassen daher gute Trenneigenschaften erwarten. In einer
vorangegangenen Arbeit[21] wurde bereits gezeigt, dass Ameisensäure mit Phenyldia-
zomethan (PDM) in Form des Rohproduktes benzyliert und gaschromatographisch
als Benzylester bestimmt werden kann, wenn der zur Benzylierung benötigte PDM-
Überschuss durch den Zusatz einer anderen niederen aliphatischen Monocarbonsäure
beseitigt und damit das Auftreten von Störpeaks von Artefakten des PDM im Chro-
matogramm vermieden wird. Der schwerflüchtige Ameisensäurebenzylester liess sich
vom Lösungsmittel trennen, das Chromatogramm zeigte gut getrennte Peaks. Die Ver-
wendbarkeit des PDM in Form seines Rohproduktes zur Analyse ist insofern von
Vorteil, als bei der Reindarstellung durch Vakuumdestillation ein Grossteil des
Reagenzes zersetzt wird und damit verloren geht. Sie wird dadurch ermöglicht, dass
das Gaschromatogramm des PDM im interessierenden Bereich keine Peaks aufweist,
welche nach Beseitigung des PDM-Überschusses Störungen verursachen[21]. In der
vorliegenden Arbeit werden die Untersuchungen auf die flüchtigen Fettsäuren von $C_1$
bis $C_5$ mit Einschluss der Milchsäure ausgedehnt in der Absicht, die Gärsäuren in

TABELLE II

RETENTIONSZEITEN (min) DER BENZYLESTER DER FETTSÄUREN $C_1$ BIS $C_5$ SOWIE
DER MILCHSÄURE BEI VERSCHIEDENEN ARBEITSBEDINGUNGEN

(A) Säule I, isotherm bei 170°, He 60 ml /min (Fig. 1); (B) Säule I, Temperaturprogramm: 120° (12
min) + 5°/min (8 min), He 60 ml/min (Fig. 2); (C) Säule II, 130° (4.7 min) + 5°/min (4 min), He 38
ml/min (Fig. 3); (D) Säule II, isotherm bei 130°, He 60 ml/min (Fig. 4 und 5); (E) Säule II, 114° (5 min)
+ 7.5°/min (4 min) He 38 ml/min (Fig. 6 und 7).

| Benzylester | A | B | C | D | E |
|---|---|---|---|---|---|
| Ameisensäure | 2.3 | 7.5 | 2.5 | 2.1 | 4.7 |
| Essigsäure | 3.0 | 10.8 | 4.1 | 2.9 | 7.0 |
| Propionsäure | 4.1 | 13.8 | 6.1 | 4.5 | 9.4 |
| Isobuttersäure | 4.9 | 15.0 | 7.1 | 5.4 | |
| n-Buttersäure | 5.7 | 16.1 | 8.2 | 6.6 | 11.5 |
| Isovaleriansäure | 6.9 | 17.4 | 9.4 | 8.4 | |
| n-Valeriansäure | 8.3 | 18.7 | 10.6 | 10.5 | |
| Milchsäure | | | 8.7 | 7.2 | 12.1 |

Silagen zusammen mit den als Silierhilfsmittel zugesetzten Säuren en bloc gaschromatographisch zu erfassen.

EXPERIMENTELLES

*Darstellung von Phenyldiazomethan*

Phenyldiazomethan wurde durch Einwirken einer methanolischen KOH-Lösung auf eine ätherische Suspension von *p*-Tosyl-N-benzyl-nitrosamid dargestellt[21], welches nach einem modifizierten Verfahren von White[21,22] durch Nitrosylierung mit Hilfe von Natriumnitrit aus *p*-Tosylbenzylamid erhalten worden war. Die letztgenannte Verbindung wurde nach Holmes und Ingold[23] aus *p*-Tosylchlorid und Benzylamin synthetisiert. Die bordeauxrote PDM-Lösung wurde mehrmals mit destilliertem Wasser gewaschen, mit Natriumsulfat getrocknet und ohne weitere Aufarbeitung durch Vakuumdestillation zum Benzylieren verwendet.

*Modellösungen*

Aus ätherischen Stammlösungen mit einem Gehalt von je 1 g Ameisen-, Essig-,
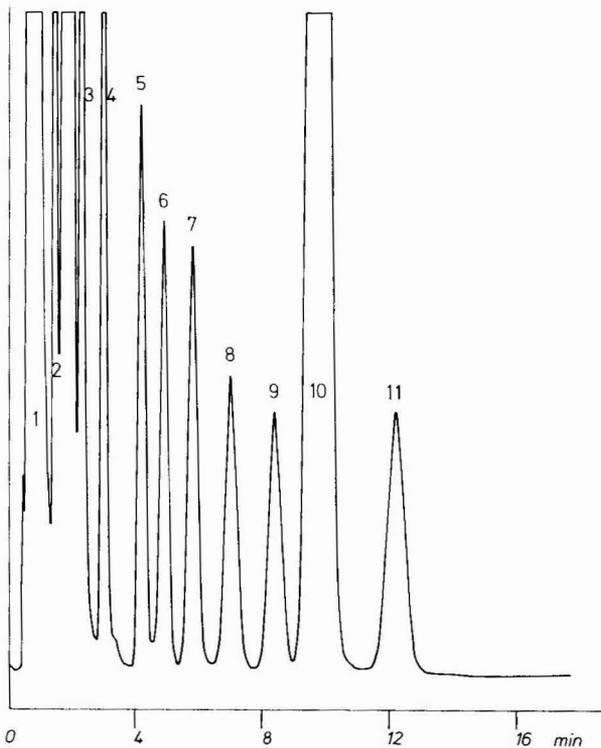


Fig. 1. Chromatogramm der Benzylester der Säuren $C_1$ bis $C_5$ in Äther. Die Benzylierung erfolgte mit PDM, dessen Überschuss mittels *n*-Hexansäure beseitigt wurde. 1 = Äther; 2 = *n*-Hexansäure; 3 = Ameisensäurebenzylester; 4 = Essigsäurebenzylester; 5 = Propionsäurebenzylester; 6 = Isobuttersäurebenzylester; 7 = *n*-Buttersäurebenzylester; 8 = Isovaleriansäurebenzylester; 9 = *n*-Valeriansäurebenzylester; 10 = Nebenprodukt der PDM-Darstellung; 11 = *n*-Hexansäurebenzylester. Trennsäule I bei 170° isotherm.

Propion-, Isobutter-, *n*-Butter-, Isovalerian, *n*-Valerian- und Milchsäure pro 100 ml Lösung wurden durch Entnahme aliquoter Volumina Modellösungen hergestellt, welche 10 bis 200 mg je Säure pro 100 ml enthielten und damit Probelösungen von Silagen mit einem Gehalt von 0.1 bis 2.0% je Säure entsprachen[5].

*Benzylierung mit PDM*

Aliquote (je 10 ml) der Modellösungen wurden mit PDM-Lösung (je 10 ml) im Überschuss versetzt und in verschlossenen Kölbchen 24 Studen bei Zimmertemperatur stehengelassen. Dann wurde der PDM-Überschuss (erkennbar an einer kräftigen Rotfärbung) durch einen Zusatz von *n*-Hexansäure (0.5 ml) beseitigt. Die farblos gewordenen Lösungen wurden der GC-Bestimmung zugeführt.

*Gaschromatographie*

Von den Benzylierungslösungen wurden je 5 μl mittels einer 10 μl-Hamilton-spritze N 75 in den Gaschromatographen, Modell GC-4 der Fa. Beckman, injiziert. Das mit einem Doppel-FID ausgerüstete Gerät wurde mit Helium als Trägergas (60 bzw. 38 ml/min) betrieben. Zur Trennung dienten eine Stahlsäule (1.80 m × 0.25 in. I.D.) mit 10% SE-30 auf Chromosorb G AW DMCS (60–80 mesh) (Säule I) sowie
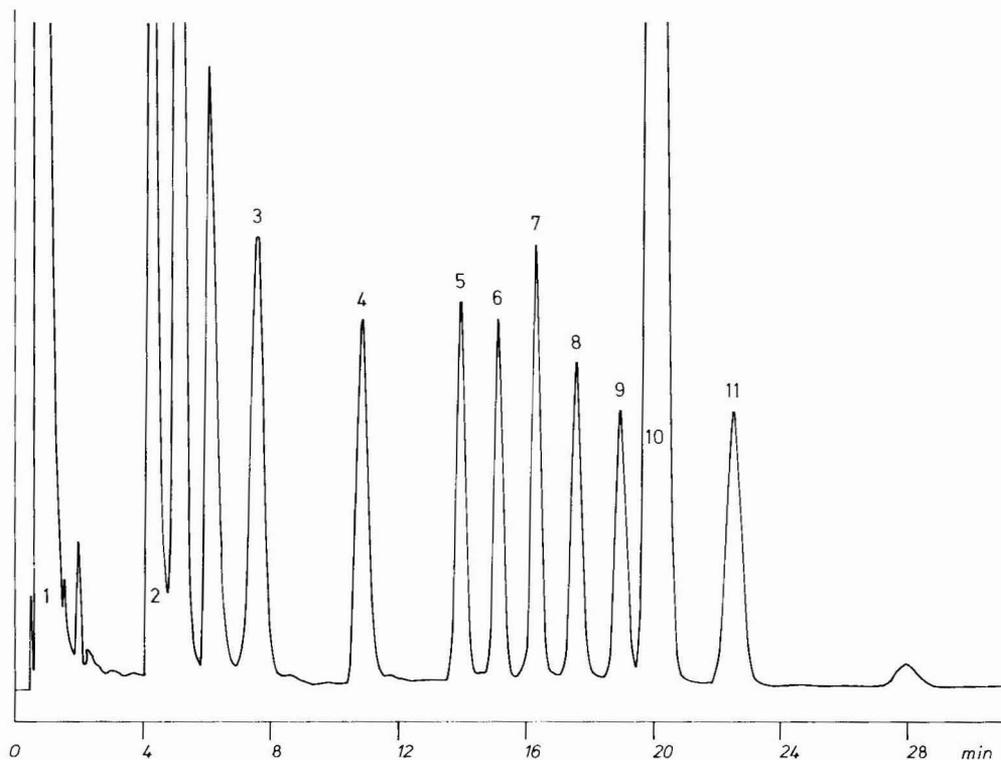


Fig. 2. Chromatogramm der Benzylester der Säuren $C_1$ bis $C_5$ in Äther. Die Bezeichnungen sind die gleichen wie bei Fig. 1. Trennsäule I. Es wurde temperaturprogrammiert gearbeitet, wobei zuerst die Säulentemperatur 12 min auf 120° gehalten und dann mit einer Steigerungsrate von 5°/min auf 160° erhöht wurde.

eine Glassäule (3.00 m × 0.4 cm I.D.) mit 10% SE-30 auf Chromosorb W AW DMCS (80–100 mesh) (Säule II). Die Glassäule wurde durch den Einspritzblock hindurchgeführt, um direktes Injizieren der Proben auf die Säule zu ermöglichen. Es wurde sowohl isotherm als auch temperaturprogrammiert gearbeitet. Die Temperaturen des Einspritzblockes und des Detektors betrugen 250° bzw. 270°, die Abschwächung lag im Bereich von 1 × 10³ bis 1 × 10⁴.

ERGEBNISSE UND DISKUSSION

Die Versuche ergaben, dass sich sowohl flüchtige Fettsäuren als auch Milchsäure im Gemisch leicht mit PDM benzylieren lassen. Es zeigt sich weiterhin, dass zur Benzylierung PDM als Rohprodukt verwendet werden kann, wodurch die mühsame und verlustreiche Reindarstellung des Reagenzes unnötig wird. Zwar taucht im Gaschromatogramm des Rohproduktes ein grosser Peak auf, welcher von einem Nebenprodukt der PDM-Darstellung stammt, doch verursacht dieser keine Störung, da er
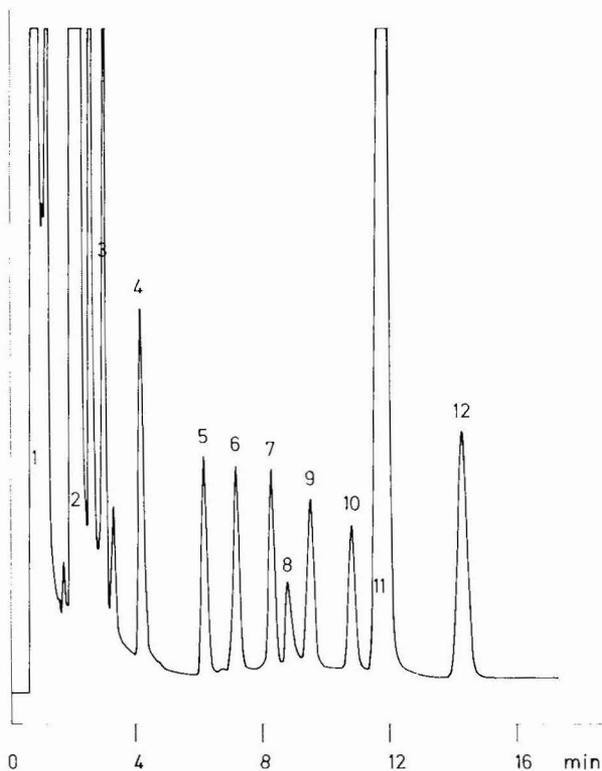


Fig. 3. Chromatogramm der Benzylester der Säuren $C_1$ bis $C_5$ sowie der Milchsäure in Äther. Die Benzylierung erfolgte mit PDM, dessen Überschuss durch einen Zusatz von *n*-Hexansäure entfernt wurde. 1 = Äther; 2 = *n*-Hexansäure; 3 = Ameisensäurebenzylester; 4 = Essigsäurebenzylester; 5 = Propionsäurebenzylester; 6 = Isobuttersäurebenzylester; 7 = *n*-Buttersäurebenzylester; 8 = Milchsäurebenzylester; 9 = Isovaleriansäurebenzylester; 10 = *n*-Valeriansäurebenzylester; 11 = Nebenprodukt der PDM-Darstellung; 12 = *n*-Hexansäurebenzylester. Trennsäule II. Temperaturprogramm: 4.7 min bei 130°, dann Steigerung auf 150° mit einer Rate von 5°/min. Trägergasstrom (He): 38 ml/min.

zwischen den Peaks des *n*-Valeriansäure- und *n*-Hexansäurebenzylesters liegt, ohne diese zu überlappen (Peak 10 in Fig. 1). Wird in Analogie zum Verfahren der Diazomethandarstellung[5] auch für die Darstellung des PDM aus *p*-Tosyl-N-benzyl-nitrosamid[21] Carbitol an Stelle von Methanol bzw. Natriummethylat verwendet, dann verschwindet dieser Peak aus dem Chromatogramm, während an einer weit entfernten Stelle ein neuer erscheint. Für die GC-Trennung erwies sich von einer Anzahl stationärer Phasen SE-30 als am besten geeignet. Polare Phasen bewirken, dass der Peak der zur Entfernung des PDM-Überschusses zugesetzten Säure in den Bereich der Benzylesterpeaks hineinwandert und deren Trennung stört, anstatt zusammen mit dem Lösungsmittelpeak am Anfang des Chromatogrammes zu erscheinen, wie es bei der unpolaren Phase SE-30 der Fall ist. Die Untersuchungen wurden mit den beiden Trennsäulen I und II unter verschiedenen Arbeitsbedingungen durchgeführt. Mit beiden Säulen konnten bei isothermem Arbeiten ausgezeichnete Trennungen der Benzylester der flüchtigen Fettsäuren $C_2$ bis $C_5$ erreicht werden (Fig. 1). Mit Hilfe eines geeigneten Temperaturprogrammes wurde auch eine scharfe Abtrennung des Ameisensäure-
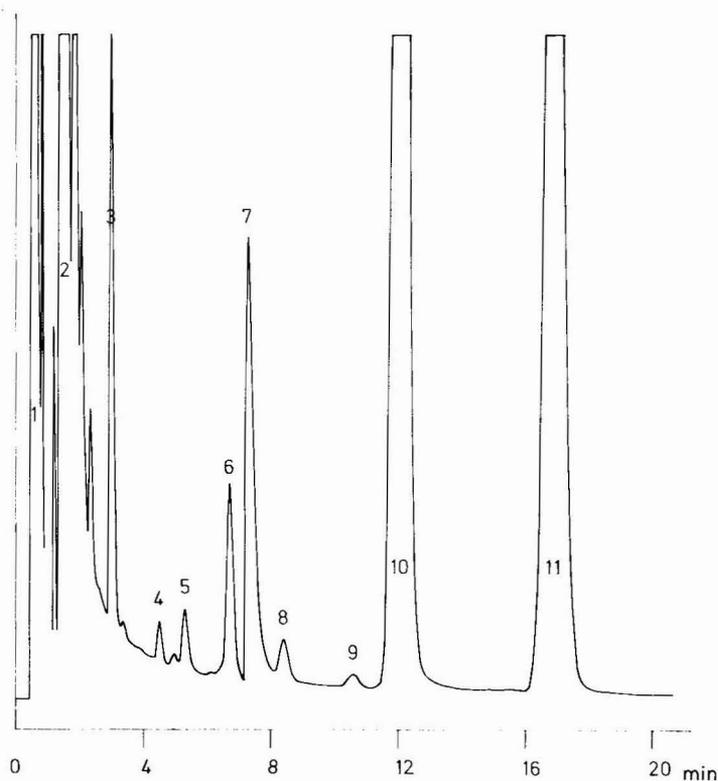


Fig. 4. Chromatogramm der Benzylester der Gärsäuren einer Silage (Sauerblatt) in Äther. Die Benzylierung erfolgte mit PDM, die Entfernung des PDM-Überschusses mit Hilfe von *n*-Hexansäure. 1 = Äther; 2 = *n*-Hexansäure; 3 = Essigsäurebenzylester; 4 = Propionsäurebenzylester; 5 = Isobuttersäurebenzylester; 6 = *n*-Buttersäurebenzylester; 7 = Milchsäurebenzylester; 8 = Isovaleriansäurebenzylester; 9 = *n*-Valeriansäurebenzylester; 10 = Nebenprodukt der PDM-Darstellung; 11 = *n*-Hexansäurebenzylester. Trennsäule II. Es wurde isotherm bei 130° gearbeitet. Trägergasstrom (He): 60 ml/min.

benzylesters ermöglicht (Peak 3 in Fig. 2). Bei Anwesenheit von Milchsäure erscheint deren Benzylesterpeak zwischen den Peaks des *n*-Buttersäure- und des Isovaleriansäurebenzylesters (Peak 8 in Fig. 3), wobei eine vollständige Trennung des Milchsäurebenzylesters vom *n*-Buttersäurebenzylester nur mit Säule II zu erzielen ist (Fig. 3). Die Anwendung des Verfahrens auf die Analyse der Gärsäuren einer Sauerblattsilage, welche mittels Ionenaustauscher isoliert und durch Perforation aus wässriger Lösung in Äther überführt worden waren[5], wird in Fig. 4 demonstriert. Es ergaben sich scharf ausgeprägte und gut getrennte Peaks. Ein Vergleichschromatogramm, welches von einer Probe nach Zusatz eines Gemisches der flüchtigen Fettsäuren erhalten wurde, diente der besseren Identifizierung der einzelnen Peaks (Fig. 5). Zur Abtrennung und Erkennung etwaiger vorhandener Ameisensäure wurde das Chromatogramm der Silage temperaturprogrammiert gefahren (Fig. 6). Der Vergleich mit einem entsprechenden Vergleichschromatogramm (Fig. 7) ergab, dass keine Ameisensäure in der untersuchten Silage vorhanden war.

Wie schon erwähnt, kann PDM in Form seines Rohproduktes, also ohne es weiteren Reinigungsoperationen zu unterwerfen, die zeitraubend und verlustreich sind, zum Benzylieren benutzt werden. Gemäss der Darstellungsmethode wird es in äthe-
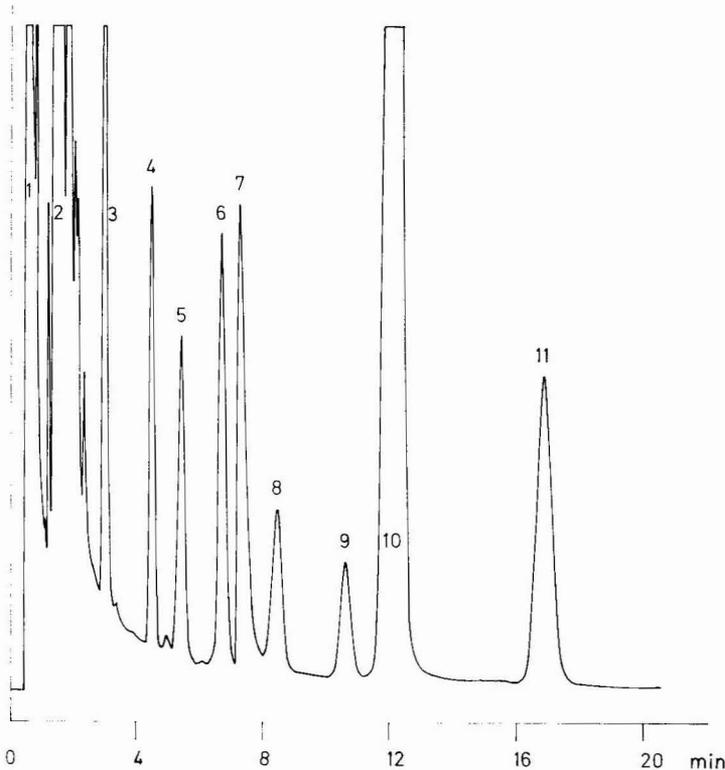


Fig. 5. Chromatogramm der Benzylester der Gärsäuren einer Silage (Sauerblatt). Zum Vergleich war der Silage ein Gemisch der Säuren $C_1$ bis $C_5$ zugesetzt worden. Benzyliert wurde mit PDM, dessen Überschuss durch Reaktion mit zugesetzter *n*-Hexansäure beseitigt wurde. Trennsäule, Bedingungen und Peakbezeichnungen wie in Fig. 4.
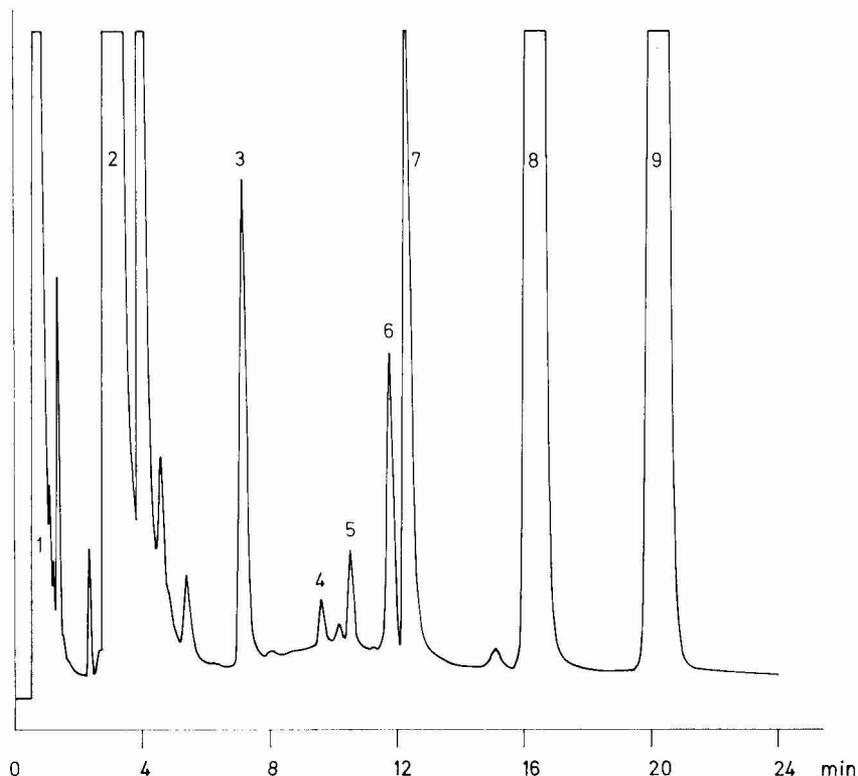
Fig. 6. Chromatogramm der Benzylester der Gärsäuren einer Silage (Sauerblatt) in ätherischer Lösung. Benzylierung mit PDM, Entfernung des PDM-Überschusses mit *n*-Hexansäure. 1 — Äther; 2 — *n*-Hexansäure; 3 — Essigsäurebenzylester; 4 — Propionsäurebenzylester; 5 — Isobuttersäure-benzylester; 6 — *n*-Buttersäurebenzylester; 7 — Milchsäurebenzylester; 8 — Nebenprodukt der PDM-Darstellung; 9 — *n*-Hexansäurebenzylester. Trennsäule II. Temperaturprogramm 5 min bei 114°, dann Temperaturerhöhung auf 144° mit einer Steigerungsrate von 7.5°/min. Trägergasstrom (He): 38 ml/min.

rischer Lösung gewonnen und so zur Reaktion gebracht. Durch Abdampfen des Äthers bei niederer Temperatur erhält man ein Konzentrat, welches aber nicht ganz so be-ständig zu sein scheint, wie das in Äther gelöste PDM, das bei —20° monatelang haltbar ist[21]. PDM reagiert mit Ameisensäure fast augenblicklich, bei den homologen Säuren nimmt die Reaktionsgeschwindigkeit aber mit zunehmendem Molekular-gewicht ab (Tabelle III). Die Verbindung verhält sich in dieser Hinsicht ähnlich wie Diphenyldiazomethan[24-26]. Für die quantitative Umsetzung ist ein ausreichender Überschuss des Reagenzes erforderlich. Bei Mangel an PDM werden, wie Versuche ergaben, nicht alle Säuren in gleichem Masse benzyliert, sondern Ameisensäure und Milchsäure werden bevorzugt; erst wenn diese beiden Säuren quantitativ verestert sind, steht das restliche PDM für den Umsatz mit den anderen Säuren zur Verfügung— ein Hinweis, dass die Reaktion von der Acidität der Säuren beeinflusst wird (vgl. Tabelle IV). Da der Verlauf der Reaktion in hohem Masse von der Reagenzmenge und, wie sich weiterhin zeigte, von der Zeitdauer ihrer Einwirkung abhängt (vgl. Tabelle III), muss die Benzylierung zur Erzielung reproduzierbarer Werte unter mög-
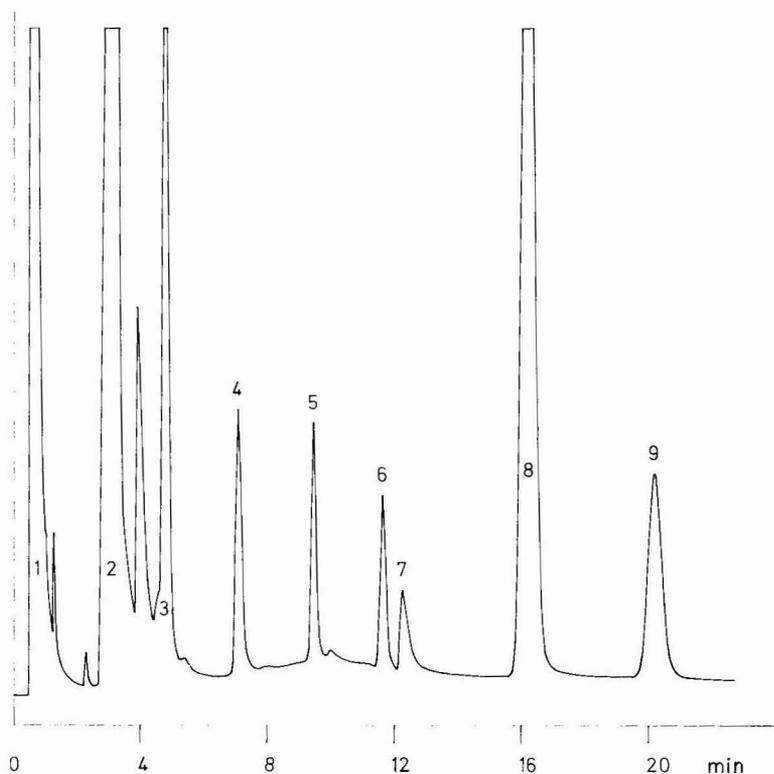
Fig. 7. Chromatogramm der Benzylester von Ameisen-, Essig-, Propion-, *n*-Butter- und Milchsäure in Äther. Benzyliert wurde mit PDM und dessen Überschuss mit *n*-Hexansäure beseitigt. 1 — Äther; 2 — *n*-Hexansäure; 3 — Ameisensäurebenzylester; 4 — Essigsäurebenzylester; 5 — Propionsäure-benzylester; 6 — *n*-Buttersäurebenzylester; 7 — Milchsäurebenzylester; 8 = Nebenprodukt der PDM-Darstellung; 9 — *n*-Hexansäurebenzylester. Trennsäule II. Temperaturprogramm und Träger-gasstrom wie in Fig. 6.

TABELLE III

PEAKHÖHEN (mm) DER BENZYLESTER BEI VERSCHIEDEN LANGER EINWIRKUNGS-ZEIT DES PDM

Peaks: Benzylester von: 1 — Ameisensäure; 2 — Essigsäure; 3 — Propionsäure; 4 — Isobuttersäure; 5 — *n*-Buttersäure; 6 — Milchsäure; 7 — Isovaleriansäure; 8 — *n*-Valeriansäure.

| Zeit (h) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| 1 | 174.3 | 51.4 | 26.7 | 22.3 | 41.1 | 46.0 | 18.0 | 10.6 |
| 2 | 175.6 | 57.1 | 29.6 | 26.9 | 42.6 | 43.8 | 19.1 | 12.4 |
| 3 | 181.9 | 66.6 | 37.1 | 29.8 | 46.6 | 45.0 | 21.8 | 14.0 |
| 4, | 182.9 | 73.8 | 40.4 | 33.8 | 49.0 | 44.1 | 22.8 | 16.0 |
| 24 | 182.2 | 115.1 | 67.0 | 54.2 | 69.0 | 45.6 | 36.8 | 26.2 |
| 48 | 181.1 | 126.1 | 78.2 | 62.8 | 74.3 | 44.3 | 39.8 | 29.4 |

TABELLE IV

p$K_a$-WERTE DER NIEDEREN FETTSÄUREN $C_1$-$C_5$ SOWIE DER MILCHSÄURE

| Säure | p$K_a$ |
|---|---|
| Ameisensäure | 3.77 |
| Essigsäure | 4.76 |
| Propionsäure | 4.88 |
| Isobuttersäure | 4.85 |
| Buttersäure | 4.82 |
| Isovaleriansäure | 4.77 |
| Valeriansäure | 4.81 |
| Milchsäure | 3.87 |

lichst gleichartigen Bedingungen vorgenommen werden. Art und Menge der nach der Veresterung zur Entfernung des PDM-Überschusses verwendeten Carbonsäure haben nur geringen Einfluss. Man wird eine möglichst flüchtige Säure wählen, um die Chromatographierdauer nicht unnötig zu verlängern. Zur Isolierung der Säuren aus Silageextrakten kann man sich der Ionenaustauscher bedienen[5]. Mit Amberlite IR-120 und IR-45 konnten nur Ameisen-, Essig-, Propion- und Milchsäure quantitativ erfasst werden. Die Überführung der Säuren aus der wässrigen in die ätherische Phase durch Perforation erfolgte dagegen durchwegs quantitativ. Im ganzen genommen erwies sich Phenyldiazomethan als ein vorzügliches Mittel, die flüchtigen Fettsäuren von $C_1$ bis $C_5$ mit Einschluss der Milchsäure in Benzylester zu überführen, welche sich unter geeigneten Bedingungen ausgezeichnet gaschromatographisch trennen und bestimmen lassen. Da bei diesem Verfahren alle für die Bewertung einer Silage massgeblichen Säuren in einer Analyse erfasst werden, dürfte es für die Bestimmung von Silagegärsäuren von Interesse sein.
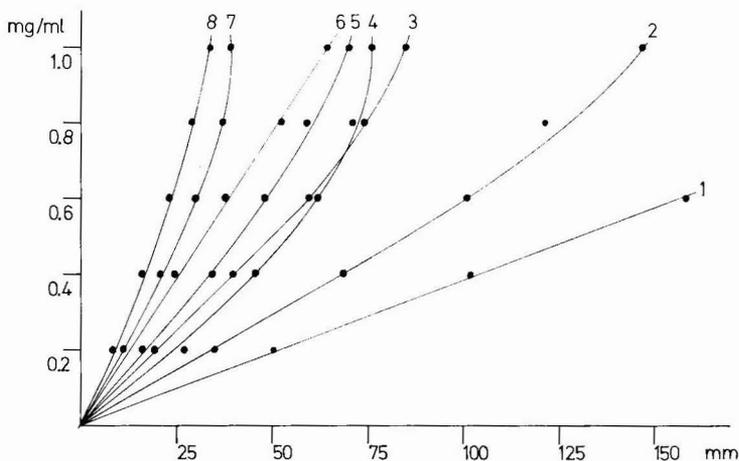


Fig. 8. Peakhöhen der Benzylester in Abhängigkeit von den Konzentrationen der Säuren in den Modellösungen. Trennsäule 1, isotherm bei 150°. 1 = Ameisensäurebenzylester; 2 = Essigsäurebenzylester; 3 = Propionsäurebenzylester; 4 = Isobuttersäurebenzylester; 5 = n-Buttersäurebenzylester; 6 = Milchsäurebenzylester; 7 = Isovaleriansäurebenzylester; 8 = n-Valeriansäurebenzylester.

Zur Auswertung wurde aus praktischen Gründen die Peakhöhenmethode gewählt[5], wobei die Werte für jede Säure im Gemisch im Bereiche 0.1–1.0 mg/ml lagen und für Ameisensäure sowie Milchsäure gute Linearität zeigten (vgl. Fig. 8). Die Werte der anderen Säuren wichen mit zunehmender Konzentration von der Linearität ab. Es ist daher zweckmässig, im linearen Bereich niederer Konzentrationen zu arbeiten. Zugleich sei auf die Möglichkeit der Auswertung von Analysendaten mittels nichtlinearer Eichkurven mit Hilfe moderner Geräte, wie z.B. das Modell SP 4000 der Fa. Spectra-Physics, hingewiesen.

ZUSAMMENFASSUNG

Es wird ein Verfahren zur gaschromatographischen Bestimmung der flüchtigen Fettsäuren von $C_1$ bis $C_5$ mit Einschluss der Milchsäure beschrieben. Mit Hilfe von Phenyldiazomethan werden die Säuren in ihre Benzylester überführt ohne vorherige Reinigung des Reagenzes durch Destillation. Die Benzylester werden sowohl vom Lösungsmittel als auch voneinander gut getrennt. Als stationäre Phase diente SE-30. Die Anwendung des Verfahrens auf Silagegärsäuren wird demonstriert.

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CHROM. 10,149

# MICRODETERMINATION OF NITRATE BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY TECHNIQUE WITH MULTIPLE ION DETECTOR

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## SUMMARY

A simple, sensitive method for the determination of nitrate in water by gas chromatography (GC) and mass spectrometry (MS) with a multiple ion detector (MID) has been developed. The principle of the method is the nitration of 1,3,5-trimethoxybenzene in sulfuric acid. In this medium, nitration is followed by the hydrolysis of the ether groups, yielding a simple nitrobenzene as the final product. This is then analyzed by GC–MS and detected with MID. Hexamethylbenzene serves as an internal standard. The interference of nitrite and chloride is prevented by using sulfamic acid and mercuric sulfate, respectively.

## INTRODUCTION

Nitrogen plays an important biological role in living organisms. Nitrate ion is one of the principal nutrients for aquatic life. In recent years, increasing concern has been focused on the formation of carcinogenic nitrosoamine from various nitrogen oxides[1,2]. The analysis of nitrate in environmental samples is, therefore, of vital importance particularly as it relates to ecology and public health.

The determination of low nitrate levels in unpolluted environmental samples has been hampered by the lack of a simple, sensitive analytical method. As part of the global baseline studies carried out in our laboratory, a spectrophotometric method has been previously reported[3]. Following the spectrophotometric method, a simpler and more sensitive method using a gas chromatographic–mass spectrometric (GC–MS) system and a multiple ion detector (MID) has been developed and is described here. The method employs the nitration of 1,3,5-trimethoxybenzene (TMB) followed by GC–MS analysis of nitrobenzene.

## EXPERIMENTAL

### Reagents

Spectroanalyzed benzene from Fisher Scientific (Pittsburgh, Pa., U.S.A.) was used as the organic solvent. TMB and HMB (99 % pure) were purchased from Aldrich

(Milwaukee, Wisc., U.S.A.) and K & K (Plainview, N.Y., U.S.A.), respectively. Eastman-Kodak electronic grade nitrobenzene was used. All other chemicals used are of reagent grade.

The nitrate solution was prepared as follows. 0.137 g of sodium nitrate was dissolved in doubly deionized water and diluted to 1 l as the stock solution. Fresh working solutions were prepared by diluting the stock solution in proper proportions.

HMB internal standard was prepared by dissolving 0.01 g of HMB in 100 ml of benzene, and diluting 0.3 ml of this solution to 100 ml with benzene to give an internal standard of 300 $\mu$g/l.

TMB reagent was made by dissolving 0.1 g of TMB in 100 ml of benzene with 300 $\mu$g/l of HMB internal standard.

Mercuric sulfate (5%) was prepared by dissolving 5 g of mercuric sulfate in 100 ml of 10% (v/v) sulfuric acid, and 5% sulfamic acid by dissolving 5 g of sulfamic acid in 100 ml of doubly deionized water. The latter solution must be made fresh every 3 months.

*Sample preparation*

The aqueous nitrate sample (10 ml) is pipetted into a 50-ml stoppered Erlenmeyer flask. Sulfamic acid reagent (0.01 ml), 0.01 ml of mercuric sulfate reagent are added, and then 20 ml of sulfuric acid are slowly added from a buret. Mix, place the flask in a cold water bath and cool to room temperature. Add, by pipet, 2 ml of TMB reagent with internal standard. Shake the mixture for 5 min. Wait for the phases to separate. Inject the upper benzene layer into the GC–MS system for analysis. A series of analyses should include a doubly deionized water blank with the same reagents from the same bottles so that any slight impurities in the reagents are cancelled out as the reagent blank.

*Instrumentation*

A Hewlett-Packard 5710A GC and 5980A MS system interfaced with a membrane and a 5974A MID were employed for the analysis. The GC parameters and MS and MID settings were listed in Table I. The chemical ionization mode in MS was

TABLE I

INSTRUMENT CONDITIONS

| Parameter | Description |
|---|---|
| GC column | Packed, 3 ft. × 2 mm I.D. glass, 2% SP-2300 or Chromosorb W HP (100–120 mesh) |
| Oven temperature | 110° to 180° at 32°/min |
| Injection port temperature | 250° |
| Auxiliary temperature | 250° |
| Carrier gas* | Methane, 12 ml/min |
| Sample size | 1 $\mu$l |
| MS gain | 11 |
| Masses on MID | 124.1 and 163.3 |
| MID gain | For mass 163.3, 100; for mass 124.1, 100 for 50 $\mu$g/l NO$_3^-$ and below, 10 for 75 $\mu$g/l NO$_3^-$ and above |

* In obtaining the mass spectra in the electron impact mode, helium at a flow-rate of 40 ml/min was used as carrier gas instead.

used. The mass settings on MID of 124.1 and 163.3 were for detecting $M+1$ ions of nitrobenzene and HMB, respectively. A 5-$\mu$l Hamilton syringe with Chaney adaptor was used for sample injections.

RESULTS AND DISCUSSION

The nitration of aromatic ring is an electrophilic substitution reaction. The electrophilic agent is the nitronium ion, $NO_2^+$, generated from the nitrate ion and sulfuric acid. The sulfuric acid, being an extremely strong acid, protonates the nitrate ion to protonated nitric acid, $H_2NO_3^+$, which loses water to form $NO_2^+$. The $NO_2^+$ ion attacks the aromatic ring to form a Wheland intermediate which in turn releases a proton to yield the final product. The full mechanism of the reaction can be written as

$$2\ H_2SO_4 + NO_3^- \rightleftharpoons 2\ HSO_4^- + H_2ONO_2^+$$

$$H_2ONO_2^+ \rightleftharpoons H_2O + NO_2^+$$
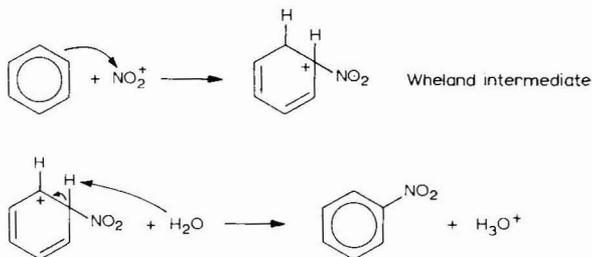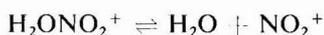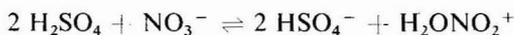




The rate determining step of the electrophilic substitution reaction is the formation of the Wheland intermediate. Benzene itself is a molecule of average reactivity as aromatic rings go. Ross et al.[4] have nitrated benzene in a sulfuric acid–water (10:1) medium at room temperature and Glover and Hoffsommer[5] in a sulfuric acid–water (3:1) medium at $75° \pm 5°$. In the present study, with the intention of carrying out an easier reaction, TMB was chosen to be nitrated. The three methoxy groups on the benzene ring, being electron donating, activate the molecule toward electrophilic substitution. This could be explained by the Wheland intermediate which contains an excess of a positive charge. The electron donating groups stabilize the positive charge, lower the energy of the intermediate and lead to a faster reaction. Consequently, nitration was carried out quantitatively in a less vigorous condition, viz. a sulfuric acid–water (2:1) medium at room temperature. The final product was found to be nitrobenzene which is discussed below. There are two possibilities for this result. One is the nitration of benzene, the other is the nitration of TMB forming 1-nitro-2,4,6-trimethoxybenzene (NTMB) followed by hydrolysis of NTMB. Based on the foresaid reasoning, the latter is most plausible.

The gas chromatogram of the reaction mixture in benzene is shown in Fig. 1. The first peak is confirmed to be nitrobenzene by retention time and mass spectra in both the electron impact (Fig. 2) and the chemical ionization (Fig. 3) modes. The mass spectrum in Fig. 2 matches well with the published spectrum of nitrobenzene[6]. Tesch
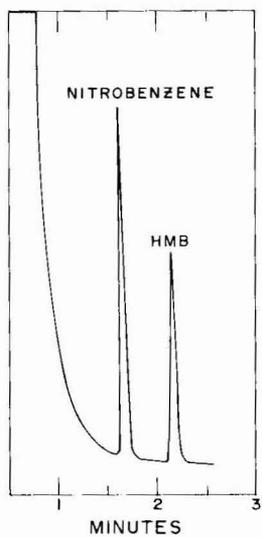
Fig. 1. Gas chromatogram of nitration mixture.
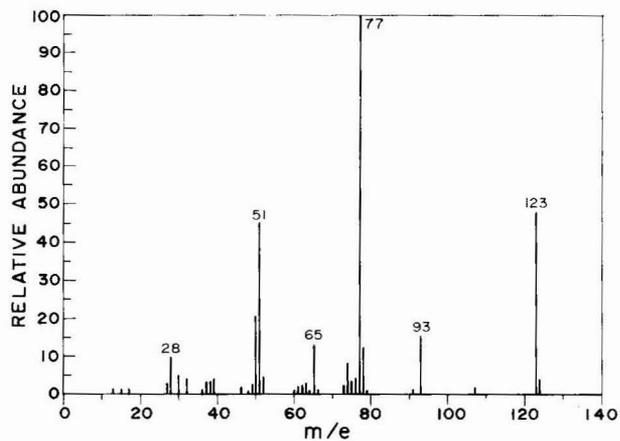


Fig. 2. Mass spectrum of nitrobenzene in electron impact mode.
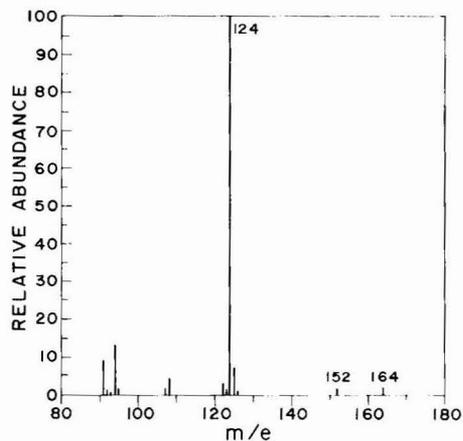


Fig. 3. Mass spectrum of nitrobenzene in chemical ionization mode.

*et al.*[7] found NTMB to be the product of nitration of TMB under their experimental conditions. However, the lack of the characteristic peaks of $(M–CH_3)^+$, $(M–CH_3–CO)^+$, $(M–CH_2O)^+$, $(M–OCH_3)^+$ and $(M–CHO)^+$ of methyl phenyl ether indicates that the NTMB is further hydrolyzed under the experimental conditions used here. Furthermore, the $M+1$, $M+29$ and $M+41$ peaks of masses 124, 152 and 164 in the chemical ionization mode in Fig. 3 provide the positive proof of molecular weight of 123 (nitrobenzene).

The hydrolysis of the three methoxy groups on TMB is a favorable phenomenon since it leads to the formation of nitrobenzene as the final product. Compared to NTMB, nitrobenzene is smaller and simpler. It elutes at a lower temperature from the GC instrument so that any taxing effect on the membrane separator at higher temperature can be avoided. It can also be detected at a lower mass by MS which in turn yields higher sensitivity.

The signals of the $M+1$ ions for nitrobenzene and HMB are shown in Fig. 4. The MID readings at mass 124.1 of nitrobenzene are calibrated against that at 163.3 of HMB, the internal standard. Linear response on the MID, corresponding to the nitrate concentration, is observed as shown in Fig. 5. Projecting from these signals, a nitrate concentration as low as a few $\mu$g/l could be observed. This is much more sensitive than the existing methods[3–5,7]. Unfortunately, the scattering along the straight line is $\pm$ 15 $\mu$g/l which interferes with the readings at low levels.
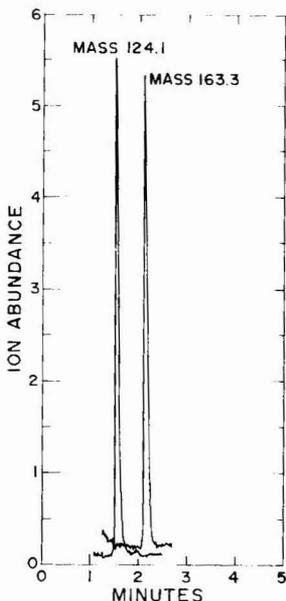


Fig. 4. MID response of nitrobenzene and HMB with 20 $\mu$g/l $NO_3^-$, 300 $\mu$g/l HMB.

The precision of MID was tested with HMB and pure nitrobenzene in benzene. Linear response was observed down to MID readings of 0, 0.1, 0.2, 0.4 and 0.6 at a gain of 100. This excludes the possibility of instrumental irregularity. Contamination while carrying out the sample preparation is then the suspect, particularly since nitric
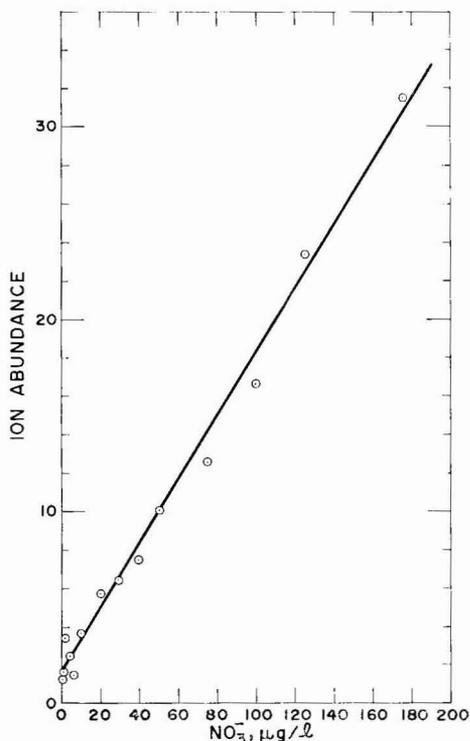
Fig. 5. Nitrate standard curve.

acid is used extensively in our laboratory for ashing and apparatus washing. Nitrogen oxides are evolved into the atmosphere during these processes. These oxides will very likely be picked up when sulfuric acid is being mixed with the aqueous nitrate sample and thus causes the contamination. The method performed under a cleaner environment should yield better accuracy, and analysis of a few $\mu g/l$ of nitrate could be possible.

## ACKNOWLEDGEMENT

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CHROM. 10,180

# GAS CHROMATOGRAPHIC DETERMINATION OF METHYLGUANIDINE, GUANIDINE AND AGMATINE AS THEIR HEXAFLUOROACETYL-ACETONATES

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## SUMMARY

Seven derivatives each of methylguanidine, guanidine and agmatine have been prepared, and the specificity and volatility of their gas chromatographic detection have been studied. The hexafluoroacetylacetonates have been found to be the most specific for the three guanidines, and are highly sensitive to alkali flame ionization and electron-capture detections. These derivatives are also fairly resistant to hydrolysis occurring in the derivatization process.

## INTRODUCTION

Methylguanidine (MG), which has long been considered to occur widely in fresh beef and several fish in fairly high concentrations ranging from 60 to 1900 mg/kg (refs. 1–4), is known to be easily converted by nitrosation under acidic conditions into highly mutagenic and carcinogenic methylnitrosocyanamide and methylnitrosourea[5–7]. MG ingested in the diet may react with nitrite in the human stomach to form these potent carcinogens. Endo *et al.*[7] speculated that these substances may be possible etiologic factors in human gastric cancer. Concerning the occurrence of MG in foods, however, most of the studies were conducted in the 1930s[1–4], and the methods used for MG separation and analysis were overly complex, might have lacked specificity and might have generated MG. The lack of suitable analytical methods has hindered attempts to identify individual guanidine derivatives in various foods.

Recently, several methods have been reported for the analysis of various guanidino compounds in biological fluids, including ion-exchange[8–13], paper [14–17] and thin-layer chromatography[18], coupled with colorimetric determinations by either the Sakaguchi or Voges–Proskauer reactions, and gas–liquid chromatography

(GLC)[19-25] after the formation of volatile derivatives. The colorimetric determination, however, lacks the specificity needed to identify each guanidino compound, and also requires considerable sample manipulation and time. Some volatile derivatives subjected to GLC analysis also lack specificity or stability, and the derivative preparation seems rather complex.

In the present study, we have prepared several volatile derivatives of guanidines, *i.e.*, MG, guanidine (G) and agmatine (AG), and have compared their specificities of derivatization and volatilities as determined by GLC with alkali flame ionization detection (AFID). It was found that the hexafluoroacetylacetone (HFAA) derivatives were the most appropriate ones among the seven different types of derivatives tested so far. Methods for the preparation and identification of the HFAA derivatives of MG, G, and AG, and the conditions for the determination of these three derivatives by GLC, are described.

EXPERIMENTAL

*Reagents*

All of the reagents employed were commercial products of analytical grade and were used without further purification. Methylguanidine hydrochloride and agmatine sulphate were obtained from Sigma (St. Louis, Mo., U.S.A.), and guanidine hydrochloride was from Wako (Tokyo, Japan). Acetylacetone (AA), trifluoroacetylacetone (TFAA), hexafluoroacetylacetone (HFAA), acetic anhydride, trifluoroacetic anhydride and carbon disulphide were obtained from Wako, and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA, specially purified grade) was from Pierce (Rockford, Ill., U.S.A.).

*Derivative preparation*

Acetyl derivatives of the three guanidines were prepared by use of acetic anhydride according to the method of Link *et al.*[26], and trifluoroacetyl derivatives using trifluoroacetic anhydride according to the method of Stalling and Gehrke[20]. AA derivatives were prepared according to Beyermann and Wisser[21], BSTFA (silyl) products according to Gehrke *et al.*[27] and isothiocyanates (isoCNS) by use of carbon disulphide according to Brandenberger and Hellbach[28]. TFAA derivatives were prepared as described for the HFAA derivatization using trifluoroacetylacetone instead of HFAA.

*Preparation of HFAA derivarives*

MG, G and AG were each prepared as solutions (1 mg/ml) in 50% ethanol. A 100-$\mu$l aliquot of a test solution was placed in a hard glass ampoule, and this was evaporated by blowing in nitrogen. Then, 50 $\mu$l each of pyridine and HFAA were added and the ampoule was heat-sealed, followed by heating at 120° for 1 h. After cooling the ampoule to room temperature, 1 ml of diethyl ether and 3 ml of 3 $N$ HCl were added to the reaction mixture and this was shaken vigorously and centrifuged. A 5-$\mu$l aliquot of the ether layer was then injected into the GLC column.

*Operating conditions for gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS)*

A Shimadzu GC-5APF gas chromatograph, equipped with an AFID (KBr monocrystal-on-detector type[29]; GLC–AFID), and a Shimadzu GC-3BE, equipped

with a $^{63}$Ni electron capture detector (GLC–ECD), were used. In order to compare the peak areas of various derivatives determined by GLC–AFID, a glass column (1 m × 3 mm I.D.) packed with 3% SE-30 on Chromosorb W (Applied Science Labs., State College, Pa., U.S.A.) was employed, and the column temperatures were varied from 70 to 200° depending on the derivatives examined. The mass spectra of the derivatives were determined by use of a Shimadzu-LKB 9000 gas chromatograph–mass spectrometer (GC–MS), equipped with a glass column (2 m × 3 mm I.D.) packed with 5% SE-30 on Chromosorb W AW DMCS.

The HFAA derivatives of the three guanidines were analyzed under the conditions shown in Table I.

TABLE I

OPERATING CONDITIONS FOR GLC AND GC–MS OF THE HFAA DERIVATIVES OF METHYLGUANIDINE, GUANIDINE AND AGMATINE

| Operating conditions | MG–HFAA and G–HFAA | AG–HFAA |
|---|---|---|
| *GLC–AFID* | | |
| Apparatus | Shimadzu GC-5APF | |
| Detector | AFID (KBr monocrystal) | |
| Carrier gas | N$_2$ | |
| Flow-rate (ml/min) | | |
| N$_2$ | 40 | |
| H$_2$ | 20 | |
| air | 700 | |
| Temperature (°C) | | |
| injection port | 210 | |
| detector | 210 | |
| column | 120 | 170 |
| Column | 20% Versamid 900 on Chromosorb W (60–80 mesh), 1 m × 3 mm I.D., glass | 3% SE-30 on Chromosorb W (60–80 mesh), 1 m × 3 mm I.D., glass |
| *GLC–ECD* | | |
| Apparatus | Shimadzu GC-3BE | |
| Detector | $^{63}$Ni ECD | |
| Carrier gas | N$_2$ | |
| Flow-rate (ml/min) | 40 | 50 |
| Temperature (°C) | | |
| injection port | 150 | 220 |
| detector | 100 | 160 |
| column | 100 | 160 |
| Column | same as GLC–AFID | same as GLC–AFID |
| *GC–MS* | | |
| Apparatus | Shimadzu-LKB, GC–MS 9000 | |
| Detector | total ion collector | |
| Carrier gas | He | |
| flow-rate (ml/min) | 30 | |
| inlet pressure (kg/cm$^2$) | 3 | |
| Temperature (°C) | 100 | 220 |
| separator | 290 | |
| ion source | 290 | |
| Electron current (eV) | 20 | 70 |
| Trap current (μA) | 60 | |
| Accelerating voltage (kV) | 8 | |
| Column | 5% SE-30 on Chromosorb W AW DMCS (60–80 mesh), 2 m × 3 mm I.D., glass | |

RESULTS AND DISCUSSION

*Selection of the most desirable derivatives of the three guanidines for GLC analysis*

Retention times, peak heights and peak shapes of seven derivatives of MG, G and AG were compared. The retention times obtained are shown in Table II. In addition, the peaks of all of the derivatives were confirmed by GC–MS. The HFAA derivatives were found to be the most suitable for the quantitative determination of the three guanidines by GLC since: (1) the derivatization with acetylacetone is highly specific for guanidines, according to the results of GC–MS analysis; (2) all of the three guanidines so far tested give derivatives with HFAA in high yield; (3) the HFAA derivatives are the most volatile of the three different acetylacetonates and (4) the HFAA derivatives exhibited the highest stability.

TABLE II

RETENTION TIMES OF VARIOUS VOLATILE DERIVATIVES OF METHYLGUANIDINE, GUANIDINE AND AGMATINE

Glass column (1 m × 3 mm I.D.) containing 3% SE-30 on Chromosorb W (60–80 mesh); carrier gas (nitrogen) flow-rate, 40 ml/min; detector, AFID.

| Derivative | Guanidine | Column temperature (°C) | Retention time (min) |
|---|---|---|---|
| HFAA | MG | 70 | 3.50 |
| | G | 70 | 3.50 |
| | AG | 170 | 4.15 |
| TFAA | MG | 90 | 2.70 |
| | G | 90 | 3.50 |
| | AG | 190 | 5.75 |
| AA | MG | 110 | 2.70 |
| | G | 110 | 2.95 |
| | AG | 70–300 | — * |
| Trifluoroacetyl | MG | 70 | 5.30 |
| | G | 70 | 5.40 |
| | AG | 190 | 1.90 |
| Acetyl | MG | 200 | 3.15 |
| | G | 200 | 4.30 |
| | AG | 70–300 | |
| BSTFA (silyl) | MG | 140 | 3.70 |
| | G | 170 | 4.90 |
| | AG | 190 | 6.90 |
| isoCNS | MG | 70–300 | |
| | G | 70–300 | — |
| | AG | 70–300 | — |

* No peak corresponding to the derivative was observed.

*Derivatization conditions for MG, G and AG with HFAA*

We initially prepared the HFAA derivatives of MG, G and AG according to the method of Erdtmansky and Goehl[24], which has been applied to the analysis of guanidino-type antihypertensive agents in the blood. However, it was found that the derivatization rates of the three guanidines were as low as 20%, and the rates were still *ca.* 50% even when the reaction mixtures were heated at 180° for 2 h. Erdtmansky and Goehl[24] used a reflux condenser during heating of the reaction mixture; when the

mixture was heated in a sealed vial instead of a condenser, it was found that the derivatization rates increased but were still insufficient. The reason for these low rates of derivatization was found to be hydrolysis of the products due to the presence of water in the reaction mixture. On the other hand, Beyermann and Wisser[21] added 100 mg of $Na_2CO_3$ and 1 ml of acetylacetone to the dry specimen in order to prepare the AA derivatives of MG and G, followed by heating under reflux at 140° for 30 min. We applied this method to the derivatization of the three guanidines with HFAA, and found that the HFAA derivatives were decomposed when more than 20 mg of $Na_2CO_3$ was added, and the yield of derivatives was markedly lowered. Mitchell[30] reported a method for the GLC analysis of Schiff bases of amino-acid methyl esters in which 1 % pyridine in methanol was added as a catalyst. In the present study, the effect of addition of pyridine, $NaHCO_3$ and $Na_2CO_3$ on the HFAA derivatization was examined. As shown in Table III, pyridine gave the highest derivatization rates. Next, the effect of the volume of pyridine on the HFAA derivatization was examined, and the results shown in Fig. 1 indicate that the highest yield of HFAA derivatives could be obtained when 50 $\mu$l of pyridine was added to the reaction mixture.

For the extraction of HFAA derivatives from the reaction mixture, Erdtmansky and Goehl[24] employed benzene as the extractant. However, it was found that

TABLE III

EFFECT OF PYRIDINE, SODIUM BICARBONATE AND SODIUM CARBONATE ON THE DERIVATIZATION OF METHYLGUANIDINE, GUANIDINE AND AGMATINE WITH HFAA

| Compound added | Relative derivatization rate* | | |
|---|---|---|---|
| | MG | G | AG |
| Control (no addition) | 1.00 | 1.00 | 1.00 |
| Pyridine | 34.5 | 87.0 | 19.3 |
| $NaHCO_3$ | 16.6 | 87.0 | 19.3 |
| $Na_2CO_3$ | 19.6 | 40.8 | 13.7 |

* The derivatization rates of the controls were expressed as 1.00.
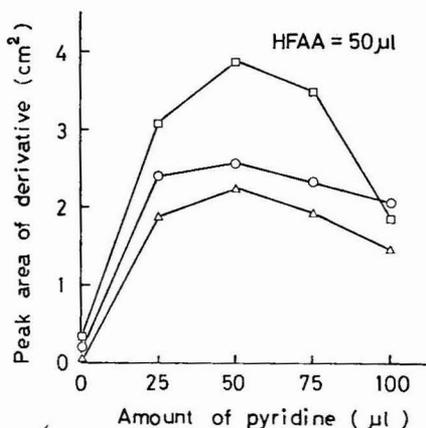


Fig. 1. Effect of the amount of pyridine on the derivatization of methylguanidine (○), guanidine (△) and agmatine (□) with HFAA. Heating condition, 120° for 60 min.

incomplete combustion of the benzene in the GLC detector chamber resulted in the production of a rather large amount of soot which apparently lowered the sensitivity of the AFID. The extraction rates of the HFAA derivatives with different solvents were thus examined. As can be seen from Table IV, diethyl ether gave the highest extraction rates among the 10 organic solvents tested.

TABLE IV

EXTRACTION RATES OF THE HFAA DERIVATIVES OF METHYLGUANIDINE, GUANI-DINE AND AGMATINE IN 3 *N* HCl WITH DIFFERENT SOLVENTS

| Solvent | Relative extraction rate* | | |
|---|---|---|---|
| | MG–HFAA | G–HFAA | AG–HFAA |
| Benzene | 1.00 | 1.00 | 1.00 |
| Toluene | 0.89 | 0.93 | —** |
| Cyclohexane | 1.00 | 0.87 | — |
| *n*-Hexane | 0.97 | 0.89 | — |
| *n*-Heptane | 0.96 | 0.72 | — |
| *n*-Pentane | 1.23 | 1.09 | — |
| Ethyl acetate | 1.33 | 1.90 | 1.05 |
| *n*-Butyl acetate | 0.91 | 1.11 | 1.07 |
| Diethyl ether | 1.69 | 1.92 | 2.04 |
| Methyl isobutyl ketone | 1.03 | 1.14 | — |

* The extraction rates with benzene were expressed as 1.00.
** Not examined.

However, not only the HFAA derivatives but also pyridine may be extracted from the reaction mixture by use of diethyl ether, and pyridine is also detected by the AFID. This must give rise to some error in the GLC–AFID determination of the HFAA derivatives. However, the acidity of the reaction mixture does not influence the extraction rates of the derivatives with diethyl ether. Thus, when 3 ml of 3 *N* HCl and 1 ml of diethyl ether were added to the reaction mixture, pyridine was completely retained in the aqueous layer.

We then examined the effects of heating temperature and time on the derivatization of the three guanidines with HFAA. As shown in Figs. 2 and 3, the highest derivatization rates were observed when the reaction mixtures were heated at 120° for 60 min.

Due to the difficulty in obtaining authentic HFAA derivatives of the three guanidines, the derivatization rates have thus far been expressed in terms of either the peak areas of the gas chromatograms (in Figs. 1, 2 and 3) or as relative derivatization rates (in Tables III and IV). Thus, the derivatization rates of the three guanidines were determined indirectly by estimating the remaining guanidines in the reaction mixtures, *viz.*, each 100 $\mu$g of MG, G or AG was derivatized with HFAA, and the remaining guanidine in the aqueous (lower) layer was determined colorimetrically using the modified Voges–Proskauer reaction according to Micklus and Stein[31]. Based on the remaining guanidines, the derivatization rates of MG, G and AG were found to be 100, 100 and 97.4 %, respectively.

The mass spectra of the HFAA derivatives of the three guanidines are shown in Fig. 4; the probable structures of the compounds are also illustrated.
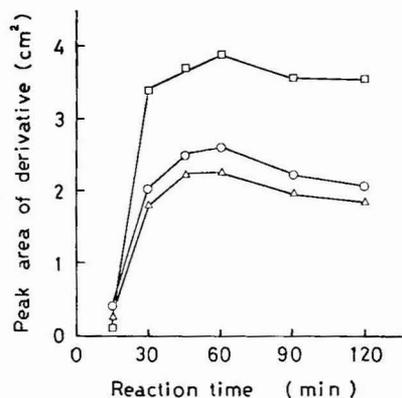
Fig. 2. Effect of the reaction time on the derivatization of methylguanidine (○), guanidine (△) and agmatine (□) with HFAA. Heating temperature, 120°.
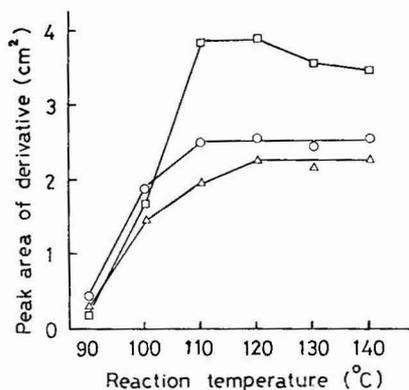
Fig. 3. Effect of the reaction temperature on the derivatization of methylguanidine (○), guanidine (△) and agmatine (□) with HFAA. Heating time, 60 min.
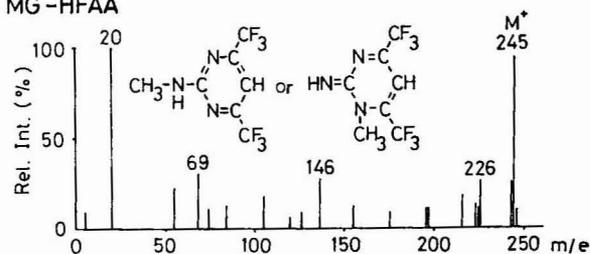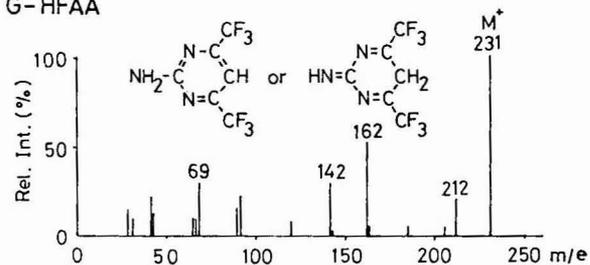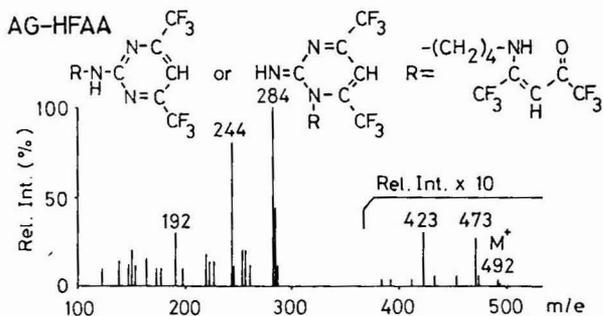


Fig. 4. Mass spectra of the HFAA derivatives of methylguanidine, guanidine and agmatine, and their probable structures.

*GC determination of the HFAA derivatives*

For the detection of nitrogenous substances, it is known that the AFID is much more sensitive than the hydrogen flame ionization detector (FID). It is also known that halogenated compounds such as HFAA derivatives are quite sensitive to the ECD as compared with the AFID. In the present study, however, most of the experiments were conducted with GLC–AFID because the derivatives of the three guanidines tested included compounds other than halogenated ones, and, moreover, the sensitivity of GLC–AFID to the test compounds was found to be almost the same as that of GC–MS.

The conditions for GC determination of the HFAA derivatives of guanidines were examined. The retention times obtained with different column packings are shown in Table V. It was found that all of the HFAA derivatives of the three guanidines could not be analyzed under the same column conditions because the molecular weight of the HFAA derivative of AG (AG–HFAA) is much larger than those of MG–HFAA and G–HFAA, and, in addition, the volatility of AG–HFAA is fairly low compared to the other two derivatives. Consequently, we selected a column containing 20% Versamid 900 for the analysis of MG–HFAA and G–HFAA, and for AG–HFAA we used a column of 3% SE-30. The gas chromatograms determined by AFID of the three guanidine HFAA derivatives are shown in Fig. 5.

TABLE V

RETENTION TIMES OF THE HFAA DERIVATIVES OF METHYLGUANIDINE, GUANIDINE AND AGMATINE OBTAINED WITH THE DIFFERENT TYPES OF COLUMN PACKINGS

Glass columns (1 m × 3 mm I.D.) were used.

| Column | | Retention time (min) | | |
|---|---|---|---|---|
| Packing | Temperature (°C) | MG–HFAA | G–HFAA | AG–HFAA |
| 3% SE-30 | 70 (170)* | 3.50 | 3.50 | 4.15 |
| 3% OV-17 | 70 (170)* | 4.35 | 4.45** | 3.70 |
| 20% Versamid 900 | 120 | 2.65 | 4.55 | – |
| 25% PEG-6000 | 120 | 8.15 | 2.05 | – |
| 5% Diethyleneglycol adipate | 120 | 2.50 | 0.95 | – |

* The column temperature for AG–HFAA analysis.
** A tailing peak was observed.

Fig. 6 shows the calibration curves for the HFAA derivatives of the three guanidines; these indicate linear relations between the peak areas and amounts of the respective derivatives over ranges of 5–40 ng for MG and G and 15–50 ng for AG. The minimum detection limits when using GLC–AFID were 5 ng for MG and G and 15 ng for AG, and when using GLC–ECD were 50 pg for MG and G and 150 pg for AG.

The proposed HFAA derivatization method may be applicable to the estimation of these guanidino compounds at levels of parts per billion ($\mu$g/kg) in foods. The conditions for the analysis of these compounds in foods are currently being investigated in further detail.
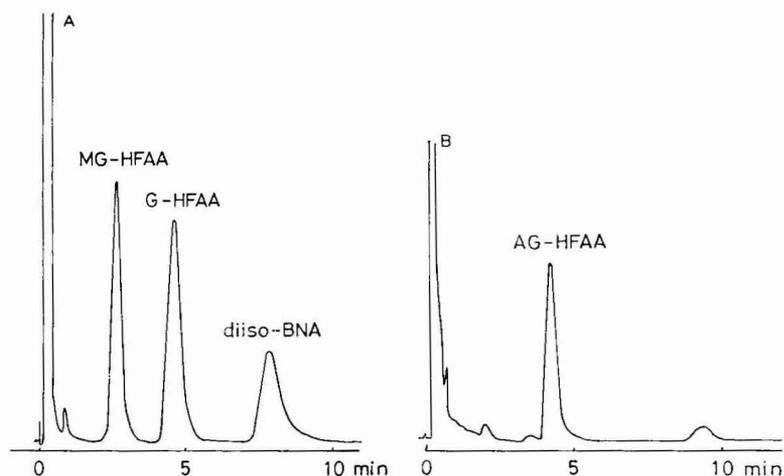
Fig. 5. Gas chromatograms of the HFAA derivatives of methylguanidine and guanidine (A) and agmatine (B). (A) Column packing, 20% Versamid 900; column temperature, 120°. (B) Column packing, 3% SE-30; column temperature, 170°. Diisobutylnitrosamine (diiso-BNA) was used as the internal standard.
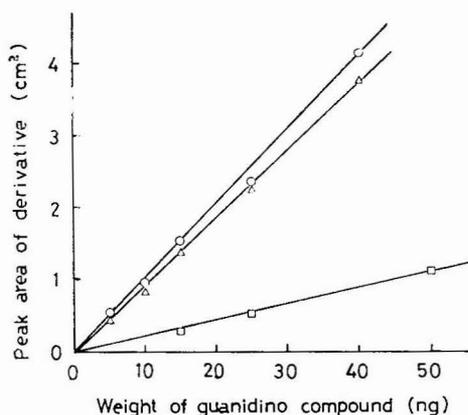


Fig. 6. Calibration curves for the HFAA derivatives of MG (○), G (△) and AG (□).

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# GAS CHROMATOGRAPHIC DETERMINATION OF APOMORPHINE IN PLASMA

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## SUMMARY

A method is described for the analysis of 1 to 10 $\mu$g/ml concentrations of apomorphine in plasma. The procedure is based on ethyl acetate extraction, a back extraction cleanup-step, derivatization with heptafluorobutyric anhydride, and gas chromatography on a 3% OV-17 column using flame ionization detection. N-*n*-Propylnorapomorphine is employed as an internal standard and quantitative relative recoveries of drug are realized with relative standard deviations of 4.6%. The method permits analysis of apomorphine in the presence of its two monomethyl ether metabolites, apocodeine and isoapocodeine. The latter compounds are also chromatographically resolved as their heptafluorobutyrate derivatives.

## INTRODUCTION

The discovery that apomorphine (I) and some of its prodrugs possess antiparkinsonism activity has led to a renewed interest in this group of compounds[1–6]. As part of a systematic examination of the metabolism of these alkaloids, it was necessary to develop a method for the analysis of apomorphine in plasma in the $\mu$g/ml range. A procedure that would permit assay of I in the presence of its potential metabolites, apocodeine (II), isoapocodeine (III) and apomorphine dimethyl ether (IV)[7–11], was also desired.

Colorimetric assay of I, based on mercuric chloride oxidation to its *o*-quinone, offers adequate sensitivity[12] but lacks the necessary selectivity as II and III can also be converted to the *o*-quinone[13]. Other colorimetric[14,15] and fluorometric[16,17] methods lack either the desired sensitivity and/or selectivity for the analysis of I in plasma.

A gas chromatographic (GC) method using 5% SE-30 and non-derivatized I[18] suffered from decomposition on the column at temperatures needed for elution[19]. GC of I as its O,O-bis(trimethylsilyl) ether derivative permitted satisfactory development but lacked needed selectivity[19]. It was proposed that derivatization of I with

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I,    R = R' = H
      APOMORPHINE

II,   R = CH₃, R' = H
      APOCODEINE

III,  R = H, R' = CH₃
      ISOAPOCODEINE
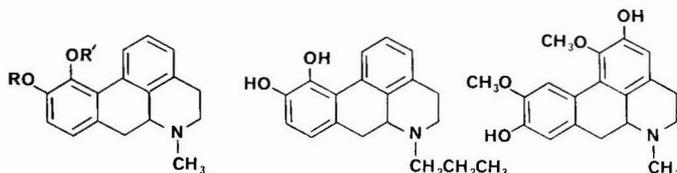
IV,   R = R' = CH₃
      APOMORPHINE DIMETHYL ETHER

V, N-n-PROPYLNOR-
   APOMORPHINE

VI, BOLDINE

heptafluorobutyric (HFB) anhydride might improve selectivity and provide the potential ability to use electron capture detection with its attendant high sensitivity.

## MATERIALS AND METHODS

### Gas chromatography

A Hewlett-Packard model 5710 A gas chromatograph equipped with dual flame ionization detectors (FID) was employed throughout. The oven and detector were maintained at 190° and 300°, respectively. Gas flow-rates were: carrier gas (nitrogen), 50 ml/min; hydrogen, 60/ml min; and compressed air, 240 ml/min. Silylated glass columns, 76 cm × 6 mm O.D., 4 mm I.D., were packed with 3% OV-17 on Chromosorb W-HP, 100–120 mesh (Analabs, North Haven, Conn., U.S.A.) and conditioned at 275° for 18 h with normal carrier gas flow. They exhibited an average continuous life of approximately 90 days.

### Materials

Apomorphine hydrochloride hemihydrate (Merck, Rahway, N.J.) U.S.A. and boldine (Nutritional Biochemicals, Cleveland, Ohio, U.S.A.) were used as purchased. N-n-Propylnorapomorphine was a gift of Sterling Winthrop Research Institute (Rensselaer, N.Y., U.S.A.). Apocodeine, isoapocodeine and 10,11-dimethoxyaporphine were prepared in our laboratory as previously described[10]. HFB anhydride and dithiothreitol were used as purchased (Aldrich, Milwaukee, Wisc., U.S.A.). Pesticide grade acetonitrile and spectroquality methanol were employed (Matheson, Coleman & Bell, Norwich, Ohio, U.S.A.); all other solvents and reagents were analytical reagent grade.

All glassware was silanized by rinsing with 2% trimethylchlorosilane in benzene solution and heating at 110° for 30 min.

### Recovery experiments

Stock solutions of I and V were prepared in 5- or 10-ml volumetric flasks by dissolving 5.0–10.0 mg of the compound in methanol or acetonitrile. Between experi-

ments, samples were stored at $-10°$. Standard GC solutions were prepared by reducing to dryness aliquots placed in Reacti-vials (Pierce, Rockford, Ill., U.S.A.) with a stream of nitrogen. Acetonitrile (50 $\mu$l) and HFB anhydride (25 $\mu$l) were added, and the solution was allowed to stand at room temperature for 30 min. Injections were made using a Hamilton 701 N 10-$\mu$l syringe. N-$n$-Propylnorapomorphine (V) was used as an internal standard to control the extraction procedure. Standard curves of the peak height ratio of I and V *versus* the concentration ($\mu$g/ml) of I in plasma were used analytically. Boldine (VI) was used as an internal standard to control the GC step when absolute recovery data were needed and was dissolved in the acetonitrile used as a derivatization solvent. Peak heights "corrected" to a "standard" boldine value were plotted *versus* $\mu$g of I (or V) per ml of plasma to yield a standard curve.

### Preparation of standards

To two sets of five 2-ml human blank plasma samples was added 2, 5, 10, 15, and 20 $\mu$g of I and 10 $\mu$g of II (in 100 $\mu$l or less of methanol). The standards were extracted and analyzed in the same manner as described for plasma samples below.

### Determination of I in plasma

Each 2-ml human plasma sample to be analyzed had 10 $\mu$g N-$n$-propylnorapomorphine and 3 ml of 0.25 $M$ phosphate buffer (pH 7.0) added[19]. Ethyl acetate (10 ml) was added, and the two phases were mixed for 10 min. The ethyl acetate layer was removed and reduced in volume to approximately 1 ml with a stream of nitrogen at room temperature. The ethyl acetate was extracted with 2 ml of 0.012 $N$ HCl. The acid layer was neutralized with 250 $\mu$l of 8% $Na_2CO_3$. Three milliliters of 0.25 $M$ phosphate buffer (pH 7.0) were added, and the aqueous layer was again extracted with 10 ml of ethyl acetate. The organic layer was reduced to dryness at room temperature by a nitrogen stream. The residue was dissolved in 50 $\mu$l of acetonitrile. HFB anhydride (25 $\mu$l) was added. Samples were injected directly into the GC after 30 min at room tempera-
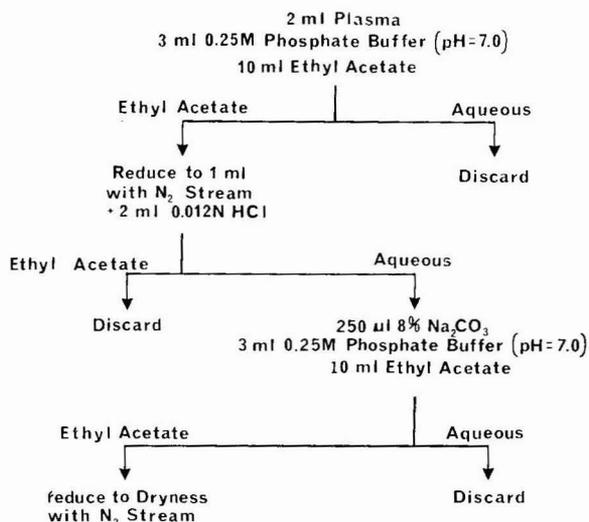


Fig. 1. The extraction of compounds I and V from plasma

ture (Fig. 1). This extraction procedure resulted in a blank plasma extract which contained no detectable peaks when chromatographed in the usual manner.

*Calculations*

The peak heights of I and II were measured. Peak height ratios were obtained by dividing the peak height of I by the peak height of II. Calibration curves were prepared by plotting peak height ratios of the standards *versus* the concentration of I in plasma expressed as $\mu g/ml$. Least squares regression was used to determine the best fit line for the data obtained from the standards. Values for the unknown concentrations were determined by calculation using the peak height ratio of I and II and the parameters determined by the regression analysis.

RESULTS AND DISCUSSION

Reaction of apomorphine (I) with acetic anhydride has been reported to yield the ring-opened product[20]. By analogy, it was anticipated that products of the reaction of I–III and V with HFB anhydride would also be ring-opened products. Mass spectra of these derivatives, however, showed that I and V contained only two equivalents of HFB acid, while the derivatives of II and III showed only one equivalent of HFB acid (Table I). It is, therefore, proposed that the derivatives formed are the phenolic esters of the non-ring-opened compounds (Table II). It is assumed that VI acts in a similar manner and that IV is unaffected by HFB anhydride. Since no extraordinary efforts
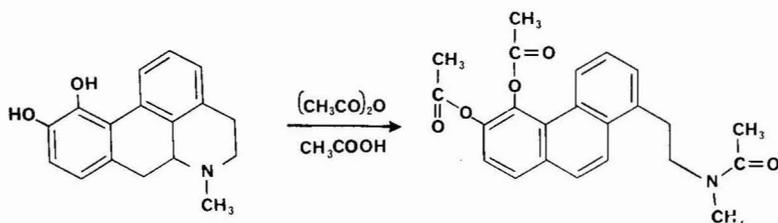


TABLE I

MASS SPECTRAL DATA FOR HFB DERIVATIVES OF COMPOUNDS I, II, III and V

| Compound | Molecular weight | Electron impact | | | Chemical ionization | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Base peak m/e | M+ m/e | % Base | Base peak (M + 1) | M + 29 m/e | % Base | M + 41 m/e | % Base |
| Apomorphine diheptafluorobutyrate | 659 | 659 | 659 | 100 | 660 | 688 | 17.3 | 700 | 6.2 |
| N-n-Propylnorapomorphine diheptafluorobutyrate | 687 | 658 | 687 | 77.1 | 688 | 716 | 22.2 | 728 | 9.8 |
| Isoapocodeine heptafluorobutyrate | 477 | 69 | 477 | 19.8 | 478 | 506 | 22.9 | 518 | 7.0 |
| Apocodeine heptafluorobutyrate | 477 | 69 | 477 | 32.2 | 478 | 506 | 30.7 | 518 | 6.3 |

TABLE II

STRUCTURES OF HFB DERIVATIVES OF I, II, III AND V
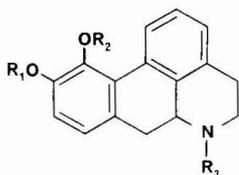


| No. | Compound | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|---|
| I | Apomorphine | $CF_3(CF_2)_2CO$ | $CF_3(CF_2)_2CO$ | $CH_3$ |
| II | Apocodeine | $CH_3$ | $CF_3(CF_2)_2CO$ | $CH_3$ |
| III | Isoapocodeine | $CF_3(CF_2)_2CO$ | $CH_3$ | $CH_3$ |
| V | N-$n$-Propylnorapomorphine | $CF_3(CF_2)_2CO$ | $CF_3(CF_2)_2CO$ | $CH_3CH_2CH_2$ |

were made to purify the HFB acid, it must be assumed that some HFB acid is present and that more is produced by the reaction of the anhydride with any residual water present in the sample. It is suggested that the weakly basic heptafluorobutyryl anion is unable to remove the proton from position 7 of aporphines, thereby preventing the rearrangement observed with acetic anhydride[20].

The time needed for derivatization was studied. I and V were found to be completely derivatized almost immediately as indicated in Fig. 2. Boldine was completely derivatized after 10 min. The derivatives were stable for at least 2 h. In the studies described our samples were developed 30 min after derivatization.

Although the developed procedure provides standard curves that are linear over three orders of magnitude (1 $\mu$g to 1 mg) only the range 1–10 $\mu$g/ml was extensively studied because other methods already exist for analyses at higher concentrations. A sample chromatogram is depicted in Fig. 3 along with a similarly processed blank plasma sample which shows no interferences in the region of interest.

Back extractions of samples were found to be necessary to eliminate two large, late chromatographing substances which did not interfere with the analytical region
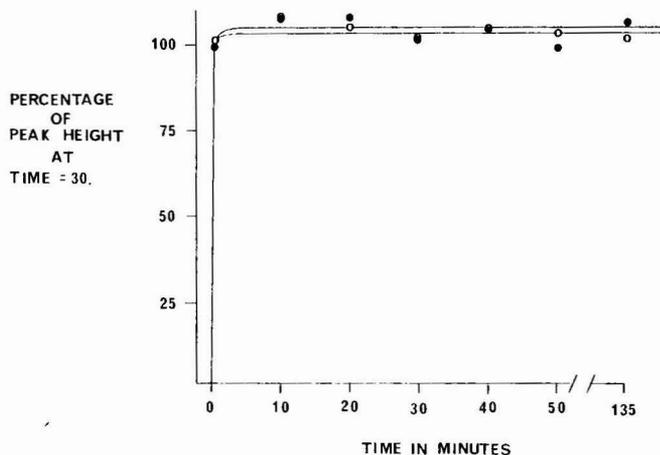


Fig. 2. Rate of derivatization of I ($\bigcirc$) and V ($\bullet$) with HFB anhydride.
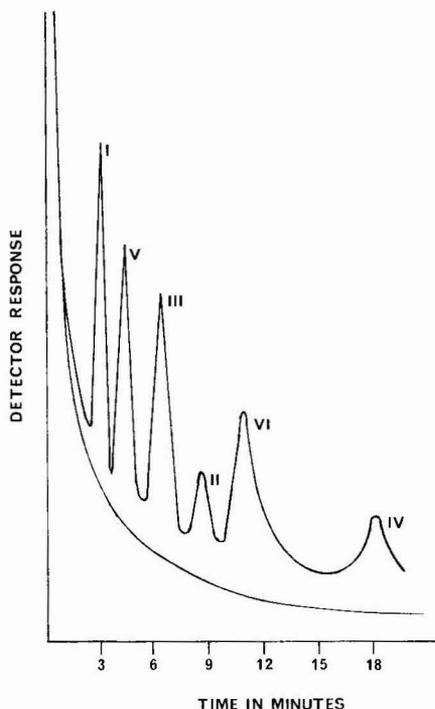
Fig. 3. GC (FID) of a mixture of aporphines using a 64 cm × 4 mm I.D. silylated glass column packed with 3 % OV-17 on Chromosorb W-HP. A Hewlett-Packard 5710A gas chromatograph was operated isothermally at 190° and a nitrogen flow-rate of 50 ml/min.

but greatly added to the time needed for repetitive samples. Boldine was used as an internal standard to control the GC step in absolute recovery experiments. The absolute recovery of apomorphine from plasma in the 1–10-$\mu$g/ml range averaged 87.9 % ($n=6$), while average absolute recovery of N-$n$-propylnorapomorphine (the internal standard) was 84.6 % ($n=6$).

In the range 1–10 $\mu$g of appmorphine per ml of plasma, correlation coefficients of 0.99 or greater were consistently observed for standard curves (Fig. 4). Over this range, an average relative standard deviation of 4.6 % was found.

When dealing with biological specimens, it is often necessary to store them for extended periods, usually by freezing. It has been found that these samples lose apomorphine as time passes. It was assumed that this was due to oxidation of the apomorphine, probably to quinone-type substances. Acid was found to slow or prevent this process[21], but the addition of sufficient acid to retard degradation tended to precipitate plasma proteins which resulted in losses of apomorphine. The acid treatment also made the adjustment of the pH prior to extraction a more tedious task. Dithiothreitol (DTT) (1 mg/ml) was found to be an effective stabilizing agent when added to the plasma prior to freezing. Analysis of DTT-treated samples that were stored for 6 weeks at −10° showed no significant loss. The DTT also did not interfere with the extraction or the actual determinations.

Compounds II, III and IV were found to separate from I and V when added to
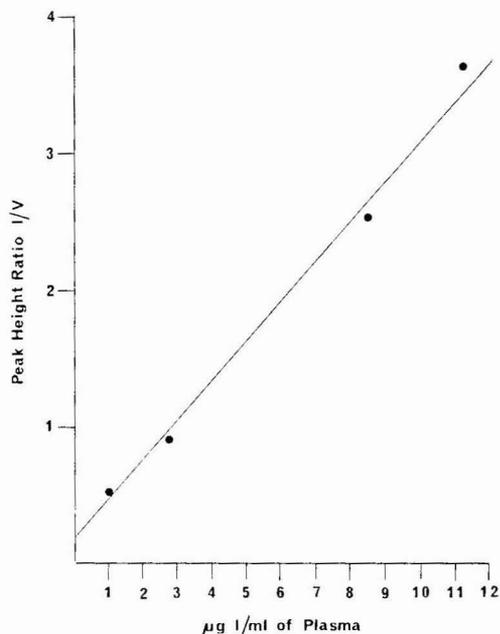
Fig. 4. Typical standard curve for the determination of I in plasma. Least squares data: slope, 0.30; y-intercept, 0.15; r, 0.996.

plasma and analyzed with the developed procedure. The analytical procedure should be applicable to all of the above named compounds, though recoveries were not specifically examined. However, from previous works[22,23], it is expected that recoveries should be excellent.

HFB derivatives offer the potential of analysis using an electron capture detector (ECD), thus, achieving significantly greater analytical sensitivity. The HFB derivative of I was reportedly used to detect apomorphine in ng/ml concentrations in horse blood plasma[24]. However, the authors of this study provided little information on recoveries or the precision of their procedure. Studies are currently being performed in our laboratories to evaluate the utility of ECD for the ppb analysis of apomorphine and its analogs as their HFB derivatives.

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CHROM. 10,135

# QUANTITATIVE DETERMINATION OF THEBAINE IN *PAPAVER BRAC-TEATUM* BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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## SUMMARY

A method is described for the quantitative analysis of thebaine from *Papaver bracteatum*, using a single high-pressure liquid chromatographic column. The procedure gives base-line separation of thebaine without the need for gradient elution equipment, and can be completed within 12 min. Thebaine isolated by this technique was shown to be pure, regardless of the age of plant or plant part from which it was obtained.

## INTRODUCTION

In recent years, as a result of a general reduction in the production of legitimate codeine from *Papaver somniferum* (Opium poppy), concomitant with an increased demand throughout the world[1,2], other sources of raw materials for the production of this alkaloid have been sought. Although *Papaver bracteatum* has been reported to contain thebaine, isothebaine, orientalidine, and codeine, in addition to some 25 other alkaloids[3,4], there is some controversy as to whether the plants investigated were actually *P. bracteatum*, or natural hybrids of *P. bracteatum* with *P. orientale* or *P. pseudo-orientale*[5]. Thebaine, however, is consistently reported to be the major alkaloid of *P. bracteatum*[1,2,5,6], the other alkaloids being present only in trace amounts. Since thebaine can be chemically converted to codeine[6,7], *P. bracteatum* is currently regarded as the most promising solution to the codeine shortage[1,2,5,6], and many procedures for the extraction, purification, and quantitation of thebaine have been reported in recent years[3,5,6,8–21].

Gas–liquid chromatography (GLC) has been used routinely to separate and quantitate thebaine from *P. bracteatum* extracts[3,8–13]. However, none of these GLC procedures has established that the thebaine peak observed during routine analysis represents only pure thebaine. It was determined in this investigation that thebaine is decomposed into a mixture of products under the conditions routinely employed in GLC analyses. Since it was not possible to determine whether the GLC thebaine peak represented pure thebaine, or a mixture of phytoconstituents and/or their

decomposition products, a high-pressure liquid chromatographic (HPLC) procedure employing milder conditions was needed.

A number of HPLC separations of thebaine and other opium alkaloids have been previously reported[14-21]. However, these methods require pre-treatment of the extract to remove polar impurities (*e.g.*, ion-exchange chromatography), gradient elution equipment, and/or they do not produce base-line separation of thebaine from other constituents. A rapid, single column separation which overcomes the disadvantages of previous methods is described in this communication.

EXPERIMENTAL

*Apparatus*

A Perkin-Elmer Model 881 gas chromatograph equipped with a hydrogen flame ionization detector and a Sargent SR recorder were used for the GLC studies. A 6 ft. × 1/4 in. O.D. spiral glass chromatographic column was packed with 2.5% OV-17 on 100–120 mesh Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.).

Liquid chromatographic separations were conducted using a Waters Assoc. (Milford, Mass., U.S.A.) Model 6000 liquid chromatograph equipped with a Beckman 25 variable-wavelength UV spectrophotometer and recorder. The separations were carried out using a Waters Assoc. 30 × 4 mm I.D. $\mu$Bondapak $C_{18}$ column.

For thin-layer chromatographic (TLC) analyses, aluminum-backed, precoated silica gel $GF_{254}$ plates (20 × 20 cm, 0.25 mm thick) or aluminum oxide $F_{254}$, type T (20 × 20 cm, 0.25 mm thick), both obtained from E. Merck (Darmstadt, G.F.R.) were used.

*Reagents*

All chemicals and solvents used in this investigation were reagent grade. Solvents for HPLC were redistilled in glass.

Standard thebaine (GLC pure) was obtained from S. B. Penick (Orange, N.J., U.S.A.).

Morphine and codeine were generated from morphine sulfate and codeine phosphate, respectively (Mallinckrodt, St. Louis, Mo., U.S.A.).

Isothebaine and orientalidine were provided by Professor E. Brochmann-Hanssen, University of California, San Francisco, Calif., U.S.A.

*Papaver bracteatum alkaloid extracts*

Dried, powdered (40 mesh) *P. bracteatum* straw (aboveground parts, excluding capsules; 1.0 g) was transferred to a 125-ml Erlenmeyer flask and 5% aqueous acetic acid (50 ml) was added. The flask was shaken for one hour by means of a rotary shaker, the mixture was filtered through a Büchner funnel, and the marc was washed with 5% aqueous acetic acid (10 ml). Following alkalinization of the filtrate with concentrated ammonium hydroxide (6 ml), the filtrate was extracted three times with chloroform (50 ml per extraction). The combined chloroform extracts were dried over anhydrous sodium sulfate, filtered, and the sodium sulfate was washed with chloroform (10–15 ml). The combined extracts were evaporated to dryness *in vacuo*, yielding a residue which was dissolved in an appropriate solvent and applied to a chromatographic column.

*Gas–liquid chromatography*

The GLC operating conditions employed were: injector temperature, 285°; detector temperature, 285°; oven temperature, 270°; flow-rate of carrier gas (helium), 48 ml/min; hydrogen pressure, 17.5 p.s.i.g.; air pressure, 50 p.s.i.g. In order to determine whether the thebaine peak represented pure thebaine, a stream splitter was installed at the elution end of the GLC column. Five repeated injections (10 $\mu$l) of *P. bracteatum* total alkaloid extract, containing approximately 6 $\mu$g thebaine per $\mu$l ethanol, were made, and the compounds represented by the two resulting peaks were collected separately.

The two resulting fractions were applied to a silica gel TLC plate and developed with toluene–acetone–ethanol–conc. ammonium hydroxide (20:20:3:1)[22]. Chromatograms were examined under both short- and long-wavelength UV light after development and then sprayed with 70% sulfuric acid in methanol. Compounds were detected by charring at 110° for 10 min. Two auxiliary TLC systems were also used *viz.*, silica gel with benzene–acetone–methanol (7:2:1)[23]; and aluminum oxide with benzene–ethanol (9:1)[24].

*High-pressure liquid chromatography*

The operating conditions for HPLC were: ambient temperature; flow-rate of eluting solvent, methanol–water containing 0.3% ammonium carbonate (4:1), 1 ml/min; wavelength of UV detector, 285 nm; recorder chart speed, 0.5 in./min. Standard solutions of thebaine, isothebaine, orientalidine, codeine, and morphine were injected onto the HPLC column and their retention times determined.

Two Beer's law standard curves were obtained by injecting different concentrations of standard thebaine onto the column in quadruplicate. The thebaine concentrations employed were 0.01, 0.02, 0.05, 0.075, and 0.10 $\mu$g/$\mu$l at 0.25 absorbance units full scale (a.u.f.s.) on the UV recorder, and 0.10, 0.20, 0.30, 0.40, 0.50, and 0.60 $\mu$g/$\mu$l at 0.5 a.u.f.s. (20 $\mu$l per injection). Peak areas were measured using the triangulation method.

For routine analyses of *P. bracteatum* alkaloids, the residue obtained from the chloroform extracts was dissolved in an accurately measured volume of chromatographic solvent and 20 $\mu$l of the resulting solution were injected in triplicate onto the HPLC column. To prevent damage to the column, it was routinely washed with redistilled water at the end of each day, until the pH of the effluent was 7. The column was then washed with methanol and stored.

In order to determine whether the thebaine peak represented pure thebaine, multiple injections of *P. bracteatum* alkaloid extract were made. The thebaine fractions were collected and combined, and a mass spectrum and UV spectrum were obtained. The isolated thebaine was also analyzed by TLC in all three chromatographic systems.

RESULTS AND DISCUSSION

Thebaine was apparently decomposed by the high temperature used during GLC, since no spot corresponding to thebaine could be observed on TLC chromatograms. The two collected GLC fractions were qualitatively similar, containing at least four compounds, but were quantitatively different. It was also observed that

the ratio of one compound to another in each fraction was a function of the GLC column temperature employed. If an oven temperature of 225° was used, only a single peak was observed on the recorder, and intact thebaine could be recovered. However, at this lower temperature, tailing of the thebaine peak was so severe as to preclude accurate quantitation. Decomposition of thebaine during GLC analysis was also noted by another research group[25].

Since thebaine is decomposed under the conditions routinely used in GLC analyses, it is impossible to determine whether the thebaine peak represents pure thebaine. Studies investigating the potential effects of chemicals on thebaine metabolism, therefore, could not be conducted using these GLC assay techniques. If, for example, a chemical being tested affected the metabolism of a plant constituent having the same retention time as the thebaine decomposition products, the effect of the chemical on thebaine metabolism would be obscured, since this phytoconstituent would be quantitated as thebaine. An HPLC procedure was developed to obviate this problem.

Under the conditions used for HPLC in this study, isothebaine, orientalidine, morphine, and codeine were all well separated from thebaine, giving the following retention times: isothebaine, 6.0 min; orientalidine, 6.0 min; morphine, 7.1 min; codeine, 7.9 min; and thebaine, 10.2 min. In addition, thebaine was well separated from all other compounds present in the total alkaloid extract of *P. bracteatum*. A typical chromatogram of this extract is shown in Fig. 1. Thebaine quantitation was also readily achieved by HPLC, since the detector response was linear for all concentrations employed. For the standard curve at 0.25 a.u.f.s. on the UV recorder, the slope was 3.02, the $y$-axis (peak area) intercept was $+0.07$, and the reliability was 0.999. At 0.5 a.u.f.s., the slope was 1.54, the $y$-axis intercept was $-0.02$, and the
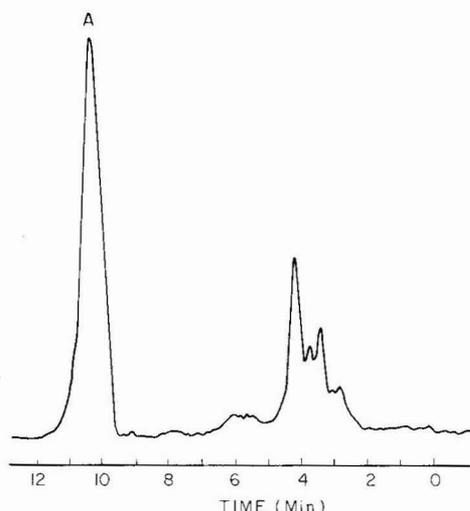


Fig. 1. Liquid chromatogram of *Papaver bracteatum* straw extract. Operating conditions: column, $\mu$Bondapak $C_{18}$; mobile phase, methanol–water containing 0.3% ammonium carbonate (4:1); ambient temperature; flow-rate, 1.0 ml/min; detector, UV spectrophotometer (285 nm). Peak A = thebaine.

reliability was 0.999. The minimum and maximum amounts of thebaine which could be accurately quantitated were 0.05 $\mu$g (0.25 a.u.f.s.) and 12 $\mu$g (0.5 a.u.f.s.), respectively.

The mass spectrum and UV spectrum of thebaine, separated from a *P. bracteatum* total alkaloid extract by this HPLC column, were virtually superimposable with those of standard thebaine. In addition, TLC analyses showed a single spot in three different systems; the $R_F$ corresponded in each case to that of standard thebaine. The $R_F$ of thebaine in the primary TLC system was 0.49, in the auxiliary silica gel system it was 0.22, and in the aluminum oxide system it was 0.45. Further, thebaine isolated from roots and straw of plants ranging in age from 6 months to 2 years, as well as from immature or mature capsules, was found in each case to be pure by TLC in all three systems. Consequently, this HPLC technique can be applied to *P. bracteatum* alkaloid extracts regardless of plant age or plant part, even though these extracts contain different phytoconstituents.

The overall recovery of thebaine from the extraction and HPLC procedures was determined by adding 5.0 mg of reference thebaine to the exhausted marc of *P. bracteatum* straw (1.0 g). Re-extraction and analysis by HPLC yielded 4.63 mg thebaine, giving an overall recovery of 93%. Twelve replicate injections of alkaloid extract from *P. bracteatum* straw were also made to determine the precision of the HPLC quantitation (Table I). These data show that the reproducibility of the quantitative procedure is 98.1% within 99.7% confidence limits. Using this technique, thebaine concentrations as low as 0.0003% can be accurately quantitated if a 1.0 g plant sample (dry weight) is used for analysis.

TABLE I

REPLICATE ANALYSES OF *PAPAVER BRACTEATUM* TOTAL ALKALOID EXTRACT

| Injection number | Peak area (cm$^2$) | Thebaine weight ($\mu$g) |
|---|---|---|
| 1 | 12.65 | 8.21 |
| 2 | 11.73 | 7.61 |
| 3 | 12.19 | 7.91 |
| 4 | 12.05 | 7.82 |
| 5 | 11.94 | 7.75 |
| 6 | 12.33 | 8.00 |
| 7 | 12.04 | 7.81 |
| 8 | 12.22 | 7.93 |
| 9 | 12.09 | 7.85 |
| 10 | 12.32 | 8.00 |
| 11 | 12.18 | 7.91 |
| 12 | 12.56 | 8.15 |
| | Average 12.19 ± 0.08* | 7.91 ± 0.05* |

* Standard error of the mean.

The only problem regarding reproducibility of this technique involves column stability. Although $\mu$Bondapak C$_{18}$ consists of a monomolecular layer of octadecyltrichlorosilane, chemically bonded to silica via a hydrolytically stable ether linkage, repeated use of the column appeared to give rise to a small number of active sites on the silica. It was therefore necessary to saturate these active sites by making a daily

injection of 20 µg thebaine standard. In addition, injections of 5 µg thebaine standard were made until successive injections gave thebaine peak areas within 2% of each other or better; usually, two injections of thebaine were sufficient.

ACKNOWLEDGEMENTS

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# SENSITIVE THIN-LAYER CHROMATOGRAPHIC METHOD FOR URINE SCREENING OF BARBITURATES[*]

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## SUMMARY

A simple and sensitive thin-layer chromatographic (TLC) method is described for the detection of barbiturates and other sedatives at therapeutic levels in the urine. The method consists of an extraction followed by TLC on a single plate in a solvent system which separates the barbiturates from most of the other drugs. The upper portion of the plate is sprayed with a new visualization reagent, N,2,6-trichloro-*p*-benzoquinone imine, followed by heating. The barbiturates appear as blue spots. This procedure is capable of detecting barbiturates at levels of 0.1 mg/dl in urine.

## INTRODUCTION

The development of poly-drug abuse in recent years has put an unusual burden on laboratories for the accurate identification of drugs in urine. Barbiturates remain one of the commonly abused drugs. The analysis of barbiturates requires procedures suitable for mass screening with rapidly available results. The method must be sensitive, give a minimum number of false positives and be low in cost.

Ultraviolet spectrophotometry (UV) at pH 10 and 13 is specific and useful as a screening tool for barbiturates and for quantitation[1] but is not sufficiently sensitive for detection of therapeutic levels. The UV method takes between 30 min and 1 h and is, therefore, not feasible for mass screening.

Colorimetric procedures can be performed in less than 10 min but are not very specific. The sensitivity of these methods is between 0.5 and 1 mg/dl[2,3] which is suitable for the detection of therapeutic levels of long-acting barbiturates in urine but is not sufficiently sensitive for the short-acting drugs. No information is possible on the identity of the barbiturate which is of importance in a hospital setting.

Gas chromatography (GC) is an extremely sensitive instrumental method for the analysis of drugs. This technique is not generally used for initial screening because

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of the expense and time involved. Rather, GC is utilized as a sensitive confirmatory tool, after preliminary identification by another technique, and for quantitation[4].

The newest development for the identification of barbiturates is immunoassay. This technique has recently been reviewed[5,6]. Critical comparisons were made of various immunoassays and thin-layer chromatography (TLC). Sensitivities of the Enzyme Multiplied Immunoassay Technique (EMIT) were 1 $\mu$g/ml in urine and for radio-immunoassay 0.5 $\mu$g/ml[7] which is acceptable. One problem with this method is the occurrence of false positives. EMIT gave 5% false positives for barbiturates as compared to TLC. Also, EMIT gave false negatives for glutethimide at therapeutic levels. Immunoassays are suitable for mass screening and they are used by the Air Force for this purpose. However, the cost per sample is moderate to high and thus may be prohibitive for commercial laboratories and hospitals. Due to non-specificity, results obtained by immunoassay should be confirmed by a non-immunological procedure.

TLC has been found to be the choice as the primary screening method for barbiturates[8]. It is a simple technique and considerably less expensive than immunoassay or GC[8]. One of the main drawbacks is the lack of sensitivity of the visualization reagent. Mercury-based sprays are the most commonly used visualizers for barbiturates. Single reagents such as mercuric sulfate or silver acetate produce white or gray spots which are difficult to see on a white TLC sheet. Spraying with mercuric salts followed by spraying with diphenylcarbazone produces pink or violet spots on a light background. This procedure gives better contrast with a sensitivity of 0.5 $\mu$g. However, at this low level the spots are fairly unstable and may disappear within minutes. In addition, mercury is expensive, presents a disposal problem, and is a health hazard due to its facility of entering the lungs in the aerosol form during spraying. The acidic nature of these visualization reagents also renders them hazardous and toxic. Another visualization method which is non-hazardous uses UV-quenching and allows barbiturates to be seen at microgram levels on TLC plates[9]. This method is not sensitive enough and not specific since many other drugs will produce spots due to UV-quenching.

We wish to report a new barbiturate visualization reagent, N,2,6-trichloro-*p*-benzoquinone imine (TCBI) for the detection of barbiturates in urine. TCBI has previously been used in combination with two other reagents as a three-spray system for the detection of barbiturates[10]. A preliminary report utilized a single spray containing TCBI[11] that gives blue colors with barbiturates and provides greater sensitivity than previously used visualization reagents.

MATERIALS AND METHODS

Human urines were obtained from the Center for Disease Control as unknowns in their proficiency testing program. Urines with known drugs and barbiturates were obtained from Warren Hospital, Phillipsburg, N.J., U.S.A. Blank urines containing no barbiturates were obtained from laboratory personnel.

*Reagents*

"Baker Analyzed" solvents and reagents were used from J. T. Baker (Phillipsburg, N.J., U.S.A.). Silica gel sheets (Bakerflex™ IB2), 200 $\mu$m, 20 × 20 cm (J. T. Baker) were used without activation. As screening solvent ethyl acetate–methanol–ammonia (100:18:1.5) was used.

The visualization reagent was prepared as follows. Dissolve 0.1 g of N,2,6-tri-chloro-*p*-benzoquinone imine in a mixture of 90 ml chloroform and 10 ml dimethyl sulfoxide. The dimethyl sulfoxide was previously saturated with sodium bicarbonate which was allowed to settle before decantation. This visualization reagent should be stored in a brown bottle in the refrigerator when not in use. The solution is stable for several months. Basic vapors such as ammonia and amines can darken the yellow colored solution which then should be discarded.

*Procedures*

*Extraction.* A single pH (9.5) liquid–liquid extraction procedure was used for the analysis[12]. Urine (10 ml) was taken through the extraction procedure and the evaporated extract reconstituted in 25 $\mu$l of methanol.

*TLC method.* Spot a half of the concentrated extract and a single barbiturate standard 1.5 cm from the bottom of the plate. Dry the spots at room temperature using a stream of air and develop 10 cm from the origin in an unsaturated tank ($8\frac{1}{2} \times 4$ $\times$ 9 in.; Kontes, Vineland, N.J., U.S.A.) using the screening solvent. Dry the plate in a 110° oven for a few minutes to remove solvents and ammonia. Cover the plate below $R_F$ 0.8 and spray the exposed portion of the plate with TCBI until just wet. Heat the plate in a 110° oven for a few minutes until the standard barbiturate is seen as a blue spot on a white background. If ammonia is not completely evaporated from the plate a light green background results which decreases sensitivity. A blue spot at, or slightly below, the $R_F$ value of the standard indicates the presence of a barbiturate. A gray-green spot indicates glutethimide.

RESULTS AND DISCUSSION

The $R_F$ values with the screening solvent for some common barbiturates and sedatives and approximate sensitivities with TCBI are shown in Table I. The barbiturates appear at $R_F$ values above 0.90 and all give blue colors with TCBI except glutethimide which is gray-green. The sensitivity of the blue color is about 0.1 $\mu$g for all barbiturates except glutethimide. TCBI has greater sensitivity than the other barbiturate visualization agents. At extremely low levels approaching the detection limit the spots are best seen by looking from the rear of the plastic-backed TLC sheet with transmitted light. The blue spots are stable indefinitely although the background does change from white to a light tan color upon standing a few days in the laboratory.

Other sedatives including carbromal, mebutamate, ethchlorvynol and carisprodal are not visualized with TCBI. These drugs can be seen as white or gray spots above $R_F$ 0.9 by dipping the plate, after spraying with TCBI, into a saturated aqueous solution of mercurous nitrate.

Forty-five drugs were tested for interferences in the method. Those drugs with $R_F$ values greater than 0.8 in the screening solvent are listed in Table II. TCBI gives colors with a wide variety of drugs[11] but the blue color is indicative of barbiturates. Only one of the possible interferences, oxazepam, gives a blue color. This drug can be eliminated as a possibility by heating the developed plate for 5 min at 110°. Oxazepam, if present, turns a brown color, barbiturates remain blue. Individual barbiturates are best identified by using a GC method with the remaining portion of the extract.

Visualization of the other classes of drugs may be accomplished by spraying the

TABLE I

$R_F$ VALUES AND SENSITIVITIES WITH TCBI FOR BARBITUTARES AND SEDATIVES

Color visualization sensitivity is the minimum amount necessary to see color. Spot visualisation sensitivity is the minimum amount needed in order to just discern a spot. The color of the spot cannot be determined at this lower level.

| Drug | $R_F$ | Color | Sensitivity ($\mu g$) | |
|---|---|---|---|---|
| | | | Color visualization | Spot visualization |
| Methyprylon | 0.86 | Blue | 1.0 | 0.5 |
| Pentobarbital | 0.91 | Blue | 0.1 | <0.05 |
| Diphenylhydantoin | 0.91 | Blue | 0.1 | <0.05 |
| Allobarbital | 0.91 | Blue | 0.1 | 0.06 |
| Phenobarbital | 0.92 | Blue | 0.1 | 0.01 |
| Barbital | 0.92 | Blue | 0.1 | 0.04 |
| Aprobarbital | 0.92 | Blue | 0.1 | <0.05 |
| Hexital | 0.93 | Blue | 0.1 | 0.05 |
| Secobarbital | 0.93 | Blue | 0.1 | 0.03 |
| Butabarbital | 0.93 | Blue | 0.1 | 0.01 |
| Butalbital | 0.93 | Blue | 0.1 | 0.01 |
| Mephobarbital | 0.94 | Blue | 0.1 | <0.05 |
| Methohexital | 0.95 | Blue | 0.1 | <0.05 |
| Amobarbital | 0.95 | Blue | 0.1 | 0.03 |
| Glutethimide | 0.98 | Gray-green | 0.5 | 0.2 |

lower portion of the plate, below $R_F$ 0.8, with the usual reagents including iodoplatinate, sulfuric acid, ninhydrin and fluorescamine. In this way, narcotics, amphetamines and tranquilizers can be detected on the same plate as barbiturates. The $R_F$ values of these other classes of drugs have been published previously[13].

Various extraction techniques were used to establish the applicability of the TLC method for urine analysis. These included a single pH (9.5) liquid–liquid extraction procedure[12], activated charcoal[14] and XAD-2 non-ionic resin[15]. All proved suitable with respect to naturally occurring interferences, *i.e.*, they have clean extracts in the barbiturate region of the plate.

A urine sample extract taken after ingestion of a therapeutic quantity of phenobarbital is shown in Fig. 1. In this case, only one-fifth of the concentrated extract from liquid–liquid extraction was used. The blue spots of the parent drug and metabolite are clearly visible on the TLC plate which was sprayed with TCBI over its entire area. Urine samples, after ingestion of other drugs including codeine, pentazocine, buta-

TABLE II

$R_F$ VALUES OF POSSIBLE DRUG INTERFERENCES AND THEIR COLORS WITH TCBI

| Drug | $R_F$ | Color with TCBI |
|---|---|---|
| Dicyclomine | 0.77 | Green |
| Anileridine | 0.79 | Brown-green |
| Oxazepam | 0.81 | Blue |
| Ibogaine | 0.90 | Brown-green |
| Benzocaine | 0.92 | Orange-brown |
| Methapyrilene | 0.93 | Brown-green |
| Lidocaine | 0.98 | Green |
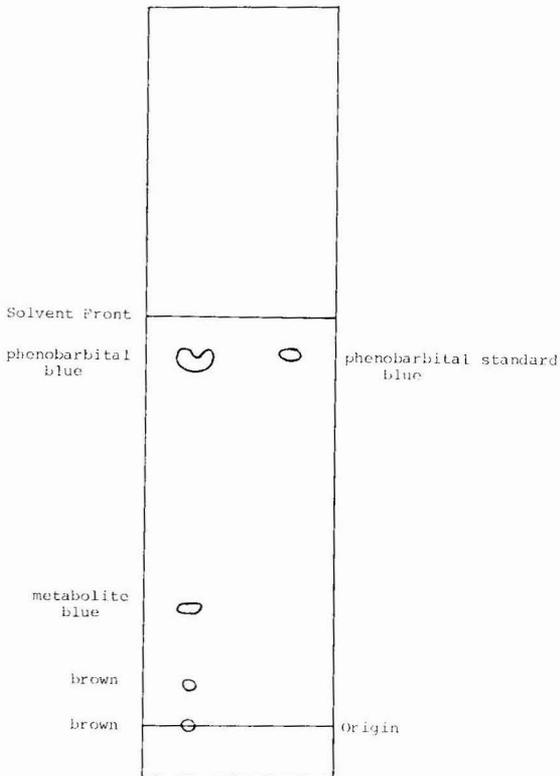
Fig. 1. TLC of urine sample extract taken 11 h after ingestion of 30 mg of phenobarbital.

zolidine, phenylbutazone and meperidine, gave no false positive results by this method. Ten proficiency urines which could contain barbiturates at levels of 1 $\mu$g/ml and above were tested using the liquid–liquid extraction and TLC. Five urines contained barbiturates and all were identified as positives by the method. No false posivites were found in the five urines containing no barbiturates but which were known to contain cocaine, amphetamines, methadone and morphine.

The detection of therapeutic levels of barbiturates by TLC is, of course, dependent on the extraction efficiency, fraction of the extract spotted, and the visualization reagent used. Great strides have been made in improving the extraction efficiency and cleanliness of the extract by the use of XAD-2 resin or charcoal. The practical limit of detection with XAD-2 and TLC in mass screening labs is about 2 $\mu$g/ml[16], although lower limits have been reported[17]. Thus, short and intermediate acting barbiturates may be difficult to detect in urine by previous methods as their levels in urine may be considerably less than 2 $\mu$g/ml.

Grove and Toseland[18] found 0.2 $\mu$g/ml of unchanged amobarbital, an intermediate acting drug, in urine by GC. The sample of urine was taken 3 days after ingestion of 200 mg of sodium amobarbital. Only GC and radioimmunoassay have been sensitive enough to detect this low level. As seen in Table III, with TCBI, very low levels of barbiturates can be detected in urine using a relatively inefficient liquid–liquid extraction technique and TCBI. Treatment of the plate with TCBI or mercurous

TABLE III

SENSITIVITY OF EXTRACTION AND TLC USING TCBI FOR THE DETECTION OF BARBITURATES IN URINE

Sensitivity was based on spiking 10 ml of urine with the drug and spotting one-half of the reconstituted extract; the concentration of drug was decreased until a dark spot was just visible on the TLC plate. The sensitivities of glutethimide and diphenylhydantoin were found by spraying with TCBI, heating the plate and dipping upper portion of the TLC sheet in a saturated mercurous nitrate solution. The resulting spots are gray.

| Drug | Urine concentration (mg/dl) |
|---|---|
| Phenobarbital | 0.1 |
| Secobarbital | 0.06 |
| Glutethimide | 0.2 |
| Diphenylhydantoin | 0.04 |

nitrate alone does not give as low a sensitivity for glutethimide and diphenylhydantoin as the combination of the two. Glutethimide and diphenylhydantoin are excreted in urine in only minute amounts[19] as the unchanged drug. Thus, the whole plate should be sprayed with TCBI and dipped in mercurous nitrate in order to see the polar metabolities which appear at $R_F$ values lower than the unchanged drug.

In summary, the TCBI visualization reagent combined with any suitable extraction procedure has been found to be a simple, selective and extremely sensitive method for the screening of therapeutic levels of brabiturates in urine.

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# ADSORPTION OF TRIVALENT IRON IN THE IONIC AND COLLOIDAL STATES ON SILICA GEL IMPREGNATED WITH MANGANESE DIOXIDE

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## SUMMARY

The sorption of trivalent iron in the ionic and colloidal states on silica gel impregnated with manganese dioxide from aqueous solution has been studied by batch equilibrations. Adsorption isotherms, for the given range of sorbent loading, can be expressed by the Langmuir adsorption equation.

The behaviour of $Fe^{3+}$ and colloidal Fe(III) on the sorbent column has also been investigated. Conditions for the sorption of $Fe^{3+}$ from nitric acid and colloidal Fe(III) from aqueous solution at different pH values are given.

## INTRODUCTION

The adsorption of different ions on hydrated manganese dioxide has been studied by many workers[1–5]. A series of separations on a column of manganese dioxide have been performed successfully, such as the following: the separation of Ba and Ra (ref. 6); $UO_2^{2+}$ and $PuO_2^{2+}$ from Zr, Nb, Ru and Cs, which remained on the column after eluting uranium and plutonium[7]; $^{51}Cr$ adsorption and $^{60}Co–^{59}Fe$ separation[4]; the separation of Tc from Mo for obtaining $^{99m}Tc$ of high purity[4]; and the elimination of Ra from liquid wastes in the uranium industry[8]. Manganese dioxide in a mixture with hydrated $Fe_2O_3$ is a very suitable scavenger for various radionuclides from aqueous solutions[9] and for $^{60}Co$, $^{65}Zn$ and $^{106}Ru$ from sea water[10].

Owing to its good adsorption properties, hydrated manganese dioxide could be suitable for larger scale adsorption, especially for the removal of various radionuclides from aqueous solutions. However, owing to difficulties in the preparation of manganese dioxide with a definite grain size and low stability of grains, *i.e.*, formation of smaller $MnO_2$ particles during long equilibration times in batch experiments, it is less suitable for studying the adsorption of ions from aqueous solutions.

For this reason, manganese dioxide was prepared by precipitation on granular silica gel used as a carrier. The sorbent thus prepared has been used in investigations of the adsorption of trivalent iron in the ionic and colloidal states from aqueous solutions.

EXPERIMENTAL

*Preparation of the sorbent*

Silica gel "for column chromatography", 0.2–0.5 mm (Merck, Darmstadt, G.F.R.) was used as a support. The silica gel was separated from powder by several decantations with water, treated with 1:1 hydrochloric acid, washed with distilled water and dried for 4 h at 110°. Impregnation was performed by pouring 150 ml of 0.23 $M$ manganese sulphate solution over 100 g of dried silica gel in a beaker. The silica gel thus prepared was then dried at 60°. The sorbent, manganese dioxide on granular silica gel, was prepared by gradual addition of the above silica gel to 1200 ml of a solution of 0.019 $M$ potassium permanganate in 1 $M$ nitric acid with constant stirring. The sorbent was separated from supernatant liquid and from manganese dioxide powder by repeated decantation, and was then washed with 500 ml of 3 $M$ nitric acid and with water until it was free from acid. After drying at 110° for about 3 h, the sorbent was treated with boiling 3 $M$ nitric acid for 5 min, washed with distilled water and again dried at 110° for 3 h. The amount of manganese dioxide on the silica gel was 29.05 mg per gram of the sorbent or 29.97 mg per gram of silica.

The procedure described above for the preparation of manganese dioxide on granular silica gel as a carrier has been used earlier[7] for the preparation of manganese dioxide identified as $\gamma$-$MnO_2$.

*Preparation of the solutions*

Experiments with $Fe^{3+}$ were carried out in nitric acid solution with practically carrier-free $^{59}Fe$ and with different concentrations of iron(III) nitrate labelled with $^{59}Fe$. The original solution of $^{59}Fe$ in the chloride form (Radiochemical Centre, Amersham, Great Britain) was converted into the nitrate form by treating with 1 $M$ nitric acid and by repeated evaporation under an infrared lamp.

Solutions of colloidal Fe(III), *i.e.*, iron(III) oxide sols, were prepared by hydrolysis of iron(III) acetate by boiling. $Fe^{3+}$ (20 ml of 0.38 $M$ $Fe^{3+}$) was precipitated as the hydroxide with ammonia solution from 0.5 $M$ nitric acid solution. The iron(III) hydroxide was washed with water (to pH *ca.* 6) and dissolved in glacial acetic acid (10 mole of acid per mole of iron). Complete dissolution was achieved after 20 h. The solution was then diluted to about 600 ml (Fe $<$ 1 mg/ml) with distilled water and boiled for 1–2 h. Colloid formation was indicated by a change in the colour of solutions from reddish to reddish brown. The colloidal solution was then evaporated on a water-bath almost to dryness, and the residue was dissolved in about 10 ml of distilled water. After several days, the colloidal solution of Fe(III) was centrifuged at 4200 $g$ for 2 h. An intensely coloured solution was then separated from the isolated gel and stored in a hermetically sealed glass vessel. This colloidal solution of iron is clear in transmitted light and turbid in reflected light (showing the Tyndall effect). The final Fe(III) colloidal solution did not contain ionic iron and was stable for several months.

Solutions of colloidal Fe(III) used in batch and column experiments were prepared under the same conditions starting from $Fe^{3+}$ solutions (0.5 ml of 0.38 $M$ Fe) labelled with $^{59}Fe$.

Part of the colloidal solution was lyophilized. The solids, which were stored in a vacuum desiccator over phosphorus pentoxide, were analyzed together with the colloidal solutions for iron and acetate content.

*Batch equilibration experiments*

Each time 100 mg of the sorbent was weighed into 6-ml glass test-tubes with stoppers and 2 ml of Fe solution in the ionic or colloidal state were added. The mixture was shaken for 24 h in a thermostat at a given temperature, then centrifuged and samples of 50–500 $\mu$l were taken for measurement. The liquid phase of sample equilibrated at 50° and 70° was transferred into other test-tubes and then centrifuged.

To avoid sorption on glass, all laboratory glassware used for storing and handling the labelled iron solutions was rinsed with a 5% solution of dichlorodimethylsilane (BDH, Poole, Great Britain) in carbon tetrachloride, then dried and heated at 250° for 2.5 h to form a water-repellent silicone layer.

*Column experiments*

The column used was 0.42 cm in diameter with a bed height of 15 cm, and was thermostated by means of a water-jacket. A given amount of iron in the ionic or colloidal state was transferred to the top of the column, pre-treated with the solution from which sorption was being investigated. $Fe^{3+}$ was eluted at 25° and 60° using nitric acid of an appropriate concentration. Colloidal iron was eluted at 25° using water, dilute nitric acid and sodium nitrate solution of desired pH.

*Analytical methods*

After dissolution and separation from silica, the content of manganese in the sorbent was determined by EDTA titration using pyrocatechol violet as indicator[11]. The density of the colloid was calculated from the weights of samples of colloidal solution and colloid-free solvent in a 10-ml pycnometer. After separation from iron, by precipitation and filtration of iron(III) hydroxide, acetate ion was determined by pH titration[12] and the iron content was determined by EDTA titration[13]. The colloidal iron was converted into the ionic state by treating the solution with concentrated hydrochloric acid. The lyophilized material was dissolved in concentrated hydrochloric acid. The charge sign of the colloidal trivalent iron was determined by electrophoretic measurements in the usual manner. The particle size of the colloid were determined by electron microscopy (JEOL JEM-7). The structure of the samples of the colloid was identified by X-ray diffraction (Simens-Kristalloflex 4 X-ray diffractometer with a Geiger–Müller counter and a recorder).

The activity of the $^{59}Fe$ was measured on a Nuclear Chicago Geiger–Müller counter with a thin window (*ca.* 2 mg/cm$^2$). The activity of the experimental solutions varied from about $10^4$ to $10^5$ counts·min$^{-1}$·ml$^{-1}$.

RESULTS AND DISCUSSION

From the dependence of the distribution coefficients $(K_d)$ of $Fe^{3+}$ $(2.7 \cdot 10^{-7} \; M)$ on the nitric acid concentration (Fig. 1) it can be concluded that the sorption of $Fe^{3+}$ depends strongly on the acidity of the aqueous solution, as would be expected for a real ion-exchange process. The $K_d$ values are independent of the $Fe^{3+}$ concentration (Henry's law) below $7 \cdot 10^{-6} \; M$.

The adsorption isotherms for $Fe^{3+}$ (Fig. 2) were also determined. The results
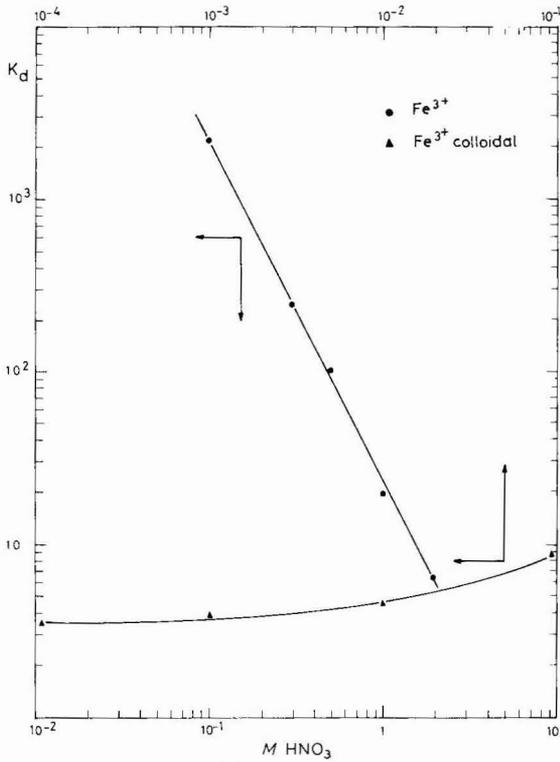
Fig. 1. Dependence of the distribution coefficients, $K_d$, of $Fe^{3+}$ (trace amounts) and colloidal trivalent iron ($3.5 \cdot 10^{-5}$ g Fe/ml) on the $HNO_3$ concentration.
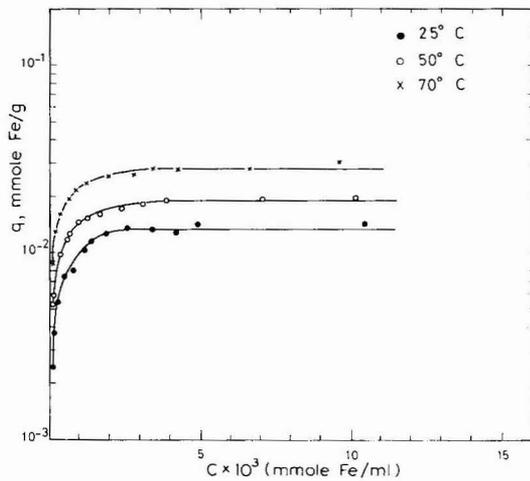


Fig. 2. Adsorption isotherms for $Fe^{3+}$ at 25 ∣ 0.2", 50 ∣ 0.5" and 70 ∣ 0.5". Liquid phase: 0.2 $M$ $HNO_3$.
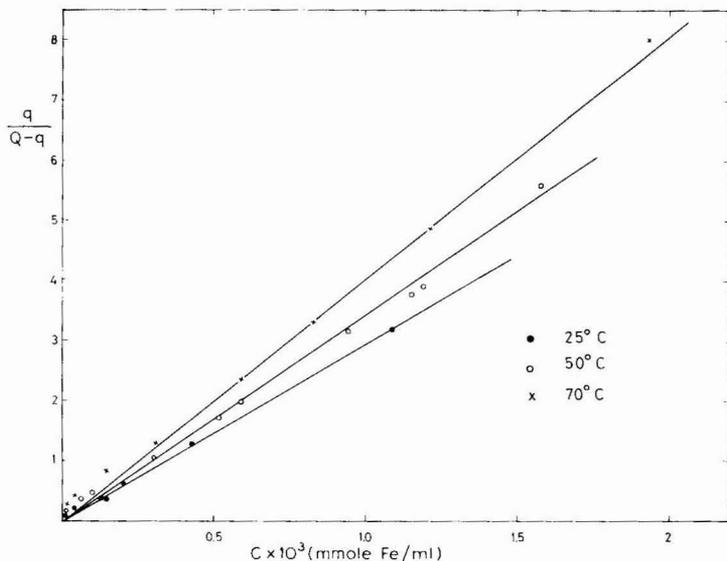
Fig. 3. Dependence of $q/(Q-q)$ on the equilibrium concentration of $Fe^{3+}$ in the liquid phase.

obtained, in the range 30–100% saturation, can be expressed by the Langmuir equation:

$$q = Q \cdot \frac{Kc}{1 + Kc} \tag{1}$$

where $q$ (mmole/g) is the amount of the element adsorbed per gram of the adsorbent, $Q$ is the same quantity at saturation, $c$ (mmole/ml) is the equilibrium concentration of the element in the solution and $K$ is the equilibrium constant.

The equilibrium constant $(K)$ is obtained from the slope of the curve $q/(Q-q)$ versus $c$ (Fig. 3).

The $K$ and $Q$ values obtained are given in Table I. The equilibrium constant and the adsorption capacity increase with increasing equilibration temperature. The capacity for $Na^+$ is 18 $\mu$equiv./g at pH ca. 6 and 25°.

It has been reported that isotherms for the adsorption of $Eu^{3+}$, $Tb^{3+}$ and $Sc^{3+}$ on $\varrho$-$MnO_2$ fit the Langmuir adsorption equation[4]. Large differences in the adsorption capacities of these ions were explained by the assumption that the number of adsorbing sites varies for the different ions[4]. From the results for $Fe^{3+}$ adsorption given in

TABLE I

COEFFICIENTS OF THE LANGMUIR ADSORPTION EQUATION FOR $Fe^{3+}$ ADSORBED ON $SiO_2$–$MnO_2$ FROM 0.2 $M$ $HNO_3$

| Temperature (°C) | Q (µequiv./g) | K |
|---|---|---|
| 25 | 39.7 | $3.0 \cdot 10^3$ |
| 50 | 57.1 | $3.5 \cdot 10^3$ |
| 70 | 84.7 | $4.0 \cdot 10^3$ |

this paper, it can be concluded that active sites on manganese dioxide, on a granular silica gel carrier, differ in their properties.

As iron belongs to the group of metals (aluminium, tin, iron) which, depending ' on the manner of preparation, may form colloids or polymers during hydrolysis[14], it was necessary to characterize the iron solution that we obtained in this way.

The $CH_3COO^-:Fe^{3+}$ molar ratios in colloidal solution and in solid material obtained by lyophilization are identical (0.32) and the composition of the lyophilized material is $FeO_{1.5}CH_3COO_{0.32}$. The specific gravity of the colloid (at 18–22°) varies from 4.6 to 5.2. X-ray analysis of samples prepared as a solid film by evaporation or by drying colloidal solutions at room temperature showed that our product is $\alpha$-$Fe_2O_3$. The structure of colloidal particles, as determined by X-ray analysis, should therefore be taken as strictly referring to gels. However, from the data given for the specific gravity of the colloid in solution and from the composition of the lyophilized product, it can be concluded that evaporation was not followed by significant changes, so that the product obtained is $Fe_2O_3$. However, the presence of some other species (amorphous hydroxide or hydrated oxides) in small amounts is not excluded. Colloidal particles are roughly spherical, 60–300 Å in diameter, *ca.* 50% of the particles being 120 Å in diameter and *ca.* 84% being 60–120 Å in diameter. The average molecular weight, calculated from the given data for particle size and specific gravity of $Fe_2O_3$ (5.3), is $3.3 \cdot 10^6$. It was reported[15] that the iron sol obtained by hydrolysis of $Fe^{3+}$ with hexamethylenetetramine at 100° is a hydrated oxide (structure rhombohedral, particle size *ca.* 250 Å).

The polymeric hydrolysis products of iron were isolated by gel filtration on a Sephadex G-25[16–18]. It is interesting that our colloidal solution of iron on a Sephadex G-25 column behaves in a similar manner to the polymer. About 93% of colloidal Fe(III) was excluded by the gel.

Adsorption of the colloidal trivalent iron on the sorbent increases very slowly on increasing the nitric acid concentration from $10^{-4}$ to $10^{-1}$ $M$ (Fig. 1). The dependence of the sorption of colloidal trivalent iron on pH is characterized by a sorption maximum in the region of weakly acidic to neutral solutions. The sorption decreases in weakly basic solutions and at pH 9.5 it is negligible (Fig. 4). A similar dependence of the sorption of radionuclides (at extremely low concentrations, below $10^{-6}$ $M$) on pH was reported earlier[19].

The colloidal particles of trivalent iron are positively charged in weakly acidic and negatively charged in weakly basic solutions, as has been determined by electrophoretic measurements. The surface charge of inorganic sorbents (of the type of hydrated oxides) also depends on the pH and concentration of the electrolytes[19,20]. In alkaline solution, the sorbent surface acquires a negative charge or changes to more negative values. Therefore, the results obtained support the assumption that the sorption of colloidal particles proceeds via a physical mechanism.

The increase in sorption on increasing the inert electrolyte concentration (Table II) can be explained by the electrolyte coagulation effect[19].

The adsorption isotherm for colloidal trivalent iron, determined in weakly acidic solution at pH 2.90 (Fig. 5), fits the Langmuir adsorption equation in the range 50–100% saturation. The adsorption capacity for colloidal trivalent iron is $5.8 \cdot 10^{-3}$ mmole of Fe per gram of sorbent.

The results obtained in the column runs are given in Tables III and IV.
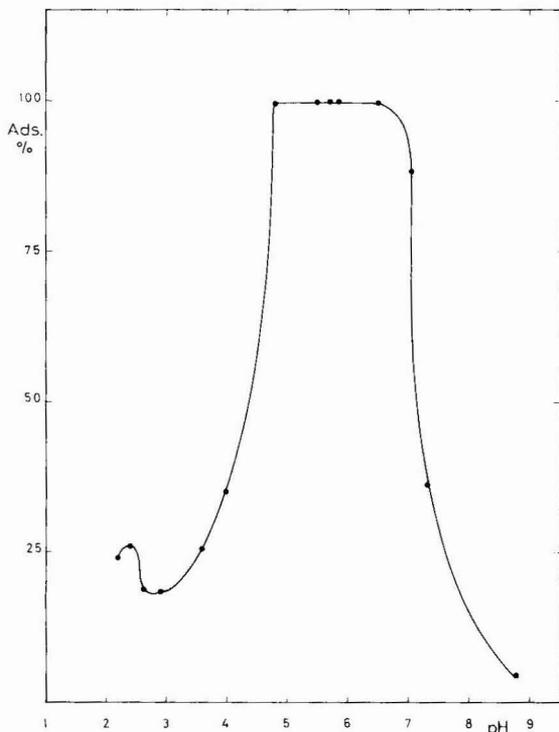
Fig. 4. Dependence of the sorption of colloidal trivalent iron ($3.5 \cdot 10^{-5}$ g Fe/ml) on pH. Solution ionic strength $\mu - 0.01$ ($NO_3^-$).

From the sorption and desorption behaviour of $Fe^{3+}$ one can conclude that $Fe^{3+}$ is adsorbed on the sorbent at very low acidity at room temperature. At higher temperatures, $Fe^{3+}$ is quantitatively sorbed at higher nitric acid concentrations. It is difficult to desorb $Fe^{3+}$ from the column, especially when desorption follows adsorption after a longer period ($> 24$ h). This behaviour indicates that, in addition to the ion-exchange process, some other kind of retention may also occur. It was reported that hydrolysed iron ions sorbed on organic cationic (weakly acidic) exchangers from dilute acid solutions (pH $>$ 1) form complexes with functional (carboxylic) groups of the exchanger[21]. Modification of FeOOH occurs in the exchanger phase as a result of ageing, which causes a low solubility of the complex[21]. From the sorption–desorption behaviour of $Fe^{3+}$ in our sorbent we consider that complexes of hydrolysed iron ions with functional OH groups of the sorbent are formed in a similar manner.

TABLE II

DEPENDENCE OF THE PERCENTAGE OF COLLOIDAL TRIVALENT IRON SORPTION ON NaNO₃ CONCENTRATION

Colloidal Fe(III): $11.4 \cdot 10^{-6}$ g Fe/ml; 0.01 $M$ HNO₃.

| NaNO₃ (mole/l) | 0.005 | 0.01 | 0.02 | 0.04 |
|---|---|---|---|---|
| Sorption (%) | 44.3 | 48.3 | 54.5 | 61.1 |
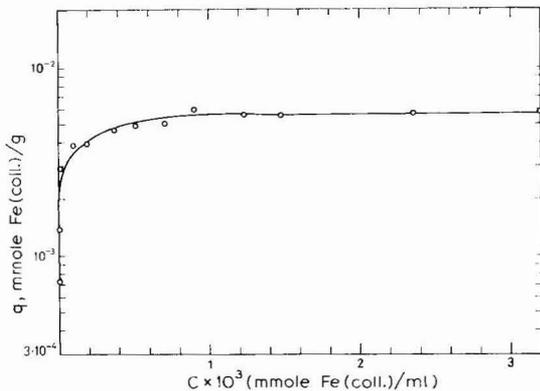
Fig. 5. Adsorption isotherm for colloidal trivalent iron at 25 ± 0.2°. Liquid phase: 0.01 $M$ NaNO$_3$, pH 2.90.

The results obtained for the colloid on the column are not in agreement with those obtained by batch equilibration. On the column the colloid was adsorbed from weakly basic solutions, which was not the case in batch experiments. No statisfactory explanation for this phenomenon has been given so far. However, the results demonstate good sorption properties of the sorbent for both the ionic and colloidal forms of iron.

TABLE III

ADSORPTION OF Fe$^{3+}$ ON SiO$_2$–MnO$_2$ COLUMN

Column, 15 cm × 0.14 cm$^2$; elution flow-rate, 1 ml/cm$^2$·min.

| Fe$^{3+}$ sorbed from HNO$_3$ ($M$) | Temperature (°C) | Percent of Fe$^{3+}$ eluted in fractions | | | |
|---|---|---|---|---|---|
| | | 0.01 M HNO$_3$ (20 ml) | 0.1 M HNO$_3$ (20 ml) | 0.5 M HNO$_3$ (20 ml) | 4 M HNO$_3$ (20 ml) |
| 0.01 | 25 | —* | | 42.7 | 32.0 |
| 0.10 | 25 | | 53.7 | 21.8 | 15.9 |
| 0.10 | 60 | | | | 99.8 |
| 0.10 | 60 | | | 41.2 | 48.7 |

   * — not eluted.

TABLE IV

ADSORPTION OF COLLOIDAL TRIVALENT IRON ON SiO$_2$–MnO$_2$ COLUMN

Column, 15 cm × 0.14 cm$^2$; elution flow-rate, 1 ml/cm$^2$·min; temperature, 25°.

| Colloidal Fe(III) sorbed from solutions | Sorbent in the form | Percent of colloidal Fe(III) eluted in fractions | | | | |
|---|---|---|---|---|---|---|
| | | Water dist. (20 ml) | 0.01 M NaNO$_3$ pH 9.8 | 0.01 M HNO$_3$ (10 ml) | 0.1 M HNO$_3$ (10 ml) | 4 M HNO$_3$ (20 ml) |
| Water dist. | Na$^+$ | —* | | | | 21.0 |
| 0.01 M NaNO$_3$ (pH 9.8) | Na$^+$ | | | | | 76.0 |
| 0.01 M HNO$_3$ | H$^+$ | | | 77.3 | 1.5 | 9.1 |

   * — = not eluted.

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## Note

Significance of the rubidium bromide thermionic detector equipped with a gate electrode in the analysis of halogenated dithiocarbamate derivatives

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During the last 10 years, the development of new and more reliable thermionic detectors has increased rapidly, and this topic has been reviewed by Krecjčí and Dressler[1], Brazhnikov *et al.*[2] and Maier-Bode and Riedmann[3].

The aim of this work was to establish a gas chromatographic (GC) detection method which could improve the identification of halogenated and non-halogenated dithiocarbamate benzyl esters. In tracing sodium dimethyldithiocarbamates and sodium diethyldithiocarbamates as metabolites of tetramethylthiuram disulphide (TMTD) and tetraethylthiuram disulphide (TETD), respectively, in serum and urine, derivatization into the corresponding dichlorobenzyl and pentafluorobenzyl esters yielded compounds which showed a different behaviour in a nitrogen flame-ionization detector (NFID) equipped with a gate electrode. The basis of this work was conceived earlier by Svojanovský and co-workers[4–6]. Using a home-made thermionic detector, they found an inversion of the thermionic system signal on changing the polarities around the jet and the collector. In studying the peak inversion phenomenon with dithiocarbamate esters, we found that all gas chromatographic peaks did not show inversion at the same gate electrode potential.

## EXPERIMENTAL

### Reagents

For the synthesis of the dithiocarbamate esters we used sodium dimethyl-dithiocarbamate dihydrate (Aagrunol, Groningen, The Netherlands), sodium diethyl-dithiocarbamate trihydrate (Merck, Darmstadt, G.F.R.), benzyl chloride (UCB, Brussels, Belgium), dichlorobenzylbromide (Fluka, Buchs, Switzerland) and pentafluorobenzyl bromide (Pierce, Rockford, Ill., U.S.A.).

*Synthesis*

To a solution of 1.00 g of sodium dialkyldithiocarbamate in 96% ethanol, 250 mg of halogenide were added. This solution was kept at room temperature overnight. The dithiocarbamate ester was extracted with dichloromethane after adding an equal amount of water to the alcoholic solution. The dichloromethane phase was washed twice with doubly distilled water and dried over sodium sulphate. After evaporation to dryness, the residue was investigated for identity and physiochemical properties by means of gas chromatographic, mass spectrometric, nuclear magnetic resonance, ultraviolet and infrared measurements. The structures of and abbreviations used for the compounds synthesized are shown in Fig. 1.
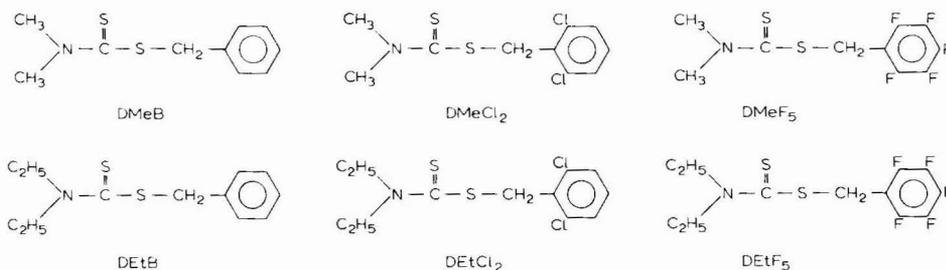
Fig. 1. Structures and abbreviations of the six compounds used.

*Standard solutions*

A standard series of each ester in methanol was prepared in the concentration range between 5 and 2000 ng/$\mu$l. The DMeCl$_2$, DEtCl$_2$, DMeB and DEtB solutions were spiked with 20 ng/$\mu$l of azobenzene and 5000 ng/$\mu$l of octadecane, which served simultaneously as internal standard and as an indicator for variations in the NFID conditions.

The DMeF$_5$ and DEtF$_5$ solutions, however, were spiked only with azobenzene (20 ng/$\mu$l), because of interference with octadecane. Daily, before operating the NFID, a test solution of 10 ng/$\mu$l of azobenzene and 5000 ng/$\mu$l of octadecane in *n*-hexane was used to monitor and to optimize the detector performance.

*Materials*

A Hewlett-Packard Type 5750 G research chromatograph equipped with a rubidium bromide Type 15161B NFID and a variable gate electrode potential was used (Figs. 2 and 3). The potential of the gate electrode can be varied manually and the digit numbers are calibrated by means of a voltmeter; gate potential readings are therefore expressed in volts. In order to quarantee long-term stability of the hydrogen flow we preferred a Type DRLS 7 reduction valve with a Type 0.2/20-RYA 2G precision valve (Air Liquide, Paris, France). Chromatograms were recorded with an HP 7128 recorder.

*GC conditions*

A Pyrex glass column (6 ft.) packed with 5% DC 200 on HP Chromosorb AW HMDS, was used. The carrier gas was helium at a flow-rate of 20–30 ml/min, with a hydrogen flow-rate of 17 ± 0.05 ml/min and an air flow-rate of 180 ± 0.2 ml/min. The
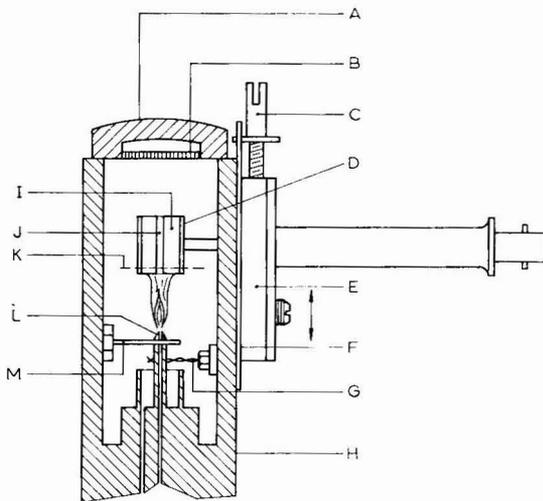
Fig. 2. RbBr NFID with gate electrode. A — Chimney cover; B — asbestos disk; C — adjustment nut; D — platina collector electrode (negative); E — collector assembly; F — guide plate; G — jet connectors; H = detector block; I — RbBr; J — central hole; K — crystal split direction; L — burner jet (positive); M — gate electrode (negative).
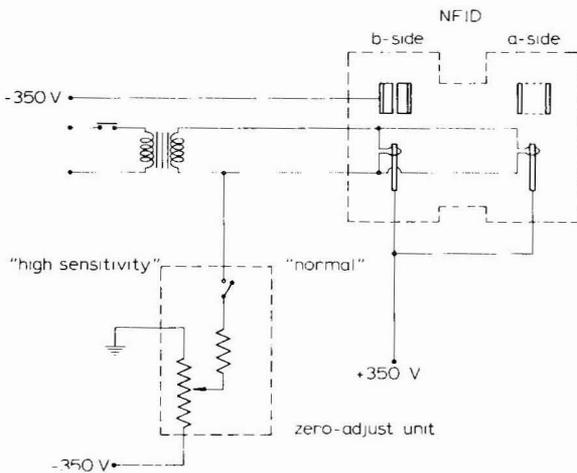


Fig. 3. Connections to gate electrode.

by-pass gas (helium) flow-rate was 40 ± 0.5 ml/min. The injection port was maintained at 260°, the column at 230° and the detector at 450°. The potentiometer setting was 48–52 and the attenuation setting 32–8·10². The zero adjust (gate electrode) was set in the high-sensitivity mode.

*Influence of the gate electrode potential on NFID response and peak performance*

By peak inversion we mean a gradual deformation of a chromatographic peak as a function of the applied voltage on the gate electrode (Figs. 4 and 5). The inversion zone is defined by the gate electrode potential range between the onset and the end of
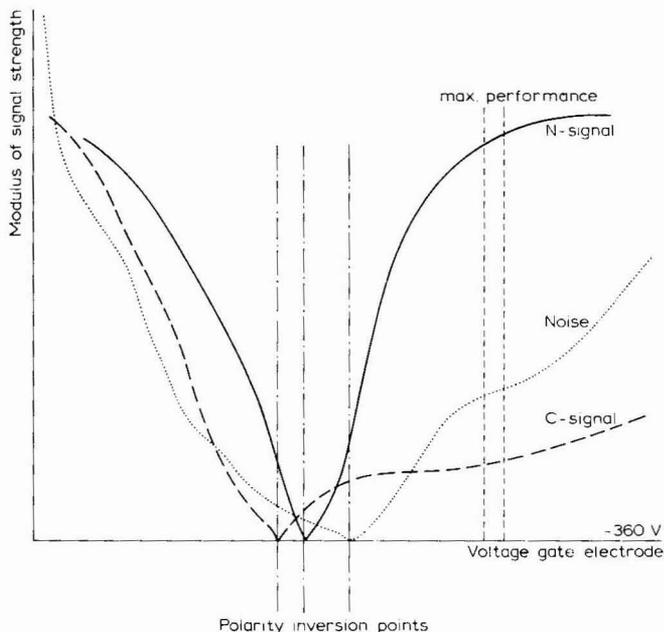
Fig. 4. Change in peak response as a function of gate electrode potential.
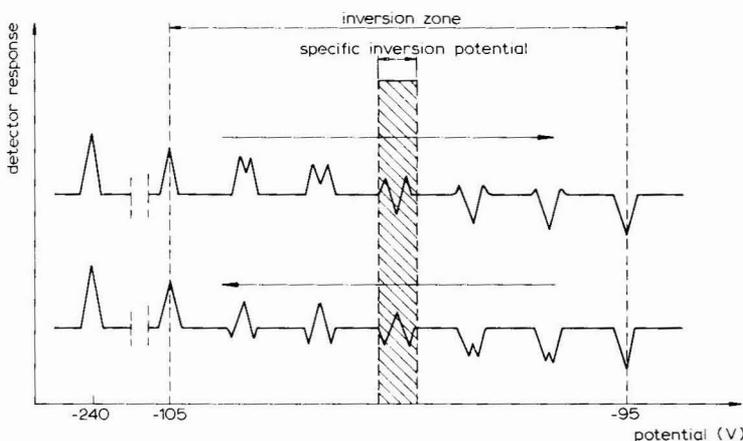


Fig. 5. Peak shape deformation and inversion as a function of gate electrode potential.

peak inversion. The arithmetic mean of this inversion zone is called the specific inversion potential and is characteristic for each test compound investigated.

Solutions of each type of ester, containing a mixture of the dimethyl- and diethyldithiocarbamate derivatives, were injected after every change in gate electrode potential. Starting from −350 V the potential was increased by 20-V steps. When a significant reduction in peak size was observed, the potential was subsequently changed in smaller steps. The peak inversion was clearly visible and, what is of major importance, the inversion of the peak of each separate test compound was observed at different potentiometer settings. In order to establish the influence of the detector parameters on

changes in a compound's specific inversion potential, a mixture of azobenzene and octadecane was added to every test solution (see *Reagents*). The ratio of the area of the azobenzene peak to the area of the octadecane peak acted as an indicator of un-  ' expected variations in the detector parameters. Before each new injection, the gate electrode potential was adjusted to $-240$ V and was kept at this potential during the elution of azobenzene and octadecane but immediately afterwards the potentiometer was re-adjusted to the desired potential (Fig. 6).
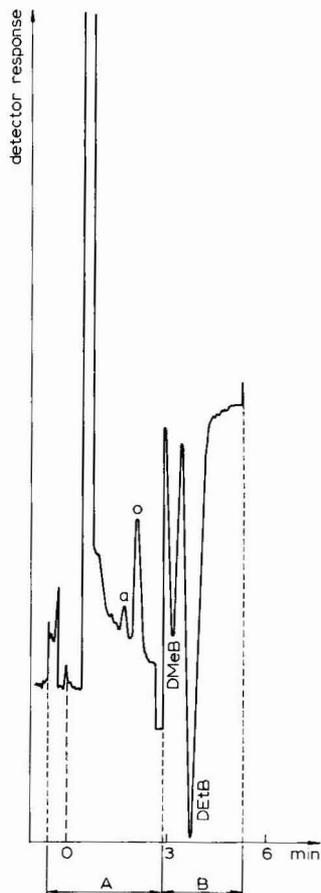


Fig. 6. Features of gas chromatogram during determination of the inversion zone. A, Gate potentia $-240$ V; B, gate potential at desired value. a, azobenzene; o, octadecane.

About 200 injections were made under the above-mentioned conditions and clearly demonstrated that the detector parameters, in relation to release of $Rb^+$, do not influence the specific inversion potential. Attempts to give each test compound a fixed specific inversion potential were unsuccessful, which can be attributed to an inadequate accuracy of the voltage supply to the gate electrode. Insertion of a digital voltmeter in the voltage supply circuit should overcome this important shortcoming. The only means of obtaining an accurate idea of the different behaviours of the six dithiocarbamate derivatives on changing the gate electrode voltage is to effect simultaneous

injection of all of the compounds. On doing so, we were able to determine the exact sequence of inversion while gradually changing the potential in the expected inversion zone after each injection. Only by observing the peak shapes an inversion sequence series was established under controlled detector conditions (Fig. 7).
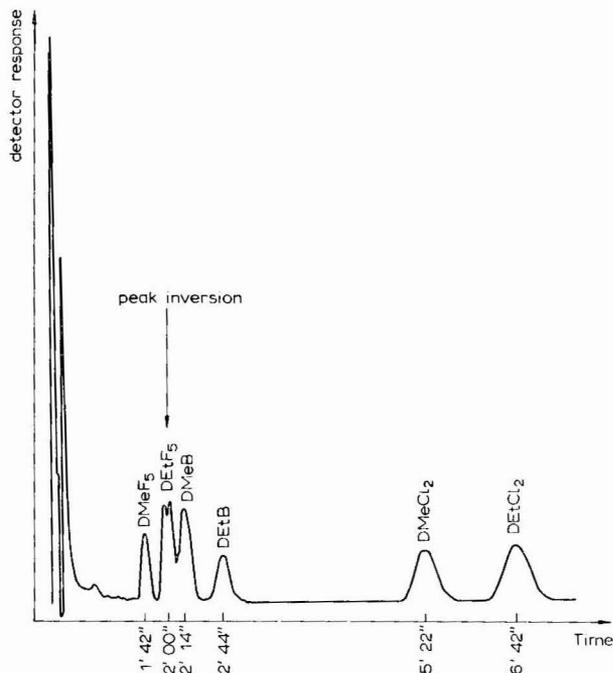


Fig. 7. Gas chromatogram showing that peak inversion takes place at a different gate electrode potential. This example was recorded at the maximum crystal position. Under these conditions and while changing the gate potential from $-130$ to $-70$V, DEtF$_5$ inverts first.

Although we found no major influence of the detector parameters on the stability of the inversion potential, there was some relationship between the crystal height and the inversion sequence. From the results obtained from about 100 injections of four compound mixtures we observed considerable changes in the inversion sequence when the rubidium bromide crystal was slowly turned downwards into the flame (Table I).

TABLE I

CHANGES IN INVERSION SEQUENCE ON CHANGING CRYSTAL HEIGHT

| Crystal position: degrees turned anticlockwise* | Inversion sequence, screening from $-130$ to $-70$ V of 4 test compounds |
|---|---|
| Max. crystal position | DEtF$_5$, DEtB, DMeCl$_2$, DEtCl$_2$ |
| Crystal position screw drive 270° | DEtB, DEtF$_5$, DEtCl$_2$, DMeCl$_2$ |
| Crystal position screw drive 450° | DEtCl$_2$, DEtB, DMeCl$_2$, DEtF$_5$ |
| Crystal position screw drive 630° | DEtCl$_2$, DMeCl$_2$, DEtB, DEtF$_5$ |

* From top to bottom in this column represents a decreasing distance between the crystal surface and the burner tip.

From further examination of the gas chromatograms, we noticed that the dimethyldithiocarbamate esters are inverted before the diethyldithiocarbamate esters at high crystal positions (low ratio of area of azobenzene peak to area of octadecane ´ peak).

CONCLUSION

Each compound, depending on its heteroatom content, inverts at a different gate electrode potential, which might be best observed by injecting a series of analogous compounds, acting as internal standards, and with a controlled release of $Rb^+$ at the crystal surface. Very accurate control of the voltage supply to the gate electrode around the flame tip would provide the possibility of collecting fixed inversion potentials, characteristic for compounds with a certain heteroatom constitution. In searching for a more specific identification of derivatized dithiocarbamates by selective GC techniques, we think that the peak inversion phenomenon may make a valuable contribution to this kind of selective analysis methodology.

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## Note

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# Gas chromatographic monitoring of the reaction of beryllium oxyacetate and beryllium oxypropionate in acetone solution

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Wynne and Bauder[1] have used proton magnetic resonance spectrometry to demonstrate that mixed beryllium oxycarboxylates undergo carboxylate scrambling in solution. Earlier claims to the preparation of pure mixed ligand beryllium oxycarboxylates by a number of investigators[2,3] were shown by Marvel and Martin[4] and Hardt[5] to be mixtures of mixed carboxylate complexes of general formula $Be_4O(RCO_2)_n(R'CO_2)_{6-n}$, where $0 < n < 6$, resulting from ligand-exchange reactions in solution.

We have established the optimum conditions for gas chromatographic (GC) elution of beryllium oxyacetate, oxypropionate, oxypivalate and oxyisobutyrate[6]; use of exhaustively silanized supports coated with 5% Apiezon L yielded more satisfactory chromatographic behaviour for beryllium oxyacetate and oxypropionate than that previously described by Barratt *et al.*[7]. Beryllium oxyacetate is eluted at 140° with a much shorter retention time than the other three beryllium oxycarboxylates.

Although GC has been used widely in the measurement of reaction kinetics and equilibrium constants for organic systems, this technique has rarely been used for kinetic studies in metal complex systems. Linck and Sievers[8] reported the use of GC to study the formation of mixed-ligand complexes in mixtures of aluminium acetylacetonate and aluminium hexafluoroacetylacetonate; however, as this work was never published in the scientific literature, kinetic data for this system are not readily available.

In view of the lack of GC investigations in the areas of kinetics and equilibria in metal complex systems, we have been interested in investigating the feasibility of monitoring ligand-exchange reactions in mixtures of beryllium oxyacetate and oxypropionate by GC. In addition, it may be possible to use GC to separate and isolate mixed-ligand complexes and thus overcome the problems outlined by previous workers[1] in the separation of complexes with similar properties.

## EXPERIMENTAL

Beryllium oxyacetate and oxypropionate were prepared by the method of Moeller[9] and purified by vacuum sublimation.

GC was carried out on a Perkin-Elmer F11 gas chromatograph using flame

ionization detection. Pyrex glass columns (1 m × 0.4 mm I.D.) were packed with 5 %
Apiezon L on DMCS-treated Universal B (60–80 mesh). The column temperature
was set at 140°, injector and detector at 200° and nitrogen flow-rate at 60 ml/min.

Ligand exchange reactions were performed by mixing beryllium oxyacetate
and oxypropionate in the molar ratio of 1:2 at 90° in acetone (0.01 $M$ solutions).
The reaction mixture was sampled (1–2 $\mu$l) using a 5-$\mu$l syringe and injected immedi-
ately into the chromatograph.

Identification of eluted samples was carried out by mass spectrometry (MS)
using a Jeol JMS D-100 mass spectrometer and a direct solids probe.

RESULTS AND DISCUSSION

Separate injections of the pure reactants, beryllium oxyacetate and beryllium
oxypropionate, yielded retention times of 4.2 and 24.2 min, respectively, at 140°.
MS analysis of the eluted samples confirmed that the complexes were eluted without
decomposition[6].

Ligand-exchange reactions were carried out at 90° because it was found that
exchange was very slow at room temperature. Typical chromatograms for sampling
of the reaction at 90° in acetone are shown in Fig. 1. As expected for ligand exchange
of two beryllium carboxylates of formula $Be_4OAc_6$ and $Be_4OPr_6$ (where Ac = acetate
and Pr = propionate), seven peaks are observed corresponding to the species
$Be_4OX_nY_{6-n}$, where $0 < n < 6$. The retention times of the first and seventh peaks
are identical to those obtained for the reactants. Although it could well be assumed
that the remaining peaks are due to the mixed-ligand complexes generated in the
reaction, confirmation of their composition was obtained by MS analysis.

Since resolution was best in the later stages of the chromatogram, fractions
of the last two mixed-ligand species in the series were collected by restricting sampling
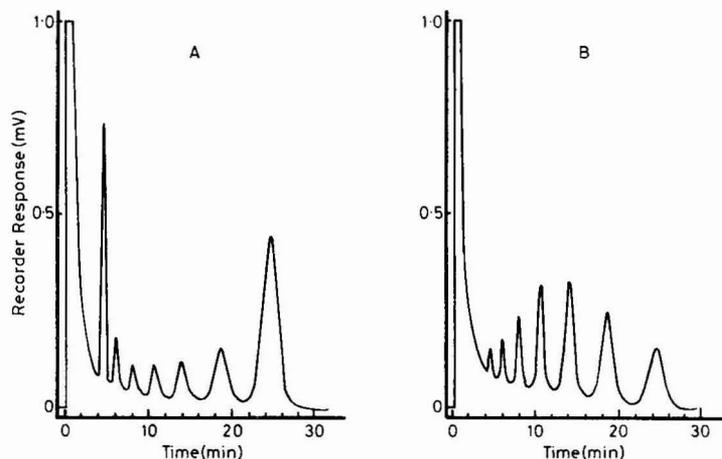


Fig. 1. Chromatograms for the reaction of beryllium oxyacetate and oxypropionate at 90° in acetone
for an initial molar ratio of 1:2. Conditions: column temp., 140°; injector and detector, 200°; Pyrex
glass column, 1 m × 0.4 mm I.D., packed with 5 % Apiezon L on silanized Universal B (60–80 mesh);
nitrogen flow-rate, 60 ml/min. Sampling time (A) 36 min, (B) 200 min.

TABLE I

MASS SPECTRAL DATA FOR SAMPLES COLLECTED FROM CHROMATOGRAPHIC PEAKS WITH RETENTION TIMES 13.7 min ($Be_4OAc_2Pr_4$) AND 18.2 min ($Be_4OAcPr_5$)

N.D. = Not detected.

| $m/e$ | Ion | Relative intensity (%) | |
| --- | --- | --- | --- |
| | | $Be_4OAc_2Pr_4$ | $Be_4OAcPr_5$ |
| 417 | $Be_4OPr_5^+$ | 2 | 100 |
| 403 | $Be_4OAcPr_4^+$ | 37 | 95 |
| 389 | $Be_4OAc_2Pr_3^+$ | 100 | N.D. |
| 375 | $Be_4OAc_3Pr_2^+$ | 6 | 6 |
| 287 | $Be_4OPr_3O^+$ | 2 | 30 |
| 273 | $Be_4OAcPr_2O^+$ | 9 | 22 |
| 262 | $Be_3OPr_3^+$ | 9 | 88 |
| 259 | $Be_4OAc_2PrO^+$ | 6 | N.D. |
| 248 | $Be_3OAcPr_2^+$ | 27 | 44 |
| 234 | $Be_3OAc_2Pr^+$ | 11 | 11 |
| 231 | $Be_4OPr_2(OH)O^+$ | 2 | 25 |
| 217 | $Be_4OAcPr(OH)O^+$ | 6 | 13 |

to coincide with the top portions of the peaks of retention times 13.7 and 18.2 min; collection times were 13.2–14.2 min and 17.6–18.8 min, respectively. The major ions observed in the mass spectra of both samples are listed in Table I.

The mass spectrum of the sample eluted at 13.7 min reveals that the high intensity peaks at $m/e = $ 403 and 389 arise from the predicted fragmentation pattern of the mixed-ligand complex, $Be_4OAc_2Pr_4$ (refs. 1, 7 and 10). The peaks from $m/e$ 287 to 217 result from further fragmentation of the ions $Be_4OAcPr_4^+$ and $Be_4OAc_2Pr_3^+$. The low intensity peaks at $m/e$ 417 and 375 are due to minor impurities from neighbouring mixed-ligand complexes in the chromatogram, viz. $Be_4OAcPr_5$ and $Be_4OAc_3$-$Pr_3$. Absence of peaks at $m/e$ 245 and 220, which would result from fragmentation of $Be_4OAc_3Pr_2^+$ ($m/e$ 375), suggests that the level of impurity from $Be_4OAc_3Pr_3$ is small. Although the peak at $m/e$ 403 is intense in the spectra of both samples, the peak at $m/e$ 417 is weak in the spectrum of $Be_4OAc_2Pr_4$ and most intense for $Be_4OAc$-$Pr_5$ (see Table I), thus suggesting that $Be_4OAcPr_5$ is only a trace contaminant in the complex eluted at 13.7 min.

The mass spectrum of the eluent at 18.2 min identifies it as $Be_4OAcPr_5$ with high intensity peaks occurring at $m/e$ 417, 403, 273, 262 and 248. The absence of peaks at $m/e$ 389 and 259 indicates that $Be_4OAc_2Pr_4$ is not an impurity, however, it is interesting to note that peaks at $m/e$ 375 and 234 suggest that $Be_4OAc_3Pr_3$ is present as a trace impurity. Contamination from $Be_4OPr_6$ is difficult to determine since peaks arising from its fragmentation ($m/e$ 417, 287, 262 and 231) are also observed for the mixed-ligand complex, $Be_4OAcPr_5$.

MS identification of two of the mixed-ligand complexes shows that the order of elution is $Be_4OAc_6$ (4.2) < $Be_4OAc_5Pr$ (5.8) < $Be_4OAc_4Pr_2$ (7.8) < $Be_4OAc_3Pr_3$ (10.2) < $Be_4OAc_2Pr_4$ (13.7) < $Be_4OAcPr_5$ (18.2) < $Be_4OPr_6$ (24.2), with the respective retention times (min) given in parentheses.

The chromatograms in Fig. 1 show clearly that it is possible to monitor the ligand-exchange reactions of metal complex systems using GC. Fig. 1A represents

the reaction after 36 min and clearly shows that the reactants are still the predominant species in the reaction mixture while the mixed-ligand complexes are continuing to be produced. On the other hand, Fig. 1B represents the state of the reaction at equilibrium which occurs after about 200 min.

As it is possible that ligand-exchange reactions may also occur in the gas phase at the column temperature employed, it was considered important to test for such behaviour. Two simple tests were performed: (i) 1-$\mu$l samples of beryllium oxyacetate and oxypropionate (as 0.01 $M$ solutions) were collected into different syringes, the oxypropionate was injected first followed immediately by the oxyacetate so that the samples would mix in the injection port and in the column; (ii) a similar procedure was used allowing a 1-min delay between injections, so that the acetate would overtake the propionate on the column because of the much shorter retention time of the former. The resulting chromatograms for both tests were similar (Fig. 2) and, in addition to peaks for beryllium oxyacetate and oxypropionate, showed the appearance of insignificant peaks due to $Be_4OAc_5Pr$ and $Be_4OAcPr_5$, thus indicating negligible reaction in the column.
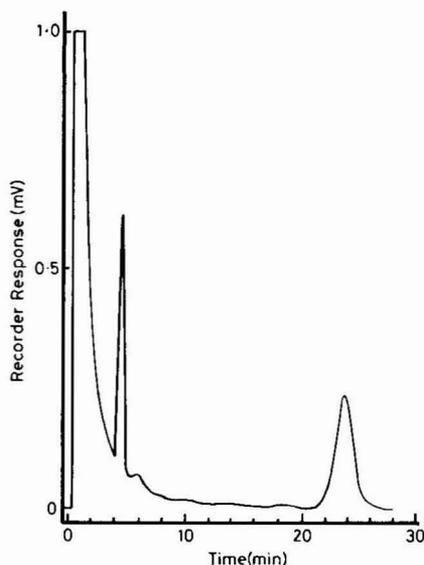


Fig. 2. Chromatogram illustrating the extent of interaction of beryllium oxyacetate and oxypropionate within the column. Column and conditions are identical to those in Fig. 1.

During the course of the present investigations, attempts were made to prepare and isolate pure samples of mixed-ligand beryllium oxycarboxylates, as described previously in the literature[2,3]. The products were analysed by GC using the conditions outlined above; chromatograms similar to Fig. 1B were obtained, showing conclusively that the products were mixtures of mixed-ligand oxycarboxylates in agreement with the findings of Marvel and Martin[4] and Hardt[5]. Fractional recrystallizations and/or column chromatography only marginally affected the chromatograms of the products.

The results presented in this paper demonstrate that GC can be used to separate mixtures of mixed-ligand beryllium oxycarboxylates of similar properties and to monitor ligand exchange reactions in these systems. An extensive investigation of the beryllium oxyacetate–oxypropionate system is currently being carried out in order to extract kinetic and equilibrium data for the ligand-exchange processes.

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## Note

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## Molecular-weight estimation of proteins using Sepharose CL-6B in guanidine hydrochloride

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It has been almost two decades since Lathe and Ruthven[1] and Porath and Flodin[2] first reported that gels can fractionate molecules on the basis of molecular size. Since then, several kinds of gel filtration media (dextran, polyacrylamide, agarose) have been introduced. Andrews[3–5] proposed that the elution characteristics of proteins are uniquely determined by molecular weight. Gel filtration techniques therefore became widely used tools not only for fractionation of macromolecules on the basis of size but also for molecular-weight determinations. However, the contention that the relative elution rates of proteins are uniquely determined by molecular weight, holds true (even approximately) only when all proteins being compared belong to the same conformational type. To avoid this discrepancy Fish *et al.*[6,7] introduced gel chromatography in presence of strong denaturants such as 6 $M$ guanidine hydrochloride (Gdn·HCl) and sodium dodecyl sulfate. Gel filtration in denaturing solvents is a useful technique for separation and molecular weight estimation of polypeptides. However, most of the soft gels in denaturants like 6 $M$ Gdn·HCl present the problems of high flow resistance causing low flow-rates and long operation periods. Further, they are not stable in 6 $M$ Gdn·HCl for long periods of time. The gel beads sometimes start breaking in this medium within a week. During the course of our search for a more stable molecular-sieving medium[8] we came across a cross-linked Sepharose gel called Sepharose CL (Pharmacia, Uppsala, Sweden), which is prepared from Sepharose by reaction with 2,3-dibromopropanol under strongly alkaline conditions. This product is claimed by the manufacturers to be more stable in Gdn·HCl (and other strong solvents) than any other gel filtration medium. However, no report exists on the separation range of these gels for proteins in 6 $M$ Gdn·HCl.

We wish to report some results showing that Sepharose CL-6B is highly stable in 6 $M$ Gdn·HCl and can be successfully used over a period of at least ten months for separation and molecular-weight determination of polypeptides. Moreover, proteins which were very poorly resolved when Bio-Glas was used to estimate their molecular weights in 6 $M$ Gdn·HCl[8] were well resolved in the present study.

EXPERIMENTAL

The proteins used and their sources are shown in Table I. The heavy chain of rabbit IgG was prepared as described[15].

TABLE I

PROTEINS USED IN GEL FILTRATION EXPERIMENTS

| Protein | Source | Molecular weight | Reference |
|---------|--------|------------------|-----------|
| B Chain of insulin | Sigma, I-2379 | 2900 | 9 |
| Insulin | Sigma, I-5500 | 5750 | 10 |
| Cytochrome $c$ | Sigma, C-2506 | 12,400 | 11 |
| Hemoglobin | Sigma, H-2500 | 15,500 | 9 |
| $\alpha$-Chymotrypsinogen | Sigma, C-4879 | 25,700 | 9 |
| Rabbit IgG, H chain | Prepared in this lab. | 49,000 | 12 |
| Bovine serum albumin | Sigma, A-4378 | 69,000 | 9 |
| Transferrin | Sigma, T-2252 | 76,600 | 13 |
| Rabbit IgG | Prepared in this lab. | 148,000 | 14 |

Sepharose CL-6B and Blue Dextran 2000 were from Pharmacia. 2,4-Dinitro-phenyl-alanine (DNP-Ala) was a Sigma product. Gdn·HCl was an ultrapure grade from Schwarz/Mann (Orangeburg, N.Y., U.S.A.) and had an absorbance of less than 0.03 at 280 nm as a 6 $M$ solution.

*Column packing and operation*

The gel was washed on a funnel with four volumes of water and suspended in the column solvent (6 $M$ Gdn·HCl plus 0.1 $M$ sodium phosphate buffer, pH 7.0). The solvent was changed several times over a 6-h period. The gel was then washed on the funnel with four volumes of the column solvent. After deaeration under vacuum, the slurry was poured into a Pharmacia column (90 $\times$ 1.5 cm), and was permitted to pack under gravity. Final equilibration was achieved by running 2–3 volumes of the column solvent through the column. The column was run at a flow-rate of 12 ml/h (6.8 ml per cm² column cross-section per h). A pressure drop of approximately 130 cm was required to maintain this flow-rate.

Proteins were reduced with 0.1 $M$ dithiothreitol and alkylated with 0.22 $M$ iodoacetamide as described previously[15]. In cases where reduction of disulfide bonds was not intended, the protein was simply dissolved in the column solvent containing 0.1 $M$ iodoacetamide.

Proteins (0.5–1.0 mg of each) were run through the column individually as well as in a mixture. The samples were brought to a volume of 200 $\mu$l and applied on to the top of the gel bed through a small tube. Fractions of 2 ml were collected and monitored by measuring absorbance at 280 nm. Blue Dextran and DNP-Ala were routinely run through the column to check the void volume ($V_0$) and the "inner" volume ($V_i$).

All chromatographic experiments were carried out at room temperature.

RESULTS AND DISCUSSION

All the results presented in this paper were obtained from a single Sepharose CL-6B column, run over a period of 10 months. Each protein was passed through the column three to six times over this period, and no significant change in the elution pattern or elution position was noticed for any of the proteins. Two typical elution profiles, one for reduced and alkylated proteins, and the other for unreduced proteins,
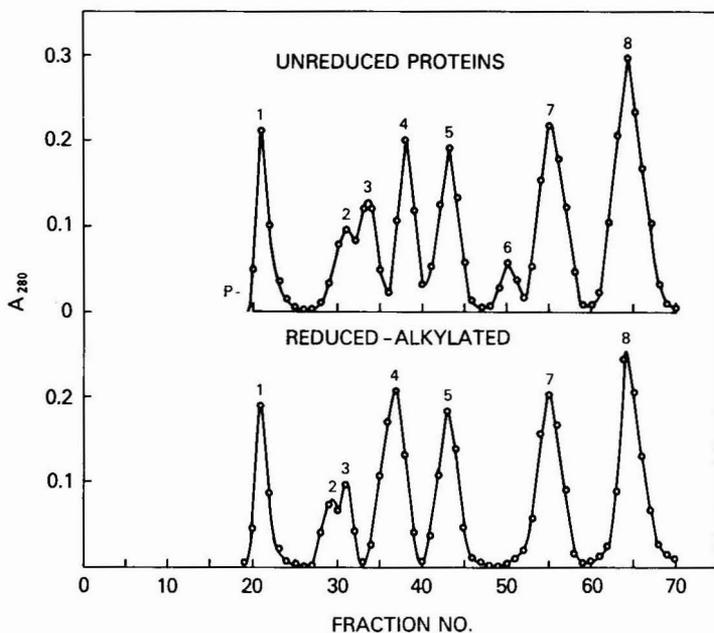
Fig. 1. Elution profiles for reduced-alkylated proteins and unreduced proteins from a Sepharose CL-6B column (90 × 1.5 cm) in 6 $M$ Gdn·HCl, 0.1 $M$ sodium phosphate, pH 7.0. 0.5–1 mg of each protein was mixed to give a total volume of *ca.* 200 $\mu$l which was applied on to the column and eluted at a flow-rate of 12 ml/h; 2-ml fractions were collected. Peaks: 1 — Blue Dextran; 2 — bovine serum albumin; 3 = rabbit IgG, H chain; 4 = $\alpha$-chymotrypsinogen; 5 — cytochrome *c*; 6 — insulin; 7 = B chain of insulin; 8 — DNP-Ala.

are shown in Fig. 1. Most evident are the sharp, symmetrical peaks and the resolving power of this gel.

Another point worth mentioning is the stability of the reduced-alkylated protein solutions. The reduced-alkylated protein samples described in our previous paper[8] were stored in 6 $M$ Gdn·HCl refrigerated for over a year. Their elution behavior from the Sepharose CL column was found to be the same as that for freshly prepared solutions of corresponding proteins. A similar check for unreduced protein solutions was not made.

Fig. 2 shows the logarithmic plots between molecular weight and distribution coefficient, $K_d$, for the reduced-alkylated and unreduced proteins. Both the plots are linear up to 80,000 daltons. Thus the use of Sepharose CL-6B in 6 $M$ Gdn·HCl permits useful molecular-weight estimates between the extreme limits of 3000 and 80,000.

In Fig. 2, the plot for reduced-alkylated proteins is deviated from the plot for the unreduced porteins, and the pattern of this deviation is in the direction that one would predict from the expected size difference between denatured proteins with disulfide bonds intact and those with disulfide bonds reduced. Even if the proteins are fully denatured in 6 $M$ Gdn·HCl, the intact disulfide bonds exert a certain amount of physical constraint on the polypeptide chain. The effect of this physical constraint is to reduce the hydrodynamic volume of the polypeptide chain in comparison to the same polypeptide chain with its disulfide bonds broken. This effect should obviously
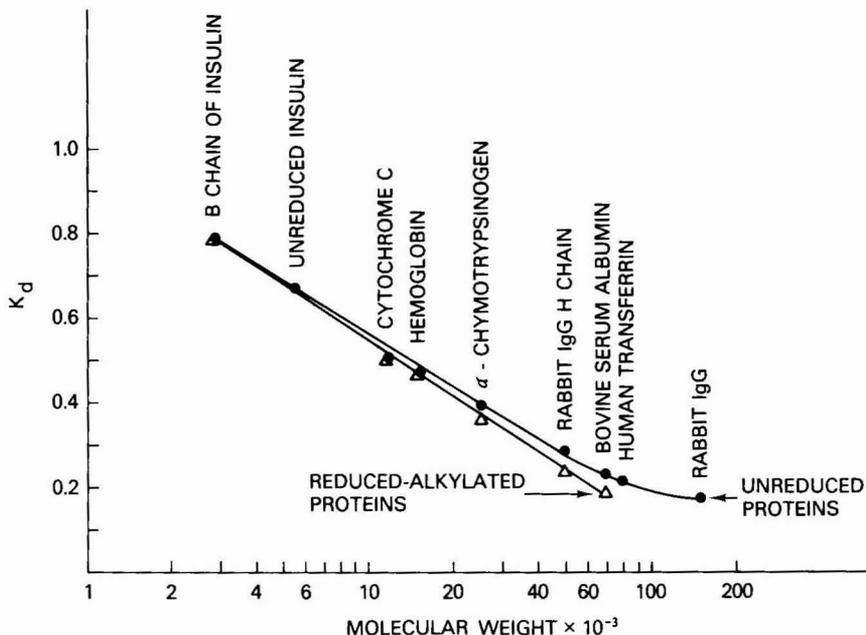
Fig. 2. Semilogarithmic plots of the molecular weights *versus* $K_d$ values obtained by running the proteins through a Sepharose CL-6B column (90 × 1.5 cm) in 6 $M$ Gdn·HCl, 0.1 $M$ sodium phosphate, pH 7.0. The distribution coefficient, $K_d$, was calculated according to equation: $K_d = (V_e - V_0)/V_i$, where $V_e$ is the elution volume of solvent at the peak concentration of eluting solute, $V_0$ is the void volume of the column determined by running Blue Dextran through the column, and $V_i$ is the volume of solvent contained within the gel bed determined by subtracting the value of $V_0$ from the elution volume of DNP-Ala.

get increasingly noticeable with increasing size of the polypeptide chain and increasing number of the disulfide bonds. This effect should also be dependent upon the size of the "loop" formed by the disulfide bond. Thus the B chain of insulin does not have any disulfide bond (ref. 16, p. 378) and cytochrome $c$ has only one disulfide bond forming a very small loop between residues 14 and 17 (ref. 16, p. 282). Therefore their $K_d$ values do not differ significantly under the two conditions. At the other end, bovine serum albumin, for instance, has 17 disulfide bonds, many of them forming large loops (ref. 16, p. 497). This causes a reduction in the hydrodynamic volume of the unreduced chain as compared to the reduced protein resulting in a high $K_d$ value.

A recent report by Nozaki *et al.*[17] has shown that anomalous retardation in gel chromatography is a general property of all large asymmetric particles, most probably because of end-on insertion into the gel pores. The fact that linear calibration curves are obtained up to a molecular weight of approximately 75,000 in Gdn·HCl, shows that the technique is suitable for molecular-weight estimations up to this molecular weight.

In conclusion, Sepharose CL-6B is highly stable in 6 $M$ Gdn·HCl, 0.1 $M$ sodium phosphate, pH 7.0, and is suitable for molecular-weight determinations in the range 3000–80,000 daltons, without presenting the problems of flow-rates and low chemical and physical stability commonly encountered with other soft gels in 6 $M$ Gdn·HCl.

This gel may also prove to be more suitable than the conventional Sepharose for making immunoabsorbents, especially when Gdn·HCl is to be used as the eluting agent.

ACKNOWLEDGEMENT

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## Note

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## Separation of ninhydrin-positive compounds on a single-column amino acid analyzer using lithium buffers

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During a study on the biosynthesis of penicillin[1], culture fluids and mycelial extracts of the mould *Penicillium* were examined with use of a single-column amino acid analyzer equipped with a cation-exchange column and with a lithium buffer gradient system as eluent. Since these analyses revealed the presence of many unidentified compounds, it was necessary to investigate the behaviour of a wide range of known materials. Although several papers[2–9] describe the separation of some amino acids using lithium buffers, extensive standardizations such as those reported for sodium buffers[10–12] are not available. Therefore, the elution behaviour of 145 ninhydrin-positive substances has been compared for two Chromobeads type-B resins of different lot numbers. Since most of these compounds are naturally occurring amino acids, the results should be of interest in the analysis (with lithium buffers) of other complex biological fluids, *e.g.*, human urine or plant extracts.

MATERIALS AND METHODS

*Amino acids*

Standard solutions of 18 and 38 amino acids (each 2.5 $\mu$mole/ml; Technicon, Brussels, Belgium) were used. S-Carbamylcysteine[13], $\alpha$-aminoadipic acid[14], $\beta$-methoxyvaline and allo-O-methylthreonine[15], $\beta$-hydroxyvaline[16], hydroxypipecolic acid[17], *threo*- and *eryhtro*-thiolbutyrine[18] and $\alpha$-amino-$\beta$-ethylvaleric acid[19] were prepared in the laboratory. Mixed disulphides were obtained by bubbling oxygen through alkaline solutions of the thiol compounds. S-Carboxymethyl derivatives were prepared by reaction with iodoacetic acid at pH 7.

*Penicillins*

The penicilloic acid of isopenicillin N was obtained by alkaline degradation of the penicillin[20]; 6-aminopenicillanic acid (6-APA) was a gift from N.V. Gist-Brocades (Delft, The Netherlands).

*Peptides*

Reduced and oxidized glutathione were products of Koch-Light Labs. (Colnbrook, Great Britain). The dipeptides L-cystinyl-bis-L-valine and L-cystinyl-bis-D-valine were prepared by a modification of the procedure of Roeske[21,22]. The tripeptides

bis-δ-(L-α-aminoadipyl)-L-cystinyl-bis-L-valine and bis-δ-(L-α-aminoadipyl)-L-cystinyl-bis-D-valine were synthesized as described elsewhere[22]. The thiol forms of these peptides (LL, LD, LLL and LLD) were obtained by reduction with dithiothreitol (DTT). Bis-δ-(L-α-aminoadipyl)-L-cystine and bis-γ-(L-glutamyl)-L-cystine were prepared by the action of carboxypeptidase A (Koch-Light) on the LLL-tripeptide and oxidized glutathione, respectively; the cysteine forms of these peptides were obtained by reduction with DTT.

## Chemicals

All reagents for the preparation of the lithium buffers and the ninhydrin reagent were obtained from E. Merck (Darmstadt, G.F.R.). No filtering of the buffers or of the lithium hydroxide solution was necessary; the former were stored at 4°. Ninhydrin reagent was prepared as described in the Technicon manual[23]. Redistilled deionized water was used throughout.

## Chromatographic conditions

The equipment consisted of a Technicon amino acid analyzer with a column (140 × 0.6 cm) filled with Chromobeads Type B (a strongly acidic cation exchange resin) in the lithium form; two batches of this resin (designated as resin I and resin II) were tested. The solutions used in the Autograd are shown in Table I.

TABLE I

GRADIENT FOR THE NINE-CHAMBERED AUTOGRAD

| Chamber No. | Buffer 1 (pH 2.75), ml | Buffer 2 (pH 3.01), ml | Buffer 3 (pH 6.50), ml |
|---|---|---|---|
| 1 | 98* | — | |
| 2 | 50 | 50 | — |
| 3, 4, 5, 6 | — | 100 | — |
| 7, 8, 9 | — | | 100 |

* Plus 2 ml of isopropyl alcohol.

Buffers of pH 3.01 and pH 6.50 were prepared as described by Vega and Nunn[5]; buffer of pH 2.75 was obtained by acidification (with 6 $M$ hydrochloric acid) of the buffer of pH 3.01.

Samples were loaded on the column in 0.20 $M$ lithium citrate buffer of pH 2.20; in the presence of thiol compounds, 5–10 mg of DTT were added. Elution was carried out at 37° with a flow-rate of 32 ml/h for 7.45 h, and at 55° with a flow-rate of 40 ml/h for the remainder of the chromatogram (back-pressure 400 p.s.i.). To accelerate elution of arginine, 100 ml of buffer of pH 6.50 was added to chamber No. 9 after 22.15 h. At the completion of each run, the column was washed with 0.3 $M$ LiOH for 2 h at 70° and regenerated at the same temperature with buffer of pH 2.75 for 1 h.

## RESULTS AND DISCUSSION

Since this work was originally intended for the separation of precursors of penicillin, which are mostly acidic peptides containing α-aminoadipic acid, the elution

system was adapted for optimal resolution in the first part of the chromatogram. The gradient of Vega and Nunn[5] was altered in three ways: 100 ml of buffer was placed in each chamber of the Autograd, a third buffer of pH 2.75 was used, and the content of the pH 6.50 buffer was lowered. For the same reason, the temperature was kept at 37° for 7.45 h, then increased to 55°.

The positions of all 145 ninhydrin-positive compounds on the chromatogram of resin I are shown in Fig. 1; the numbers associated with the various peaks refer to the compounds listed in Table II. As compared with the system of Vega and Nunn[5], the elution orders of cystine and valine, of phenylalanine and β-alanine and of the cystathionines and methionine are reversed; also, the total analysis time up to arginine is lengthened to over 25 h.

A number of compounds not tested by Vega and Nunn[5] were well separated. Allo-γ-hydroxyglutamic acid was eluted before allo-β-hydroxyglutamic acid. The three amino sugars tested (glucosamine, mannosamine and galactosamine) were well resolved. In addition, good separations were obtained for most of the diastereo-
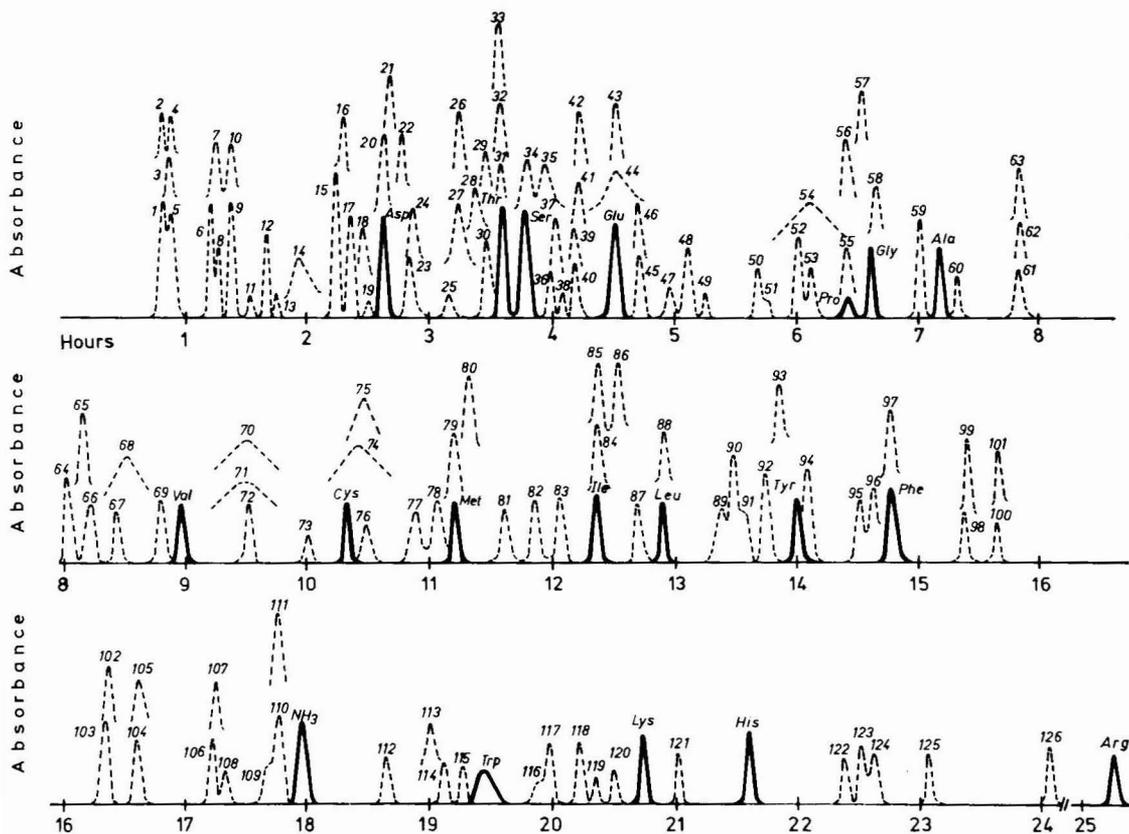


Fig. 1. Elution positions of 145 ninhydrin-positive compounds on resin I. The 18 physiological amino acids, and ammonia, are indicated by solid lines. For identification of numbered peaks (broken lines) see Table II.

TABLE II

IDENTIFICATION OF PEAKS IN FIG. 1

| Peak No. | Compound | Peak No. | Compound |
|---|---|---|---|
| 1 | Cysteic acid | 50 | Cysteine |
| 2 | Homocysteic acid | 51 | *threo*-Thiolbutyrine |
| 3 | Cysteinesulphinic acid | 52 | S-Methylcysteine |
| 4 | O-Phosphothreonine | 53 | $\alpha$-Aminoadipic acid |
| 5 | O-Phosphoserine | 54 | Glutathione (oxidized) |
| 6 | Taurine | 55 | *erythro*-Thiolbutyrine |
| 7 | Penicillaminic acid | 56 | S-Carboxymethylhomocysteine |
| 8 | *threo*-$\beta$-Hydroxyaspartic acid | 57 | $\beta$-Hydroxyleucine |
| 9 | Phosphoethanolamine | 58 | Penicillamine (reduced) |
| 10 | Levulinic acid | 59 | Isoserine |
| 11 | Dithiothreitol | 60 | Lanthionine (peak 1) |
| 12 | *erythro*-$\beta$-Hydroxyaspartic acid | 61 | Citrulline |
| 13 | Urea | 62 | Lanthionine (peak 2) |
| 14 | S-Carboxymethylglutathione | 63 | $\alpha$-Aminoisobutyric acid |
| 15 | Allo-$\gamma$-hydroxyglutamic acid | 64 | Glucosamine |
| 16 | S-Methylcysteine sulphoxides | 65 | S-Ethylcysteine |
| 17 | Allo-$\beta$-hydroxyglutamic acid | 66 | $\alpha$-Aminobutyric acid |
| 18 | Cephalosporin C | 67 | Mannosamine |
| 19 | 3-Hydroxypipecolic acid | 68 | Bis-$\gamma$-(L-glutamyl)-L-cystine |
| 20 | S-Carboxymethylcysteine | 69 | Galactosamine |
| 21 | S-Carboxymethylpenicillamine | 70 | Bis-$\delta$-(L-$\alpha$-aminoadipyl)-L-cystine |
| 22 | Diaminosuccinic acid (peak 1) | 71 | Bis-$\delta$-(L-$\alpha$-aminoadipyl)-L-cystinyl-bis-L-valine |
| 23 | Glutathione (reduced) | 72 | $\alpha$-Aminopimelic acid |
| 24 | S-Methylglutathione | 73 | Pipecolic acid |
| 25 | 4-Hydroxyproline | 74 | Bis-$\delta$-(L-$\alpha$-aminoadipyl)-L-cystinyl-bis-D-valine |
| 26 | Diaminosuccinic acid (peak 2) | 75 | 6-Aminopenicillanic acid |
| 27 | Penicilloic acid of isopenicillin N | 76 | Homocysteine |
| 28 | $\gamma$-(L-Glutamyl)-L-cysteine | 77 | Phenylglycine |
| 29 | Methionine sulphoxide (peak 1) | 78 | Homocitrulline |
| 30 | Methionine sulphone | 79 | Norvaline |
| 31 | Methionine sulphoxide (peak 2) | 80 | Mixed disulphide of L-cysteine and D-penicillamine |
| 32 | Allo-threonine | 81 | Allo-isoleucine |
| 33 | $\beta$-Hydroxyvaline | 82 | Ethionine |
| 34 | $\delta$-(L-$\alpha$-Aminoadipyl)-L-cysteine | 83 | Djenkolic acid |
| 35 | $\delta$-(L-$\alpha$-Aminoadipyl)-L-cysteinyl-L-valine | 84 | Penicillamine (oxidized) |
| 36 | O-Methylthreonine | 85 | Cystathionine |
| 37 | O-Methylserine | 86 | Allo-cystathionine |
| 38 | Allo-4-hydroxyproline | 87 | $\alpha$-Amino-$\beta$-hydroxybutyric acid |
| 39 | Muramic acid | 88 | 3,4-Dihydroxyphenylalanine |
| 40 | Asparagine | 89 | Isoglutamine |
| 41 | Allo-4-hydroxypipecolic acid | 90 | $\alpha,\varepsilon$-Diaminopimelic acid |
| 42 | S-Carbamylcysteine | 91 | Norleucine |
| 43 | $\beta$-Methoxyvaline | 92 | Cycloserine |
| 44 | $\delta$-(L-$\alpha$-Aminoadipyl)-L-cysteinyl-D-valine | 93 | $\alpha$-Amino-$\beta$-ethylvaleric acid |
| 45 | Glutamine | 94 | Mixed disulphide of L-cysteine and DL-homocysteine |
| 46 | Homoserine | 95 | $\beta$-Alanine |
| 47 | 4-Oxopipecolic acid | 96 | Mixed disulphide of DL-homocysteine and D-penicillamine |
| 48 | Sarcosine | 97 | O-Benzylserine |
| 49 | 5-Hydroxypipecolic acid | | |

TABLE II *(continued)*

| Peak No. | Compound | Peak No. | Compound |
|---|---|---|---|
| 98 | $\beta$-Aminoisobutyric acid | 112 | $\delta$-Aminovaleric acid |
| 99 | $\delta$-Aminolevulinic acid | 113 | Valinol |
| 100 | L-Cysteinyl-L-valine | 114 | 5-Hydroxylysine |
| 101 | L-Cysteinyl-D-valine | 115 | Allo-5-hydroxylysine |
| 102 | Argininosuccinic acid | 116 | Creatinine |
| 103 | Homocystine | 117 | $\alpha,\gamma$-Diaminobutyric acid |
| 104 | $\gamma$-Aminobutyric acid | 118 | Ornithine |
| 105 | S-Benzylcysteine | 119 | Valinamide |
| 106 | 5-Hydroxytryptophan | 120 | $\varepsilon$-Aminocaproic acid |
| 107 | $\alpha$-Aminocaprylic acid | 121 | 1-Methylhistidine |
| 108 | Ethanolamine | 122 | 3-Methylhistidine |
| 109 | Kynurenine | 123 | Carnosine |
| 110 | L-Cystinyl-bis-L-valine | 124 | Homocarnosine |
| 111 | L-Cystinyl-bis-D-valine | 125 | $\alpha$-Amino-$\beta$-guanidinopropionic acid |
| | | 126 | Homocysteine thiolactone |

isomers of amino acids and peptides tested: *e.g.*, *threo-* and *erythro-*$\beta$-hydroxyaspartic acid; hydroxyproline, isoleucine, 5-hydroxylysine and their allo-forms; *threo-* and *erythro-*thiolbutyrine and the LLL- and LLD-isomers of oxidized and reduced $\delta$-($\alpha$-aminoadipyl)-cysteinylvaline. However, some of the compounds were not resolved in our system. The strongly acidic amino acids (cysteic acid, homocysteic acid, phospho-serine and phosphothreonine) always eluted with the front. Identical elution times were obtained for threonine and allo-threonine, for aspartic acid and S-carboxymethyl-cysteine, for the second peak of lanthionine and $\alpha$-aminoisobutyric acid, for methionine and norvaline, for 3,4-dihydroxyphenylalanine and leucine, and for $\beta$-aminoiso-butyric acid and $\delta$-aminolevulinic acid. The LL- and LD-epimers of cysteinylvaline and of cystinyl-bis-valine always ran together.

The elution pattern with resin II differed from that with resin I in several respects. Reduced glutathione was eluted after aspartic acid on resin I, but the two were only marginally separated on resin II. Methionine sulphone and threonine were only separated with resin I. The resolution of $\delta$-(L-$\alpha$-aminoadipyl)-L-cysteine and the LLL-thiol tripeptide was better with resin I than with resin II. Glutamic acid and glutamine were well resolved on resin I, but had the same retention time on resin II. In contrast, the LLD-thiol peptide and glutamic acid were only separated on resin II. Also, separations of cystine and 6-APA, allo-isoleucine and ethionine, and 3-methyl-histidine and carnosine were better on resin I, whereas those of citrulline and lanthio-nine (peak 2), galactosamine and valine, homocitrulline and methionine, $\alpha,\varepsilon$-diamino-pimelic acid and norleucine, and O-benzylserine and phenylalanine were better on resin II. The differences between the two resins remained constant, although both columns were re-packed several times. Differences in separation on the two resins can be advantageous for the identification of unknown compounds; in this way, gluta-thione and the LLD-thiol tripeptide (labelled with sulphur-35) could be detected in cultures of *Penicillium chrysogenum*[1].

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## Note

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## Thin-layer chromatographic analysis of oxo and thio compounds

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When aliphathic alcohols are oxidized with silver compounds and bromine[1-4], the products are ketones and tetrahydrofurans. Our present study has indicated that significant quantities of other products such as aldehydes and acids may result from oxidation of cyclic alcohols. Gas–liquid chromatography (GLC) is an ideal technique for the determination of ketones and some aldehydes. Acids, however, are very difficult to analyze by GLC unless they are derivatized first[5-8]. In general, in the published thin-layer chromatographic (TLC) methods[9-13] ketones are derivatized by a reaction with 2,4-dinitrophenylhydrazine before or during development.

The aim of this study was to develop a TLC procedure that would be sensitive enough to detect ketones and possibly acids and aldehydes without prior derivatization. After unsuccessfully testing 22 different chromogenic reagents that had been reported previously for conjugated and specific ketones, we turned our attention to 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INPTC) as a possible chromogenic reagent. This compound is sensitive to ketones, aldehydes and acids as indicated by tests performed on 26 compounds containing carbonyl or thio-carbonyl groups.

### EXPERIMENTAL

*Materials*

The solvents used were purchased from either Burdick & Jackson Labs., (Muskegon, Mich., U.S.A.) or Fisher Scientific (Silver Spring, Md., U.S.A.). The ketonic pesticides and metabolites were obtained from the Pesticide Reference Standard Section, Registration Division, Environmental Protection Agency (Washington, D.C., U.S.A.). All of the remaining ketones, acids and aldehydes were obtained from commercial sources.

The TLC plates (aluminum oxide and silica gel) with a thickness of 100 $\mu$m and dimensions 20 × 20 cm were obtained from J. T. Baker (Phillipsburg, N.J., U.S.A.).

The chromogenic reagent was prepared by dissolving 0.5 g INPTC in 100 ml of methanol. The basic spraying solution was prepared by dissolving 5 g of potassium hydroxide in 10 ml of water in a 100 ml volumetric flask and then diluting to volume with methanol.

*Thin-layer chromatography*

About 0.1 to 1.0 g of each ketone, acid or aldehyde was placed in separate 10-ml volumetric flasks and then diluted to volume. Water was used for the dilution of ethyleneurea and hydantoin, methanol for kepone and ethylenethiourea, and methylene chloride for 4-methylcyclohexanone, ordram, cycloheximide and 4,4'-bis(dimethylamine) benzophenone. All of the remaining compounds were diluted with hexane. Using a template, the compounds were applied as spots at 2 cm intervals on an imaginary line 1 in. from the bottom of the TLC plate. Another line was drawn across the plate 12 cm above this line to mark the solvent front for propanol-2–isooctane (1:3). A line at 10 cm was used for the other solvent systems.

A TLC chamber (4.5 × 23 × 23 cm) was lined with blotting paper and saturated with the developing solvent mixtures. About 25 ml of the developing solvent were poured into a trough at the bottom of the tank. The plates were then developed until the solvent front reached the drawn line (about 30 min). The plate was air dried for 5 min in a hood, and then sprayed with INPTC. The plate was then allowed to stand in the hood for 5 min and then heated for 5 min at 75° in an oven. Characteristic colored spots were observed as indicated in Tables I and II. When the plate was sprayed with a basic solution, the spots changed colors as indicated in Tables I and II. Fig. 1 represents several ketones including some with ketonic impurities also detected by the procedure.
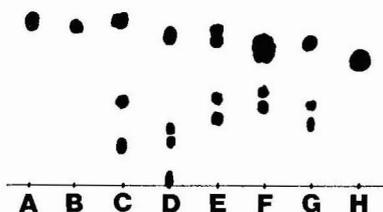


Fig. 1. TLC on silica gel. Solvent system, acetonitrile–water (9:1). A = 0.5 mg pentanone-2; B = 0.5 mg pentanone-3; C = 0.5 mg cyclobutanone; D = 0.5 mg cyclopentanone; E = 0.25 mg cyclohexanone; F = 0.25 mg 4-methylcyclohexanone; G = 0.25 mg 3-methylcyclohexanone; H = 0.25 mg octanone-2. Note: commercial products contained impurities.

RESULTS AND DISCUSSION

There are three major problems in the TLC of many low-molecular-weight compounds: volatility, unsatisfactory color formation and solubility. Low-molecular-weight samples are generally more soluble than high-molecular-weight samples. We felt that there is enough affinity between the low-molecular-weight compounds and aluminum oxide and silica gel adsorbents to make TLC analyses possible, provided that a suitable mobile phase is selected. For example, when the propanol-2–isooctane (1:3) mixture was used as the mobile phase for 3-pentanone, the ketone could not be detected. With hexane–isooctane (1:1), a streak was observed extending from the origin to about 60 mm. However, when the polar solvent mixture acetonitrile–water (9:1) was used, a compact spot with a $R_F$ value of 0.66 was observed.

To find a sensitive and practical chromogenic reagent for low-molecular-weight non-conjugated aliphatic and cyclic ketones has always been a problem. In fact, we tested 22 different chromogenic reagents, including many which had been reported as

TABLE I

TLC OF ALIPHATIC AND CYCLIC KETONES, ETHYLENETHIOUREA AND SOME
ACIDS AND ALDEHYDES ON ALUMINA SHEETS

Solvent systems: A, propanol-2–isooctane (1:3); B, acetonitrile–water (9:1). Distance developed:
A = 12 cm, B = 10 cm. ND = Not detected; NA = not analyzed.

| Compound | Amount $(\mu g)^{\star}$ | $R_F$ | | Color observed | |
|---|---|---|---|---|---|
| | | A | B | INPTC | KOH |
| Pentanone-2 | 400 | ND | 0.84 | pink | blue |
| Pentanone-3 | 400 | origin | 0.69 | pink | blue |
| Cyclopentanone | 400 | ND | 0.76 | pink | blue |
| 4-Methylcyclohexanone | 200 | 0.67 | 0.83 | pink | greenish blue |
| Octanone-2 | 200 | 0.54 | 0.77 | pink | yellowish green |
| Menthone | 200 | 0.65 | 0.82 | yellow | yellowish orange |
| 3-Methylcyclohexanone | 200 | p.63 | 0.81 | pink | blue |
| 5-Methyloctanone-2 | 200 | 0.69 | 0.77 | pink | blue |
| Cyclooctanone | 100 | 0.68 | 0.80 | yellow | green |
| Cyclohexanone | 100 | 0.57 | 0.82 | pink | blue |
| Cholestan-6-one | 60 | 0.64 | NA | pink | blue |
| 4-Androsten-17$\beta$-<br>ol-3-one-17-acetate | 40 | 0.59 | 0.79 | pink | blue |
| Decachlorooctahydro-1,3,4-<br>metheno-2H-cyclobuta($cd$)-<br>pentalen-2-one | 20 | origin | NA | pink | blue |
| 4,4'-bis(dimethylamine)<br>benzophenone | 100 | origin<br>to 40 mm | origin<br>to 80 mm | pink | blue |
| Cyclobutanone | 500 | NA | 0.76 | pink | blue |
| 2-Ethylhexanoic acid | 150 | NA | origin<br>to 25 mm | pink | blue |
| $n$-Heptanoic acid | 150 | NA | origin<br>to 10 mm | pink | blue |
| $n$-Heptaldehyde | 150 | NA | 0.80 | pink | blue |
| S-Ethylhexahydro-<br>1H-azepine-1-carbothioate | 60 | NA | 0.78 | pink | blue |
| Hydantoin | 20 | origin | NA | pink | blue |
| Ethylene thiuram monosulfide | 2 | NA | 0.63 | pink | blue |

$^{\star}$ Amount easily detectable.

useful for conjugated ketones. In most cases, large sample sizes were needed and in some instances even with large samples, certain ketones would not yield a color reaction.

However a color reaction was possible with the chromogenic reagent INPTC and as little as 20 $\mu$g could be detected as indicated in Tables I and II. For spot testing the amounts can be about ten times lower. For example 10 $\mu$g of cyclohexanone is detectable by spot testing.

To gather more information on the specificity of the procedure, several cyclo-alcohols were included in some of the TLC experiments. The alcohols were not detectd by the INPTC spray as illustrated in Fig. 2 (areas F and G).

The characteristic color for each compound after spraying with INPTC is shown in the tables. For cyclooctanone, a yellow spot was observed after the INPTC spraying, whereas for cyclohexanone, a pink spot is observed. The results have triggered our interest in the nature of the product formed on TLC plates by INTPC and

TABLE II

TLC OF ALIPHATIC AND CYCLIC KETONES, ETHYLENETHIOUREA AND SOME ACIDS AND ALDEHYDES ON SILICA GEL SHEETS

Solvent systems: A, propanol-2–isooctane (1:3); B, acetonitrile–water (9:1); C, hexane–isooctane (1:1). Distance developed: A = 12 cm, B = 10 cm, C = 10 cm. ND = Not detected; NA = not analyzed.

| Compound | Amount (µg) | $R_F$ | | | Color observed | |
|---|---|---|---|---|---|---|
| | | A | B | C | INPTC | KOH |
| Pentanone-2 | 200–500 | ND | 0.68 | ND | pink | blue |
| Pentanone-3 | 200–500 | ND | 0.66 | origin to 60 mm | pink | blue |
| Cyclopentanone | 100–500 | ND | 0.58 | 0.63 | pink | blue |
| 4-Methylcyclohexanone | 100–500 | 0.50 | 0.58 | 0.09 | pink | blue |
| Octanone-2 | 200–250 | 0.40 | 0.52 | 0.23 | pink | blue |
| Menthone | 100–300 | 0.18 | 0.84 | 0.30 | pink | blue |
| 3-Methylcyclohexanone | 100–250 | ND* | 0.58 | 0.50 | pink | blue |
| 5-Methyloctanone-2 | 250 | NA | 0.48 | 0.22 | pink | blue |
| Cyclooctanone | 100–150 | 0.46 | 0.86 | 0.48 | greenish yellow | yellow |
| Cyclohexanone | 100–250 | 0.52 | 0.65 | 0.55 | pink | blue |
| Cholestan-6-one | 60–100 | 0.48 | origin to 80 mm | 0.55 | pink | green |
| 4-Androsten-17β-ol-3-one-17-acetate | 40–60 | 0.41 | 0.83 | 0.25 | dark pink | reddish brown |
| Decachlorooctahydro-1,3,4-metheno-2H-cyclobuta(cd)-pentalen-2-one | 20–40 | origin | 0.79 | NA | no color | pink |
| 4,4′-bis(dimethylamine) benzophenone | 100–150 | NA | origin to 85 mm | origin | pink | blue |
| Cyclobutanone | 500 | NA | 0.15 | ND | pink | blue |
| 2-Ethylhexanoic acid | 200 | NA | 0.71 | 0.38 | no color | blue |
| n-Heptanoic acid | 200 | NA | 0.69 | 0.28 | no color | pink |
| n-Heptaldehyde | 200 | NA | 0.81 | 0.52 | pink | orange |
| S-Ethylhexahydro-1H-azepine-1-carbothioate | 50–60 | NA | 0.84 | 0.40 | pink | dark pink |
| Ethyleneurea | 40 | NA | 0.43 | NA | pink | purple |
| Ethylenethiourea | 40 | NA | 0.67 | NA | pink | purple |
| Endrin ketone | 40 | NA | 0.79 | NA | no color | pink |
| Cycloheximide | 40 | NA | 0.70 | NA | pink | brown |
| n-Butyraldehyde | 80 | NA | 0.85 | NA | pink | orange |

* None detected at low level.



**A  B  C  D  E  F  G**
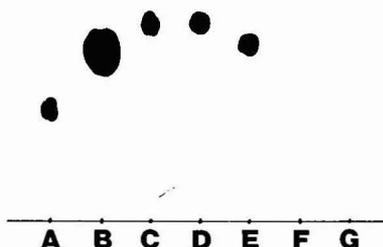
Fig. 2. TLC on silica gel. Solvent system, acetonitrile–water (9:1). A = 40 µg ethyleneurea; B = 40 µg ethylenethiourea; C = 40 µg endrin ketone; D = 40 µg kepone; E = 40 µg cycloheximide; F = 0.3 mg cyclohexanol; and G = 0.3 mg 3-methylcyclohexanol.

the carbonyl compound. Initial studies indicate that the solid adsorbent is a necessary component of the reaction and that different types of product are formed in the various cases. Preliminary evidence suggests that the highly colored compounds are 1:1 adducts, whereas the carbonyl compounds forming yellow products are of an entirely different nature. Studies are currently underway to determine the structure of these products.

## ACKNOWLEDGEMENTS

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## Note

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## Liquid chromatography assay of the calcium salt of citrovorum factor

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The calcium salt of citrovorum factor (1, CF·Ca) has been found to reverse the toxicity associated with a high dose of methotrexate (2) in the treatment of osteogenic sarcoma[1]. The high cost of purification of the salt by column chromatography prompted the investigation of the use of partially purified, but well characterized, samples of the salt in biological studies. Previously, a high-performance liquid chromatography (HPLC) method was described for the identification of CF and the impurities generated during its preparation[2]. These impurities included the pterins, *p*-aminobenzoylglutamic acid (PABGA), 10-formyl-7,8-dihydrofolic acid (10-CHO-DHF), and 10-formylfolic acid (10-CHO-FA). In this system, however, the CF and 10-CHO-FA peaks overlapped and limited the usefulness of the procedure for the determination of the amounts of these two components. In contrast, the use of a microparticulate reversed-phase packing with a mobile phase consisting of pH 4 citrate buffer and dioxane gave baseline separations for 10-CHO-DHF, 10-CHO-FA, and CF. This system coupled with elemental analyses provided a method for the assay of CF·Ca samples.



R = 4-C$_6$H$_4$CONHCH(CO$_2$H)CH$_2$CH$_2$CO$_2$H
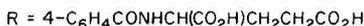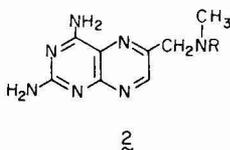
## EXPERIMENTAL

### Liquid chromatography

All separations were carried out with an ALC-242 liquid chromatograph equipped with a UV detector (254 nm), an M-6000 pump, and a column (30 cm × 4 mm I.D.) packed with μBondapak C$_{18}$ (Waters Assoc., Milford, Mass., U.S.A.) at room temperature with an eluting solvent of pH 4 citrate buffer–dioxane (94:6) (Burdick & Jackson, Muskegon, Mich., U.S.A.) at a flow-rate of 1 ml/min. All chromatograms were recorded at a chart speed of 1 cm/min and an attenuation of 16 a.u.f.s. at a pressure of about 1000 p.s.i. Solutions of the samples were injected with

a Hamilton Model 701 syringe (10 $\mu$l capacity, 0.0185 in. O.D. needle). For the preparation of the buffer, citric acid monohydrate (21.01 g) and sodium citrate dihydrate (29.41 g) were dissolved separately in distilled water (1000 ml). After filtration of each solution through a Millipore filter (type HAWP04700, 0.45 $\mu$m), a mixture of the citric acid solution (330 ml) and the sodium citrate solution (170 ml) was diluted to 1000 ml with filtered water.

*Standard compounds*

Pterin was purchased from Aldrich (Milwaukee, Wisc., U.S.A.), and pterin-6-methanol was prepared from 2,4,5-triaminopyrimidin-6(1H)-one and 1,3-dihydroxy-acetone[3]. The calcium salts of PABGA and 10-CHO-FA were prepared in an aqueous solution and precipitated by the addition of ethanol. Reaction of 5,10-methenyl-5,6,7,8-tetrahydrofolic acid chloride with aqueous base (pH 11) at room temperature gave mainly 10-formyl-5,6,7,8-tetrahydrofolic acid and trace amounts of CF, 10-CHO-DHF, PABGA, and pterins. Further treatment of this sample in water (pH 7.4) in the presence of air resulted in a lowering of the pH to 6.7 and the precipitation in low yield of a 2:1 mixture of the calcium salts of 10-CHO-DHF and 10-CHO-FA. In contrast, treatment of a neutral, aqueous solution of the methenyl compound at 100° gave the impure CF·Ca, which was purified by Florisil chromatography[4].

*Standard curves*

Stock solutions were prepared in volumetric flasks by dissolving weighted samples of the calcium salts of PABGA, 2:1 10-CHO-DHF:10-CHO-FA, 10-CHO-FA, and CF in distilled water. Aliquot portions from each stock solution were diluted in volumetric flasks to give a series of concentrations from which the chromatograms were determined on 10-$\mu$l portions. These operations were carried out as rapidly as possible to minimize errors resulting from oxidative decomposition of the samples. Standard curves were obtained by plotting either peak height (PABGA) or peak area (peak height $\times$ peak width at one-half peak height) *versus* the equivalent amount of the *anhydrous* calcium salt injected. In the preparation of the standard curve for 10-CHO-DHF, the amount of 10-CHO-FA in each dilution was determined from its standard cuvre and subtracted from the weight of the mixture. The standard curves were linear in the following concentration ranges: PABGA·Ca (0–0.6 $\mu$g), 10-CHO-DHF·Ca (0–0.5 $\mu$g), 10-CHO-FA·Ca (0–0.4 $\mu$g), and CF·Ca (0–7 $\mu$g). Before an assay, the standard curves were checked by injection of a known amount of a fresh solution of the standards. Over a two-day period, an error of less than 5% was observed.

RESULTS AND DISCUSSION

A sample of unpurified CF·Ca was dried to constant weight *in vacuo* over $P_2O_5$ and analyzed for C, H, N, Ca, ash (CaO). This sample dissolved in water gave the chromatogram shown in Fig. 1. The amount of each identifiable component was determined from a chromatogram in which the sample weight was 10.23 $\mu$g (Table I). The results indicated that this sample contained about 78% CF·Ca, which on an anhydrous basis corresponded to about 86% CF·Ca and 14% impurities. The only major unidentified component ($<$3%) appeared as a shoulder on the side of the
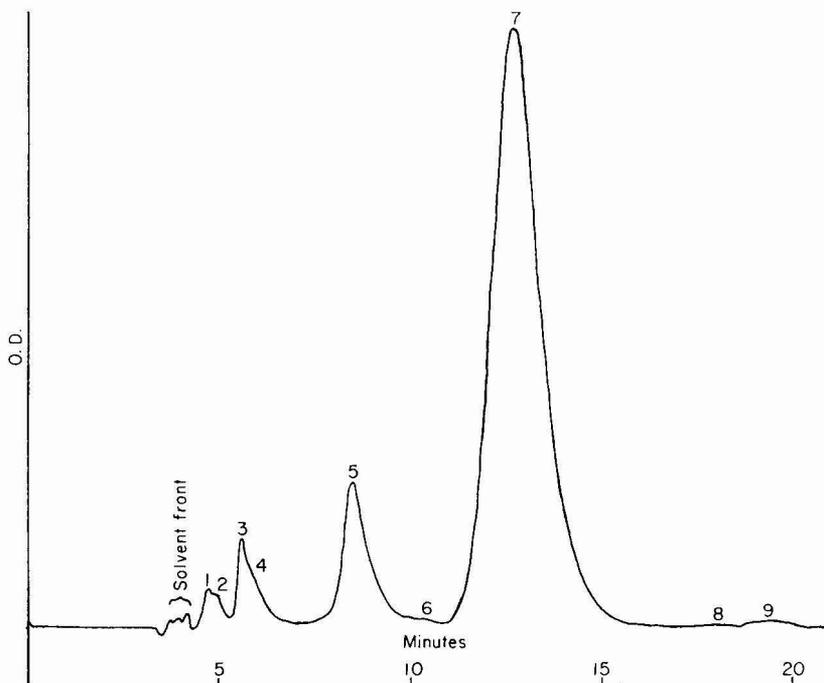
Fig. 1. HPLC of unpurified CF·Ca. Solvent: pH 4 citrate buffer–dioxane (94:6); sample weight, 8.26 μg (in water). 1 = Pterin-6-methanol; 2 = pterin; 3 = p-aminobenzoyl-L-glutamic acid; 4 = unidentified; 5 = 10-formyl-7,8-dihydrofolic acid; 6 — 10-formylfolic acid; 7 = CF; 8 and 9 = unidentified.

TABLE I

HPLC ASSAY RESULTS

This assay represents an analysis of a solution of the sample because the presence of either 10-formyl-5,6,7,8-tetrahydrofolic acid or 5, 10-methenyl-5,6,7,8-tetrahydrofolic acid in the solid would probably be detected as 10-formyl-7,8-dihydrofolic acid in the solution.

| Components* | Amount (%) |
|---|---|
| Anhydrous calcium salts | |
|   p-Aminobenzoyl-L-glutamic acid | 3.1 |
|   10-Formyl-7,8-dihydrofolic acid | 4.6 |
|   10-Formylfolic acid | <0.5** |
|   Citrovorum factor | 78 |
| Pterins | |
|   Pterin-6-methanol | 0.6** |
|   Pterin | 0.4** |
| Other components*** | |
|   Ethanol | 4.1 |
|   Water | 5.7 |
| Unidentified material | |
|   Peaks 4, 8, and 9 (Fig. 1) and undetected material | 3.0 |

    * Elemental analysis of the impure CF sample gave the following results: Calcd. for $C_{20}H_{21}N_7O_7 \cdot Ca \cdot 0.5C_2H_6O \cdot 1.8H_2O$ (MW 567): C, 44.49; H, 4.91; N, 17.29; Ca, 7.07; ash (CaO), 9.89. Found: C, 44.49; H, 4.97; N, 17.29; Ca, 7.18; ash (CaO), 9.65.
    ** Estimated from peak height.
    *** Calculated from elemental analyses.
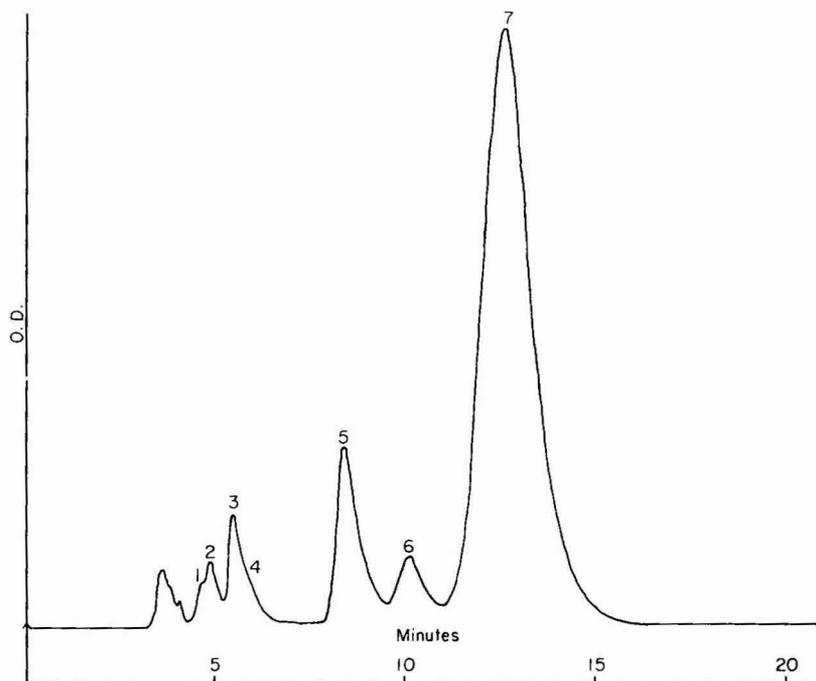
Fig. 2. HPLC of a mixture of components at concentrations similar to those found in Fig. 1. Solvent: pH 4 citrate buffer–dioxane (94:6). 1 = Pterin-6-methanol (0.059 $\mu$g); 2 = pterin (0.056 $\mu$g); 3 = calcium $p$-aminobenzoyl-L-glutamate (0.197 $\mu$g); 4 = unidentified; 5 = calcium 10-formyl-7,8-dihydrofolate (0.403 $\mu$g); 6 – calcium 10-formylfolate (0.207 $\mu$g); 7 = CF·Ca (6.82 $\mu$g).

PABGA peak. Although the error for each component is considered to be within ±5%, a lower error should be obtainable if the assay were used routinely.

Peak assignments were confirmed by the determination of the chromatogram of a mixture of the components (Fig. 2) at concentrations similar to those found in the assayed sample (Fig. 1). In the chromatogram of the known mixture, the peak area was 27% high for PABGA probably because this compound was present in small amounts in many of the other components of the mixture. This CF sample was as effective as a commercial sample in reversing the toxicity of a high dose of methotrexate in mice.

ACKNOWLEDGEMENT

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## Note

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## Separation of naturally occurring adenine nucleosides and nucleotides by anion-exchange chromatography

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Since the discovery of poly(A) sequences at the 3′ ends in mRNA[1], an intensive study of poly(A) metabolism has been made both at the analytic and enzymic levels. The poly(A) sequence is usually obtained by digestion of the purified mRNA with RNase A and $T_1$ (ref. 2). Recently, there have been some investigations on the enzymes which hydrolyze poly(A); the determination of the substrate specificity of these enzymes both for exoribonucleases[3] and for endoribonucleases[4–7] depends on methods available for the characterization of the products. In the present study we describe a chromatographic technique which could be used for the separation and identification of adenine nucleosides and nucleotides formed after enzymatic hydrolysis of poly(A). The basic principles of this method were described some time ago[8,9].

### EXPERIMENTAL

Anion-exchange chromatography of synthetic mixtures of adenine nucleosides and nucleotides was carried out on DEAE-Sephadex A-25 (particle size, 40–120 $\mu$m; Pharmacia, Uppsala, Sweden). Adenosine, 2′,3′-AMP, 5′-AMP, ADP and ATP were obtained from Boehringer (Mannheim, G.F.R.), 2′-AMP and 3′-AMP from Serva (Heidelberg, G.F.R.) and 1-methyladenosine (1-m-Ado) and $N^6$-m-Ado from P-L Biochemicals (Milwaukee, Wisc., U.S.A.). A column (27 × 0.8 cm) with a cooling jacket was used for the separation. The DEAE-Sephadex gel was equilibrated with 0.05 $M$ ammonium bicarbonate (pH 8.0). An amount of 1.0 ml of a mixture containing 100 $\mu$g of each of the nucleosides and nucleotides was applied to the column. After washing with the equilibration buffer, the column was eluted with a linear salt-and-pH gradient (200 ml) of 0.05 $M$ $NH_4HCO_3$ (pH 8.0) to 0.4 $M$ $NH_4HCO_3$ (pH 9.0, adjusted with ammonia). Fractions of 3 ml were collected; the flow-rate was 6 drops/min. The column was monitored with an Uvicord II at 254 nm.

### RESULTS AND DISCUSSION

A typical elution profile of the chromatographic separation at 20° on DEAE-Sephadex A-25 with a linear salt-and-pH gradient is shown in Fig. 1. The eluted nucleosides and nucleotides could be identified on the basis of their previously deter-
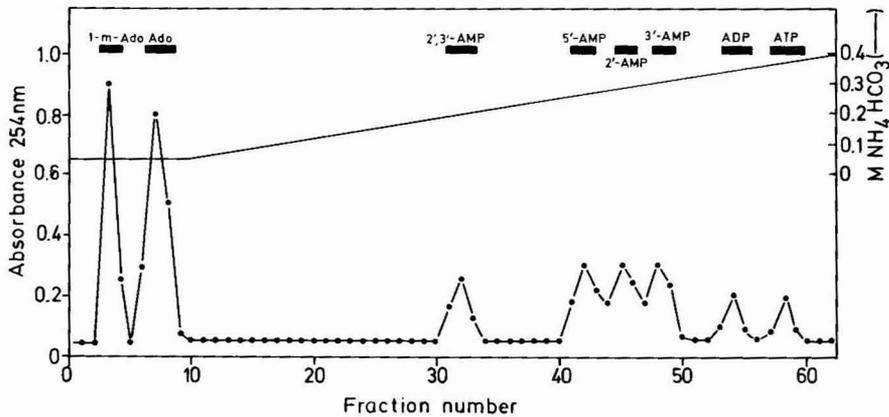
Fig. 1. Separation of adenine nucleosides and nucleotides on DEAE-Sephadex A-25 in an $NH_4HCO$ gradient. The solid bars indicate the positions of the authentic compounds.

mined elution positions. In the presence of 0.05 $M$ $NH_4HCO_3$ the nucleotides are bound to the anion exchanger, while the nucleosides are eluted. The first peak contains 1-m-Ado, whereas $N^6$-m-Ado is always eluted at the same position as Ado. The nucleosides can be recovered by increasing the concentration of $NH_4HCO_3$. For the separation of the monophosphates it was necessary to combine the use of the salt with a pH gradient. Under these conditions a resolution of 2′,3′-AMP, 5′-AMP, 3′-AMP and 2′-AMP was obtained. The separation of these monophosphates allows identification of each suspected product of a poly(A)-degrading exoribonuclease. The advantage of the volatile bicarbonate buffer system used is the possibility of converting the separated nucleic acid component into a salt-free state[8].

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**Note**

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## Chromatography of natural phenolic cinnamate derivatives on Sephadex LH-20 and G-25

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Extracts from higher plants contain mixtures of phenolic cinnamic acid derivatives, often of the chlorogenic acid type. In the course of efforts to apply chromatography on Sephadex to separation of these compounds from natural samples, difficulties were encountered with peak shape, purity and variability. Substances of the chlorogenic acid type were adsorbed from water and were generally well separated by continued elution with water from preceding and following impurities lacking the typical ultraviolet (UV) absorption spectra of cinnamates. Individual chromatographic peaks were obtained for cinnamate derivatives, but they were often slow to reach maximal absorbance with an abrupt decline after the maximum. When rechromatographed, peak fractions expected to be pure were contaminated by cinnamates concentrated in other peaks and, depending upon conditions, multiple peaks in various quantitative proportions could be produced from the same fraction.

Reasons for such behaviour have been clarified by this research and the value of Sephadex chromatography to study *cis–trans* isomerism in these compounds illustrated.

EXPERIMENTAL

Sephadex LH-20 or G-25, as obtained from Pharmacia (Uppsala, Sweden), was swelled in water for at least one hour and slurry-packed into an LKB 4200 precision column, 12 × 300 mm, under a flow-rate of about 0.5 ml/min produced by an LKB varioperpex 12000 peristaltic pump to give a packed length of about 240 mm. Effluents were monitored at 280 nm with an LKB Uvicord II 8300 having a 3-mm light-path flow cell and recorded by an LKB type 6520-6 recorder, but converted to absorbance by an Infometrics PSU 15/50 Linalog converter giving 0.5, 1.0 or 2.0 A full scale.

A repurified commercial sample of 5-chlorogenic acid and 4-p-coumaroyl-quinic acid isolated from apples[1] were used as the test compounds. UV irradiation

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was by a 125-W Hanovia medium pressure Hg arc lamp. Absorption spectra were obtained with a Unicam SP-800B spectrophotometer.

RESULTS AND DISCUSSION

Fig. 1 shows the elution chromatograms obtained when equal portions of the same solution of 4-*p*-coumaroylquinic acid were developed on Sephadex LH 20 with water as the eluent. The freshly dissolved crystalline material (*trans*) in A and the isomerized (*cis* + *trans*) in B. The characteristically skewed curves are shown plus evidence of partial separation of the *cis* and *trans* isomers. Addition of 0.2 % (v/v) of glacial acetic acid in the eluting solvent gave the comparable chromatograms shown in Fig. 2. Note that the *trans* derivative now produces a slower eluting, Gaussian-shaped peak (Fig. 2A) and the *cis* form produced by irradiation is completely separated (Fig. 2B, baseline between) from the *trans*. These columns, the exit tubing and the receivers were completely darkened during the run by covering with aluminium foil. That the earlier peak is the *cis* form and the later the *trans* was verified by the absorption spectra (*cis* maximum 5 nm shorter wavelength and extinction about 50 % of the *trans*[1,2]) and paper chromatography in 2 % acetic acid of the separated fractions.
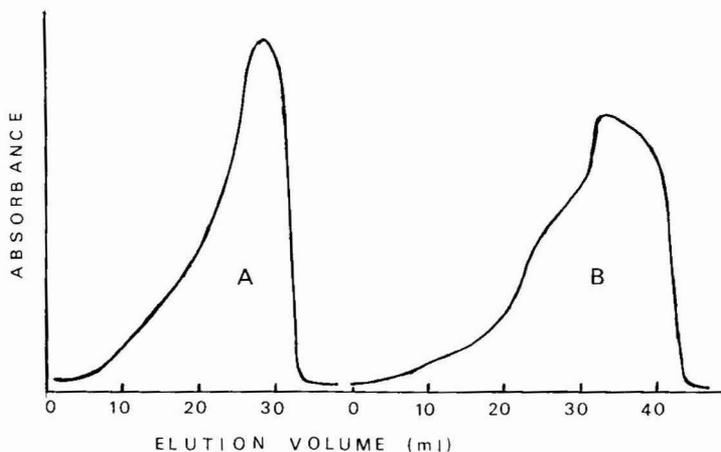


Fig. 1. 4-*p*-Coumaroylquinic acid chromatographed in the dark on Sephadex LH 20 developed with water. A, *trans*-4-*p*-coumaroylquinic acid (2 mg/ml); B, an equal portion of the same solution as in A UV irradiated during 30 min.

The exposure of the *trans*-4-*p*-coumaroylquinic acid in dilute solution in ordinary laboratory light for 9 h (on a cloudy day and with only about 2 h of artificial lighting) gave apparently complete isomerization as indicated by a chromatogram very nearly identical to Fig. 2B and by the lack of further change in the absorption spectrum of the solution upon UV irradiation.

The effect of the acetic acid in the eluting solvent is believed to be primarily to suppress ionization of the quinic acid carboxyl, a principle well established in chromatography. However, the results indicate that the carboxy anion form is considerably less strongly adsorbed on Sephadex LH 20 than is the un-ionized carboxyl.
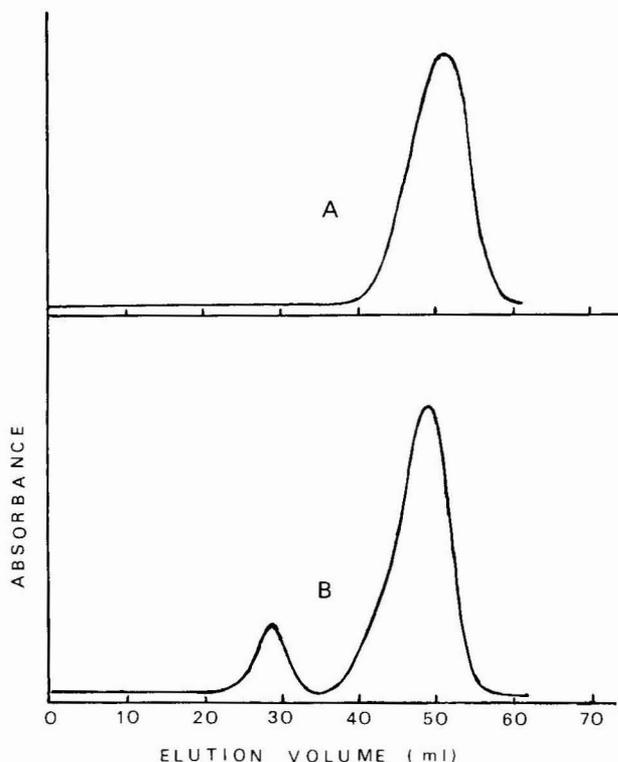
Fig. 2. 4-*p*-Coumaroylquinic acid, (A) *trans* and (B) *cis* + *trans*, chromatographed in the dark on Sephadex LH-20 and developed with 0.2% acetic acid in water.

A hydrogen-bonding mechanism is suggested with the acidic carboxyl hydrogen forming an especially strong bond, presumably with the oxygen H-bond acceptors in the Sephadex. The effect of the acetic acid is not limited to this, however, because it should also suppress the conversion of the *trans* forms to the *cis* forms. It has been shown that in the cinnamate series the equilibrium favours the *trans* form more at low pH than at pH 5–7[3]. This can be a factor even during a column run if the column is not protected from light. Fig. 3 shows that an isomerized *cis–trans* mixture did not resolve when the column was continuously irradiated but rather the continued isomerization gave a broader, flatter peak representing the average mobility of the rapidly intraconverting two forms.

     A third benefit of the acetic acid should be the conversion of any salts of the phenolic quinates and related compounds to the free acids. In fact, we have found with certain plant extracts that acetic acid appears insufficiently acidic to quickly displace all the complexes and therefore recommend addition only to the first void-volume of the eluent of 0.05 N HCl or other strong acid. Without this the major peaks are unaffected, but more rapidly moving (highly water-soluble), cinnamate-bearing peaks may occur which appear to be base-salts of the acid function of the cinnamoyl ester (quinate, etc.) rather than additional parent cinnamate derivatives.

     Similar ability to resolve the *cis* and *trans* forms of 5-chlorogenic acid was found and Fig. 4 shows a chromatogram of a mixture of the *cis* and *trans* isomers of
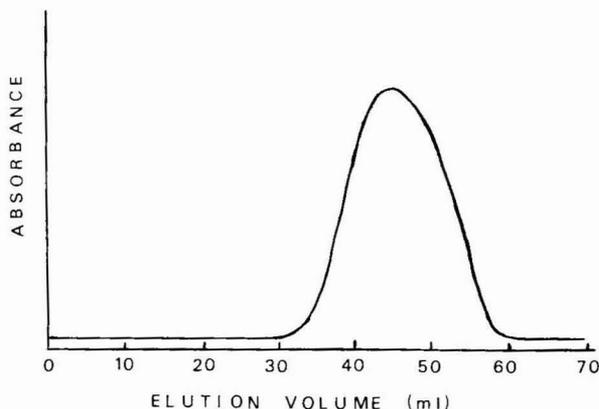
Fig. 3. Chromatogram of 4-*p*-coumaroylquinic acid under conditions identical to Fig. 2B except that the column was continuously irradiated with UV light during the run.

4-*p*-coumaroylquinic acid and 5-chlorogenic acid. Note that the separation is more greatly affected by *cis* or *trans* rather than by an additional phenolic OH-group with the result that the elution sequence was the two *cis* forms followed by the two *trans* forms. It thus becomes evident why a peak fraction from a chromatogram not protected from light and especially if run without suppression of carboxyl ionization often contains other members of the phenolic cinnamoyl family of compounds; the *cis* or *trans* form of homologues may overlap, equilibria can occur on the column and the slowly rising leading edge can cause overlap during a considerable elution volume. This isomerization in the sample or on the column by light may also help explain observed difficulties in reproducing chromatograms from the same plant extract sample.

Chromatograms run on Sephadex G-25 of similar samples showed similar effects —the *cis* forms preceded and were well separated from the *trans* forms, the *cis–trans* effect on separation was larger than the effect of one more phenolic OH, the
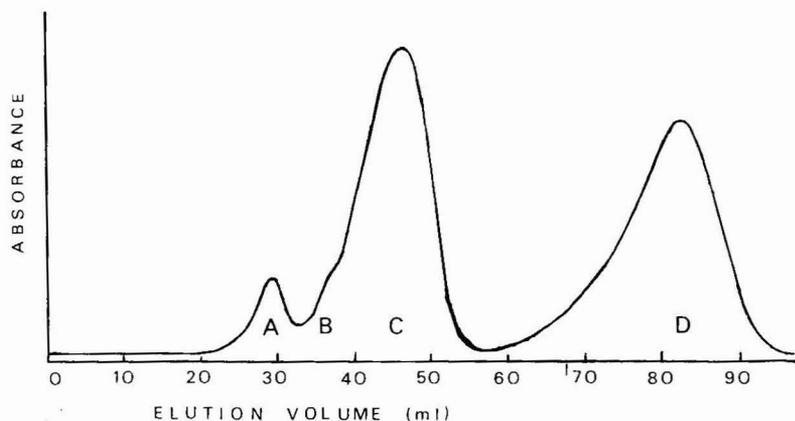


Fig. 4. Chromatography in the dark on Sephadex LH-20 with 0.2 % acetic acid in water of a mixture of (A) *cis*- and (C) *trans*-4-*p*-coumaroylquinic acid and (B) *cis*- and (D) *trans*-5-chlorogenic acid.

sequence of elution with dilute acid was similar and with both adsorbents the free acids were held tighter than the quinate esters; *e.g.*, caffeic followed chlorogenic acid.

Chromatography on Sephadex LH 20 or G 25 with proper attention to the effects of light and acidic aqueous eluents can be, therefore, not only a very satisfactory technique for isolation and purification of chlorogenic-like compounds, but clearly it can also be used to study the *cis* and *trans* isomerization. We have been able to produce separate crystalline *cis* and *trans* derivatives of *p*-coumaroyltartaric acid by separation on LH 20 and concentration etc. in the dark. As far as we are aware this has not been done before since crystallization from an equilibrating mixture produced the *trans* forms of these compounds. Other systems, of course, will separate *cis* and *trans* forms of such compounds, but none seems to be as effective and as simple. Paper chromatography in aqueous solvents is low in capacity and perhaps more difficult to protect from light. Silica gel chromatography does not seem to separate certain pairs[4] and gas chromatography requires derivative formation plus smaller samples[5].

The method can be used to get very pure individual compounds by taking advantage of their *cis–trans* isomerization. For example, *cis*-chlorogenic acid isolated in the dark as a simple peak can be isomerized and rechromatographed to separate the *trans* form. Since the newly formed *trans* isomer would now fall in a portion of the chromatogram known to have been "base-line empty", it should be free of contamination.

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## Note

Separation of 4-N,N-dimethylaminoazobenzene-4'-thiohydantoins of amino acids by thin-layer chromatography on silica gel

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We have developed a sensitive coloured reagent 4-N,N-dimethylaminoazo-benzene-4'-isothiocyanate (DABITC) for the sequence determination of peptide and proteins[1,2]. The identification of the released N-terminal amino acids as 4-N,N-dimethylaminoazobenzene-4'-thiohydantoins (DABTH-AAs) on polyamide sheets (2.5 × 2.5 cm) is sensitive and efficient and has so far been the preferred method for the identification of DABTH-AAs. Two shortcomings in the chromatography of DABTH-AAs on polyamide have become apparent; DABTH-Leu and DABTH-Ile remain unresolved; and recovery of DABTH-AAs from the polyamide sheets is difficult unless a strong acidic solvent [such as 6 *N* HCl–ethanol (1:2)] is used to dissolve both DABTH-AAs and the polyamide simultaneously[3].

We report here the use of silica gel for the separation of DABTH-AAs. A satisfactory resolution of DABTH-Leu and DABTH-Ile is obtained. The recovery and quantitation of DABTH-AAs could be achieved by extracting DABTH-AAs from the silica gel by ethanol or methanol.

### EXPERIMENTAL

DABTH-AAs were prepared by the reaction of DABITC with excess of the amino acid in alkaline solution according to our previous report[1,4]. The final product was separated from unreacted amino acid by extraction into ethyl acetate.

Less than 0.2 nmole of each DABTH-AA was applied to a 10 × 10 cm pre-coated plate of silica gel (Merck, G60 without fluorescent indicator, 0.25 mm) with the diameters of the applied spots confined within 1.5 mm. The plate was developed by the solvents indicated in Fig. 1. DABTH-AAs can be identified directly as yellow spots, however, greater sensitivity can be obtained by spraying the dried plate with 6 *N* HCl–methanol (1:30) whereby the derivatives are obtained as intense red spots against a white background.

### RESULTS AND DISCUSSION

Fig. 1 shows the one-dimensional separation of DABTH-AAs on silica gel plates developed by chloroform–methanol or ethanol mixed solvents. The solvent
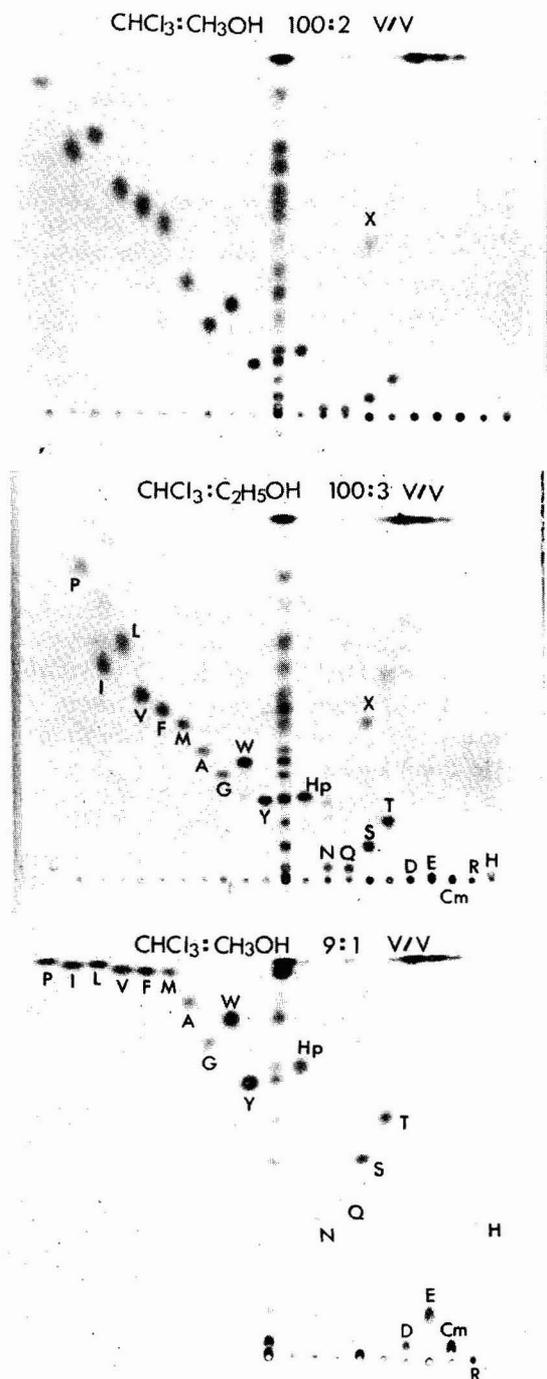
Fig. 1. Photographs of one-dimensional separation of DABTH-AAs on silica gel plate developed by the indicated solvent systems. Approximately 100 pmole of each DABTH-AA was applied. The DABTH-AAs appeared on the plates as red coloured spots after exposire to HCl vapour. Abbreviations: P = proline; I = isoleucine; L = leucine; V = valine; F = phenylalanine; M = methionine; A = alanine; G = glycine; W = tryptophan; Y = tyrosine; Hp = hydroxyproline; N = asparagine; Q = glutamine; S = serine; T = trheonine; D = aspartic acid; E = glutamic acid; Cm = Carboxymethylcysteine; R = arginine; H = histidine; × = unidentified by-product of DABTH-Ser (see Fig. 2).

chloroform–methanol (9:1) is suitable for separation of DABTH-Asn, DABTH-Gln, DABTH-Ser, DABTH-Thr, DABTH-Asp and DABTH-Glu. The system chloroform–ethanol (100:3) is used to separate the remaining commonly occurring DABTH-AAs except DABTH-Asp and DABTH-CmCys which cannot be distinguished in either solvent. The $R_F$ value of DABTH-Arg is zero in both solvents. The major by-product ($\times$) which is usually found during the formation of DABTH-Ser by the reaction of DABITC with serine, serylalanine and triserine can be identified in the system, chloroform–methanol (100:2). The relative position of this by-product on the polyamide sheet is shown in Fig. 2. The $R_F$ value of $\alpha$-DABTH-($\varepsilon$-DABTC)-lysine (Bis-Lys) is 0.27 (cf. glycine $= 0.29$) in chloroform–ethanol (100:3) and 0.91 (cf. alanine $= 0.89$) in chloroform–methanol (9:1).
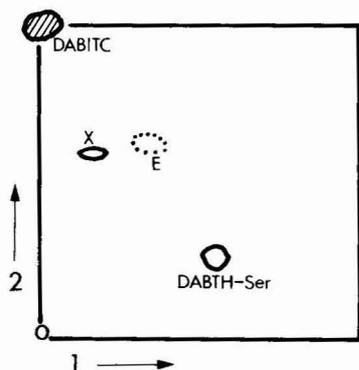


Fig. 2. Position of $\times$, an unidentified by-product during the formation of DABTH-Ser, on a two-dimensional polyamide sheet developed with water–acetic acid (2:1) (solvent 1) and toluene–n-hexane–acetic acid (2:1:1) (solvent 2). This by-product is believed to possess a thiohydantoin structure since it appeared as a red spot after exposure to HCl vapour. E is the authentic blue marker 4-N,N-dimethylaminoazobenzene-4′-thiocarbamoyl diethylamine.

Cheng Chin (Taipei, Taiwan) polyamide sheets have provided one of the most elegant mediums for the qualitative detection of amino acid derivatives[1,5-8]. The sturdy structure of the absorbent, however, makes the recovery of DABTH-AAs tedious. For quantitation using spectrophotometrical measurement, we have used a strongly acidic solvent, 6 N HCl–ethanol (1:2) to dissolve both DABTH-AAs and the polyamide absorbent simultaneously[3]. This procedure however could possibly cause the destruction of some acid labile DABTH-AAs. The recovery of DABTH-AAs from silica gel is relatively simple. A number of solvents, such as ethanol, methanol or acetone, can be used to extract DABTH-AAs, the silica being easily removed by centrifugation.

Perhaps the most significant result of DABTH-AAs separation on silica gel plates is the resolution of DABTH-Leu and DABTH-Ile which we have failed to achieve by using polyamide sheets. The silica gel plates, therefore, should be used in combination with polyamide sheets whenever discrimination between leucine and isoleucine or quantitation if DABTH-AAs is needed.

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## Note

## Improved procedure for peptide characterization using thin-layer chromatography and a fluorescamine indicator

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Fluorescamine and *o*-phthalaldehyde are highly sensitive and rapidly reacting reagents used to detect peptides and amino acids[1,2]. The optimal conditions for the intensity and stability of fluorescent spots have already been reported[3,4], and fluorescamine has also been utilized in the quantitative analysis of amino acids[5].

We have extended the fluorescamine technique to the detection of tryptic peptides on thin-layer chromatograms. These visualized fluorescent tryptic peptides could be further characterized by the determination of their amino acid composition using a conventional amino acid analyzer. The recovery of the N-terminal amino acid of a once-fluorescamine-stained peptide has been shown to be as good as that of a purified identical peptide without staining. We here report an improved procedure for peptide characterization using fluorescamine staining.

EXPERIMENTAL

Fluorescamine (Fluram), 4-phenylspiro(furan-2(3*H*),1'-phthalan)-3,3-dione (Hoffmann-La Roche, Basel, Switzerland) was used as a freshly prepared solution in acetone. Silica gel 60 thin-layer plates (without fluorescent indicator; 20 × 20 cm, thickness 0.25 mm) were purchased from E. Merck (Darmstadt, G.F.R.).

The lyophilized tryptic digests were dissolved in water and 1–1.2 mg of protein were applied to a silica gel plate. Separation in the first dimension was achieved by electrophoresis in 2 *M* acetic acid–0.6 *M* formic acid (pH 1.9) at 50 V/cm for 60 min. Separation on the second dimension was by ascending chromatography in pyridine–acetic acid–*n*-butanol–water (40:14:68:25) for 15 h.

First the plates were sprayed with 0.2 *M* borate buffer (pH 8.0)–acetone (1:1) and then with a solution of fluorescamine in acetone (50 mg per 100 ml). Fluorescent spots were scraped off the plates during the first 3–4 min after the tryptic peptides had been visualized under long-wavelength (366-nm) UV light. The fluorogenic reaction with scraped tryptic peptides was stopped by adding 5.7 *N* hydrochloric acid and then hydrolyzing at 110° for 20 h.

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RESULTS AND DISCUSSION

In the course of sequence analysis of the $\alpha$ subunit of DNA-dependent RNA polymerase from *Escherichia coli*, we purified a tryptic peptide by conventional methods, using column chromatography and then silica gel thin-layer chromatography. The amino acid composition was determined using the amino acid analyzer. In a separate experiment, we isolated an analogous peptide from tryptic fingerprints on silica gel thin-layer chromatograms after staining either with fluorescamine or with ninhydrin. The amino acid composition of samples of this peptide isolated in the three ways described are compared in Table I. Although the N-terminal amino acid determined by the dansyl chloride technique in each case was threonine, the stoichiometric amount of threonine after staining with ninhydrin was 30% lower than the value obtained after fluorescamine staining. Thus, the fluorogenic reaction with the N-terminal amino acid does not have an adverse effect on the amino acid composition.

TABLE I

AMINO ACID COMPOSITION OF AN IDENTICAL TRYPTIC PEPTIDE

The values represent the number of amino acid residues per peptide. The background of a silica gel blank was deducted from the amount of each amino acid residue. Values for threonine were extrapolated to zero time.

| Amino acid | Purified peptide without staining | After staining with fluorescamine | After staining with ninhydrin |
|---|---|---|---|
| Lysine | 1.0 | 1.0 | 1.2 |
| Threonine | 1.0 | 1.0 | 0.7 |
| Glutamic acid | 2.0 | 2.0 | 2.2 |
| Valine | 0.9 | 0.8 | 0.9 |
| Leucine | 2.1 | 2.2 | 2.0 |

Although the fluorescamine reagent is known not to react with secondary amino acids such as proline and hydroxyproline, the tryptic peptides of various proteins containing these residues can still be characterized, since trypsin does not cleave lysine–proline or arginine–proline bonds. For example, it was used to find an amino acid substitution in the $\alpha$ subunit of a mutant RNA polymerase[6], or for the investigation of the phylogeny of lactate dehydrogenase molecules[7].

The fluorescamine-treated tryptic peptides could be further purified by rechromatography on thin-layer plates and, after a second fluorescamine spraying, it is possible to repeat the analysis as described above.

ACKNOWLEDGEMENT

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## Note

Analysis of trace amounts of acetylsalicylic anhydride in acetylsalicylic acid

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Considerable interest has been generated in acetylsalicylic anhydride (ASN) as one of the significant impurities in acetylsalicylic acid (ASA): ASN may be a potential cause for allergic reactions to ASA[1,2]. The anhydride has been purported to be a highly immunogenic substance[1] in minute amounts and therefore a sensitive, specific analytical technique is required to determine quantitatively trace levels of ASN in ASA.

De Weck[1] described an extraction technique followed by thin-layer chromatography for the separation of ASN from ASA. His extraction technique has the disadvantage that 0.5 $N$ sodium hydroxide must be added dropwise continuously over a period of 90 min to a phosphate buffer–benzene system, in order to maintain the desired pH. Bundgaard and Bundgaard[2] modified this extraction system so as to eliminate the dropwise addition of caustic solution. Their final analysis is more sensitive and is based on a spectrophotometric determination employing an $\alpha$-benzamidocinnamate–pyridine reagent which they had to synthesize. Sethi[3] has modified the Bundgaard technique, and recently Ali[4] reported the use of gas and liquid chromatography for the determination of ASN in ASA.

The purpose of this paper is to describe a relatively simple, inexpensive, quantitative technique that appears to be specific for trace amounts of ASN in ASA.

EXPERIMENTAL

*Materials*

All solvents were analytical-reagent grade. The solvent systems employed for the chromatographic separations were methyl ethyl ketone–cyclohexane (50:50) and (70:30). Silica gel plates with fluorescent indicator (Silica Gel GF, Analtech, Newark, Del., U.S.A.; 10 × 20 cm and 20 × 20 cm, 250 $\mu$m) were activated at 105° for 1 h. The sample and standard solutions were spotted with the aid of an automatic spotting device (Analytical Instrument Specialities, Libertyville, Ill., U.S.A.).

The chromatograms were developed by ascending chromatography in a Gelman chromatography chamber (Gelman, Ann Arbor, Mich., U.S.A.), saturated with the vapor of freshly prepared solvents 15 min prior to development. A Chromata-Vue (Ultra-Violet Products, San Gabriel, Calif., U.S.A.) was used at 254 nm for the detection of spots after chromatography.

*Procedure*

Weigh 5.0 g of ASA into a 250-ml glass-stoppered erlenmeyer flask. Add 75 ml of ethyl acetate and stir with the aid of a magnetic stirring bar until the sample is dissolved. Add 125 ml of saturated sodium bicarbonate, cap and stir the two-phase system vigorusly for 30 min. Place the contents into a 250-ml separatory funnel. Wash the erlenmeyer flask with 10 ml of ethyl acetate and transfer this to the separatory funnel. Discard the lower aqueous phase from the separatory funnel. Wash the ethyl acetate layer twice with 10-ml portions of water, discarding the aqueous layer each time.

Add about 2 g of anhydrous sodium sulfate (granular) to the ethyl acetate and shake. Pour off the resulting clear solution into a 150-ml beaker. (One also has the option of filtering the ethyl acetate solution into the beaker through filter paper). Wash the separatory funnel with 10 ml of ethyl acetate and transfer it to the beaker. Evaporate the solution to about 10 ml on the edge of a steam-bath and air evaporate the remainder to dryness in a hood without the aid of heat.

Add 5.0 ml of acetone to dissolve the faint residue and spot 100 $\mu$l onto the silica gel plate. Standards of ASN are prepared in acetone so that, for example, 20, 10, 5, 2 and 1 $\mu$g of ASN can be spotted along with the samples. Place the plate in the saturated chamber, let the solvent front move a distance of 10 cm from the origin, and remove the plate. Place the plate under a stream of cool air until nearly dry and then place the plate in the Chromata-Vue under UV light and compare the ASN spot of the sample to the standard ASN spots. The low-level spot (1 $\mu$g) tends to fade with time, therefore one should procede with the visual interpretation quickly.

RESULTS AND DISCUSSION

An important step in obtaining quantitative results for ASN in ASA is the order of addition of the extracting solvents. It is necessary to dissolve the ASA in the ethyl acetate before the addition of bicarbonate solution. This places the desired ASN into the organic solvent first (the more favorable phase because of its low solubility in water) and allows the ASA and any salicylic acid (SA) to be easily extracted out of the organic phase after the addition of the bicarbonate solution. Since Garrett[5] has demonstrated that the hydrolysis of ASN is 9 min at 26° and pH 8.0, then having the ASA intially in the ethyl acetate should decrease the possibility of hydrolysis of ASN by the sodium bicarbonate solution.

Recovery studies were performed by spiking samples of ASA with known amounts of ASN ranging from 0.003% to 0.05%. Recoveries were $\pm$ 0.001% ASN at the low levels and $\pm$ 0.005% ASN at the higher levels.

Visual observation under UV light showed one major spot to be present besides the ASN. This spot which has an approximate $R_F$ value of 0.4 tails significantly and appears to coincide with ASA in its chromatographic profile and $R_F$ value. The methyl ethyl ketone–cyclohexane solvent systems separate the ASN ($R_F = 0.6$) satisfactorily from the potential trace amounts of ASA ($R_F = 0.4$) and any possible SA ($R_F = 0.2$).

It appears from the chromatogram that a minute amount of ASA remains in the organic phase. This is quite conceivable considering the large sample of ASA used in the analysis. Since ASA can contribute to the oxazolone formation with the

Bundgaard reagent, one could possibly expect somewhat higher results for ASN in a sample of ASA. The technique of solvent extraction followed by thin-layer chromatography helps to increase the specificity of the method.

Several solvent systems as described by De Weck[1] were evaluated for the simultaneous separation of SA, ASA and ASN. However, the methyl ethyl ketone–cyclohexane mixtures provided the best results. Separation was slightly better with the 50:50 mixture than with the 70:30 mixture, although either ratio is quite acceptable.

Some concern was originally generated over the possibility of decomposition of ASN during the evaporation step under warm conditions. Chromatographic analysis of ASN with the warming (evaporation) technique was compared to a totally air-dried sample of ASN and to a control of ASN which did not undergo any evaporation step. The resulting chromatograms showed no salient difference in the chromatographic profiles or intensity of the spots. Likewise, it was established that standards can be used the next day as ASN prepared in acetone and kept for 24 h at room temperature showed no difference in intensity or chromatographic profile as compared to a freshly prepared standard.

Six different ASA samples, each of which was considered to have a low ASN content, were analyzed according to the procedure described in this article. Two samples were found to contain 0.002% ASN, one contained 0.005%, two contained 0.010% and the last one was found to contain 0.020% ASN. The results obtained are in the same order of magnitude as those published previously.[2–4]

One should keep in mind that the relative sensitivity of the method may be increased significantly. For example, one can easily reduce the amount of acetone to dissolve the residue from the evaporation step and/or increase the sample weight or volume of sample solution applied to the silica gel plate. Likewise, if only a limited amount of ASA is available, one should be able to decrease the sample weight as long as the ratio of the extracting solvents to sample size is maintained.

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## Note

## Dünnschichtchromatographischer Nachweis von Pemolin im Harn nach Einnahme therapeutischer Dosen

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Pemolin (5-Phenyl-2-imino-4-oxazolidon) wird als Psychoenergetikum verwendet[1]. Für den Pemolinnachweis nach Körperpassage sind papier- und dünnschichtchromatographische Verfahren beschrieben worden[2,3]. Auch die Hochdruckflüssigchromatographie ist für den Nachweis und die quantitative Bestimmung von Pemolin im Harn benutzt worden[4]. Die beschriebenen Verfahren eignen sich aber nur für Untersuchungen, die gezielt auf diese Substanz oder auf Stimulantia vorgenommen werden.

Die vorliegende Arbeit befasst sich mit der Möglichkeit, eine Pemolineinnahme nachzuweisen, auch wenn die Harnanalyse im Rahmen einer allgemeinen chemisch-toxikologischen Routineuntersuchung durchgeführt wird.

METHODIK

Für die Untersuchung standen fünf freiwillige Versuchspersonen zur Verfügung. Sie erhielten morgens nach dem Frühstück $1^1/_2$ Tabletten Tradon (entsprechend 30 mg Pemolin) *per os*; unmittelbar vor Versuchsbeginn wurde die Blase entleert und die erhaltene Harnprobe —als Blindprobe— genauso aufbereitet und untersucht wie die nachfolgenden. Nach Medikamenteneinnahme wurden die über 24 h zwanglos gelassenen Urinproben getrennt aufgehoben und untersucht.

*Extraktion des Harnes*

Der Spontanurin (jeweils die Gesamtmenge) wurde mit 10 %iger HCl auf pH 1–2 gebracht und dreimal mit Chloroform extrahiert; die wässrige Phase wurde dann mit 10 %iger NaOH-Lösung alkalisiert (pH 13–14) und erneut dreimal mit Chloroform ausgeschüttelt. Die Chloroformextrakte wurden dann über $Na_2SO_4$ getrocknet, filtriert und das Lösungsmittel bei *ca.* 40° unter vermindertem Druck destilliert. Die Rückstände wurden anschliessend dünnschichtchromatographisch untersucht.

*Dünnschichtchromatographie*

Es wurden Platten von 200 × 200 mm benutzt, die nach dem Standardverfahren von Stahl[5] mit Kieselgel G beschichtet wurden. Es wurde die Technik der zwei-dimensionalen Trennung angewandt. Fliessmittel waren: (1) Chloroform–Methanol–25 % Ammoniak-Lösung (90:10:0.5) (Richtung 1), und (2) N,N-Dimethylformamid–Essigsäureäthylester–*n*-Octanol (1:9:3 Tropfen)[3] (Richtung 2). Vor der Behandlung

der Chromatogramme mit den Reagenzien wurden die Platten im Trockenschrank bei ca. 120° 5–10 min getrocknet.

*Nachweis.* Zuerst wurde mit einer 20%igen methanolischen KOH-Lösung besprüht und nach Trocknung mit dem kalten Föhn mit 1%iger methanolischer *m*-Dinitrobenzollösung nachbehandelt[6]. Für die Bestimmung der Nachweisgrenze wurden die Substanzlösungen mit Hilfe einer Mikropipette von 10 µl Fassungsvolumen (Einteilung 1 µl) auf die Platte aufgetragen.

ERGEBNISSE UND DISKUSSION

Pemolin ergibt mit dem Kaliumhydroxyd–*m*-Dinitrobenzol-Reagenz eine rot-violette Färbung, die nach *ca.* 19 min bei Raumtemperatur in einen orangen Farbton umschlägt. Mit diesem Reagenz kann man 1 µg Pemolin nachweisen.

Bei der Untersuchung der Harnproben zeigte sich, dass unverändertes Pemolin bei allen 5 Probanden in dem ersten Spontanurin, der etwa 2 bis knapp 4 h nach Einnahme abgegeben wurde, nachweisbar war. In den meisten Fällen war es auch noch 24 h nach Versuchsbeginn auf dem Chromatogramm zu sehen. Pemolin trat immer in dem bei saurer Reaktion erhaltenen Extrakt auf und manchmal zusätzlich in dem bei pH 13–14 erhaltenen Chloroformextrakt.

Ausser Pemolin wurde in dem sauren Extrakt eine Substanz beobachtet, die sich mit dem Kaliumhydroxyd–*m*-Dinitrobenzol-Reagenz rot-violett anfärbte und wie Pemolin nach *ca.* 10 min eine orange Farbe annahm; diese Substanz trat nicht in den medikamentenfreien Harnproben auf. Spätere Untersuchungen haben ergeben[7], dass es sich bei dieser Substanz um 5-Phenyloxazolidin-2,4-dion handelt. Das Auftreten von 5-Phenyloxazolidin-2,4-dion im Harn kann ein wichtiger Hinweis auf eine Pemolineinnahme sein. In manchen Fällen tritt es allein auf, vielfach z.B., wenn die Harnprobe nach dem Stas–Otto-Verfahren aufbereitet wird. Es zeigt mit dem Fliessmittel 1 einen $R_F$-Wert von 0.15 (Pemolin $R_F = 0.38$), mit dem Fliessmittel 2 einen $R_F$-Wert von 0.73 (Pemolin $R_F = 0.60$). Die Spezifität des Kaliumhydroxyd–*m*-Dinitrobenzol-Reagenz wurde an 20 Substanzen, die aus sauren Lösungen extrahierbar sind, geprüft; nur Methyprylon war anfärbbar[6]; diese Verbindung ergibt aber eine blau-violette Färbung und weist mit den hier verwendeten Fliessmitteln einen ganz anderen $R_F$-Wert als Pemolin auf und kann somit von Pemolin und 5-Phenyloxazolidin-2,4-dion ohne weiteres unterschieden werden.

Die vorliegenden Untersuchungen zeigen, dass eine Pemolineinnahme im Rahmen einer allgemeinen Routineanalyse von Harn mit der angegebenen dünnschichtchromatographischen Methode festgestellt werden kann.

LITERATUR

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## Book Review

*HPTLC—High performance thin-layer chromatography* (Journal of Chromatography Library, Vol. 9), edited by A. Zlatkis and R. E. Kaiser, Elsevier, Amsterdam, Oxford, New York, and Institute of Chromatography, Bad Dürkheim, 1977, 240 pp., price Dfl. 110.00, US$ 43.95, ISBN 0-444-41525-4.

High-performance thin-layer chromatography (HPTLC) —to quote from the preface of the present book— is defined as the combined action of several variables which include an optimized coating material, a new method of feeding the mobile phase, a novel procedure for layer conditioning, an improved dosage method and a competent data acquisition and processing system. Or, in other words, HPTLC is recommended as a fast and inexpensive preliminary technique for high-performance liquid chromatography, which will also be the method of choice for certain analytical problems.

Six authors have contributed to the present volume of the Journal of Chromatography Library, which essentially is a considerably enlarged version of *Einführung in die Hochleistungs-Dünnschicht-Chromatographie*, edited by Kaiser and published in 1976 by the well-known German Institute for Chromatography at Bad Dürkheim. The book is written in a slightly aggressive style and the authors are firmly opposed —and rightly so!— to those who consider TLC as one of the less important analytical tools.

The first (R. E. Kaiser) and second (J. Blome) chapters deal with the simplified theory of TLC and discuss aspects such as the $R_F$, real $R_F$ and $k$ values, the flow function in HPTLC, separation capability, separation power and the separation number in linear and circular TLC. A chapter on the advantages, limits and disadvantages of the ring developing technique (J. Blome) follows, which considers the prerequisites for this technique, application and gradient problems, and flowing-around and solvent atmosphere effects.

The fourth and fifth chapters (R. E. Kaiser) are the heart of the matter. The author extensively discusses the U-chamber, which allows electronically controlled flow of the mobile phase, which moreover is isolated from the surrounding atmosphere, application of gradient techniques, control of the gas phase, and continuous-flow HPTLC. The chapter on dosage techniques stresses that only dosage volumes below 10 nl are compatible with HPTLC and reviews the instrumentation for nano dosage.

Two chapters (H. Halpaap and J. Ripphahn) are devoted to the development of pre-coated HPTLC plates and their application in quantitative TLC, and to aspects such as the influence of the type of chamber, sorbent and solvent activity, and temperature. The activity of the stationary phase is the main topic of D. Jänchen's considerations on the reproducibility of TLC separations. Lastly, the use of UV-absorption and fluorescence measurements is discussed by U. B. Hezel, who also records a number of practical examples.

The book, which contains many figures and tables and an adequate index, is well produced. It is an important book for the practical chromatographer, because it presents a complete system and procedure for an advanced thin-layer technique that will certainly see increasing application. However, much work remains to be done, before the combined action of speed, precision and sensitivity will really have turned HPTLC into the second generation of TLC.

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1 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
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