The ANALYST

The Journal of the Society for Analytical Chemistry

A monthly International Publication
dealing with all branches of
Analytical Chemistry

Published by the
SOCIETY FOR ANALYTICAL CHEMISTRY

Volume 97

No. 1161, Pages 929–1008 **December, 1972**

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Published by The Society for Analytical Chemistry
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Volume 97, No. 1161 December, 1972

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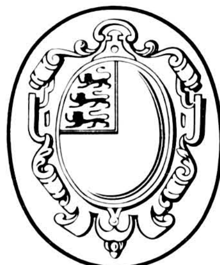

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Summaries of Papers in this Issue

A Rapid, Inexpensive Coulometric Determination of Sulphur in Petroleum Products

A method for the determination of sulphur in oils in the range 0·01 to 1·0 per cent. *m/m* is described. A simple and inexpensive constant-current coulometer is used. A result can be obtained in 2 minutes by using a 0·5-μl sample. The coefficient of variation is 1·4 per cent. at the 0·2 per cent. *m/m* of sulphur level.

J. M. CARTER

Laporte Industries Limited, Widnes, Lancashire.

Analyst, 1972, **97**, 929–936.

The Determination of Sulphur Dioxide in Meats

Several methods were compared in determining sulphur dioxide in meat samples that contained no added sulphur dioxide but that had various pH values, in order to determine whether or not false positive results were obtained owing to the presence of hydrogen sulphide in the meat. The recovery of sulphur dioxide that had been added to meat samples was also studied.

An iodimetric method gave false positive results for sulphur dioxide. A modified Monier-Williams method did not give false positive results, but it has limited sensitivity, giving a possible error of ±6·4 p.p.m. with a 10-g sample. The relative merits of each method are discussed.

D. BARNETT

CSIRO Division of Food Research, P.O. Box 52, North Ryde, N.S.W. 2113, Australia.

Analyst, 1972, **97**, 937–939.

The Determination of Organocarbon in Clay Materials

A dry-combustion method is described for the determination of organocarbon in clay materials. The essential features are the use of purified oxygen; slow insertion of the sample into the furnace to avoid rapid dehydroxylation of the kaolinite and attendant loss of pyrolysis fragments in the atmosphere of steam that is generated; and the use of traps to remove gaseous fluorine compounds, steam and sulphur dioxide. Extensive tests on the apparatus with calcium carbonate and with mixtures of kaolin with glucose and with tannic acid are described. Tests with a commercial kaolin containing organic matter equivalent to about 550 μg of carbon per gram of material indicated a standard error of 8 μg, or ±1·5 per cent. A published method in which the kaolin is heated in a stream of oxygen containing 2 per cent. *V/V* of ozone has been re-investigated; the organocarbon was only partially oxidised so that the method has no analytical value. Two wet-combustion methods involving the separate use of potassium dichromate and potassium persulphate, which are used in soil analysis, were evaluated. The organic matter on the clay was again only partially oxidised to carbon dioxide under the experimental conditions.

A. P. FERRIS and W. B. JEPSON

Central Laboratories, English Clays Lovering Pochin & Co. Ltd., St. Austell, Cornwall.

Analyst, 1972, **97**, 940–950.

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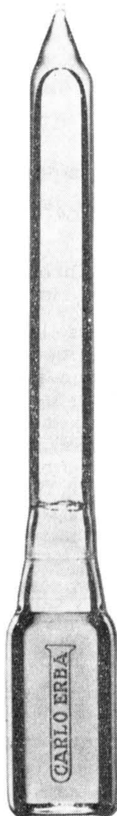
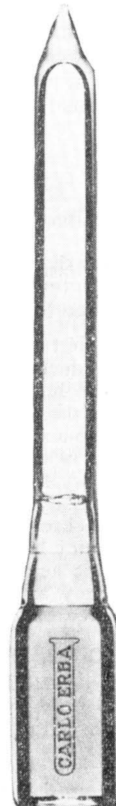


Hydrocarbons

This list includes all the normal chain paraffinic hydrocarbons from C4 to C40 and the principal isomers from C4 to C9, as well as some of the main cycloparaffinic, olefinic, diolefinic, cycloolefinic and aromatic hydrocarbons.

Fatty Acids

This list includes all the saturated fatty acids from C4 to C24, C9 pelargonic acid and the principal C16, C18, C22 unsaturated acids. All these products are straight-chain compounds.



Methyl esters of Fatty Acids

This list includes the Methyl esters of the above mentioned Fatty acids, as well as the C3, C5, C7.

Alcohols

This list includes the normal C4 and C5 Alcohols, together with their secondary, tertiary and branched chain isomers; moreover the normal C6, C7, C8, C9, C10, C11, C12.

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Derivatives for the Identification and Quantitative Determination of Some Keto and Aldo-carboxylic Acids by Gas - Liquid Chromatography

Studies on the derivatives of some physiologically and pathologically important keto and aldo-acids (pyruvic, glyoxylic, 2-oxoglutaric, oxaloacetic and 4-hydroxyphenylpyruvic) have shown that the *O*-ethoxime trimethylsilyl esters are the most suitable for quantitative gas - liquid chromatography. The *O*-trimethylsilyl oxime trimethylsilyl esters were thermally unstable under the conditions used. The *O*-methyl and *O*-benzyl oxime trimethylsilyl esters are also suitable for gas - liquid chromatography and would be useful in identification studies. The *O*-benzyl oxime trimethylsilyl derivatives are suggested as being especially suitable when only the keto and aldo-acids are to be studied, and it is not necessary to identify the lower boiling trimethylsilyl esters of other carboxylic acids on the same chromatogram.

Two aldehyde-specific reagents, dimedone and *NN'*-diphenylethylenediamine, were also examined for use with glyoxylic acid. The trimethylsilyl ester of 1,3-diphenylimidazolidine-2-carboxylic acid (the derivative of glyoxylic acid with *NN'*-diphenylethylenediamine) has a high boiling-point and is suitable for quantitative gas - liquid chromatography. This derivative should be especially useful in studies that are particularly concerned with the metabolism of glyoxylic acid or possibly other aldehydes.

The retention times of the derivatives relative to the retention times of n-tetracosane and n-hexacosane are compared with the corresponding values for the trimethylsilyl derivatives of some physiologically related carboxylic acids that do not contain keto or aldo groups.

RONALD A. CHALMERS and R. W. E. WATTS

Division of Inherited Metabolic Diseases, Medical Research Council, Clinical Research Centre, Watford Road, Harrow, Middlesex, HA1 3UJ.

Analyst, 1972, **97**, 951–957.

The Quantitative Extraction and Gas - Liquid Chromatographic Determination of Organic Acids in Urine

A method for the quantitative extraction and gas - liquid chromatographic determination of organic acids in aqueous solution and biological fluids has been developed. The acids are extracted by a DEAE-Sephadex anion-exchange column, neutral and basic compounds are removed by washing with water and the acids are eluted with a pyridinium acetate buffer. The ethoximes of oxo-acids are formed in the eluate to stabilise these compounds and the eluate is then freeze-dried under carefully controlled conditions. The residue of dry free acids and their pyridinium salts is trimethylsilylated and the trimethylsilyl derivatives are separated and quantitatively determined by gas - liquid chromatography with the use of internal standards. The method would not be suitable for the determination of formic, acetic, propionic or butyric acid because of their volatility.

The accuracy and precision of the method, which can be applied to other protein-free biological fluids as well as urine, are discussed.

RONALD A. CHALMERS and R. W. E. WATTS

Division of Inherited Metabolic Diseases, Medical Research Council, Clinical Research Centre, Watford Road, Harrow, Middlesex, HA1 3UJ.

Analyst, 1972, **97**, 958–967.

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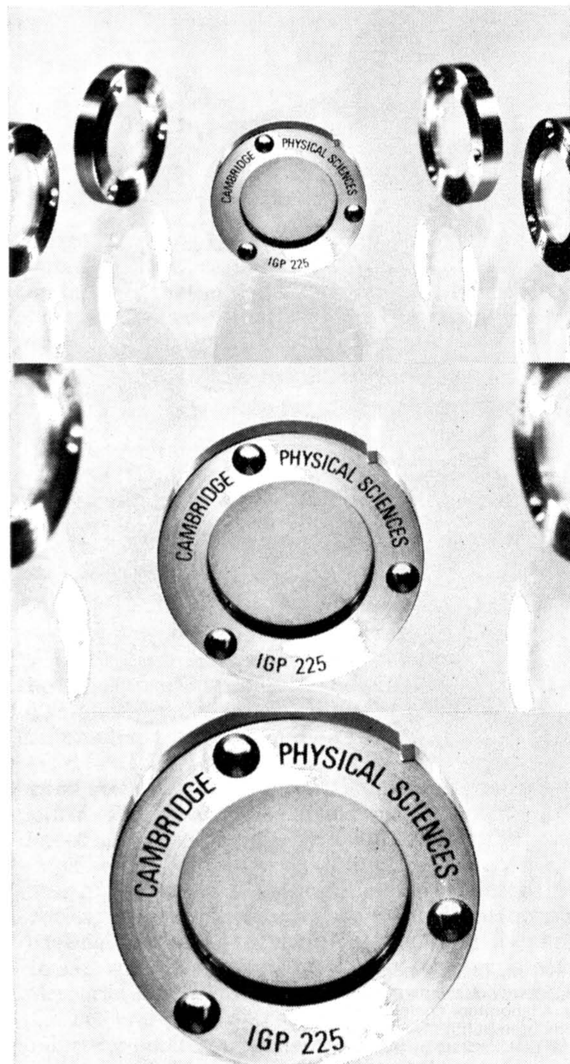

Cambridge Physical Sciences has developed a new photolithographic process for making fine grids of periods of less than 0.4 microns. The resulting near infrared polarisers are inexpensive enough to be used as accessories for laboratory instruments such as scanning spectrophotometers and interferometers.

The polarisers consist of a fine aluminium grid on a 2 millimetre thick KRS-5 substrate. KRS-5 transmits out to 35 microns, is mechanically strong and easy to handle, insensitive to ambient light and chemically stable.

The addition of the near infrared polariser to the range means that Cambridge Physical Sciences now produces efficient grid polarisers to cover the whole infrared region, working wavelength range extending from 2 to 1000 microns. The polarisers are all mounted in stainless steel rings for ease of handling.

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DECEMBER, 1972 Vol. 97, No. 1161

THE ANALYST

A Rapid, Inexpensive Coulometric Determination of Sulphur in Petroleum Products

By J. M. CARTER*
(*Laporte Industries Limited, Widnes, Lancashire*)

A method for the determination of sulphur in oils in the range 0·01 to 1·0 per cent. *m/m* is described. A simple and inexpensive constant-current coulometer is used. A result can be obtained in 2 minutes by using a 0·5-µl sample. The coefficient of variation is 1·4 per cent. at the 0·2 per cent. *m/m* of sulphur level.

ACTIVITY testing of dehydrosulphonation catalysts involves the determination of the sulphur content of hydrocarbon oils at the 0·01 to 1·0 per cent. *m/m* level. Previously, a quartz-tube combustion method[1] has been used. However, this method is extremely time consuming and the number of samples that can be analysed per day is limited. A replacement method needs to meet the following criteria—

(*a*) An analysis should be rapid, preferably taking less than 10 minutes.

(*b*) As little operator skill and experience as possible should be required.

(*c*) The coefficient of variation of the method should not exceed a few per cent.

The Dohrmann coulometric system[2–5] meets all these requirements. However, this system is far too sophisticated for the present task and the cost is of the order of £4000. These factors led to the examination of a simpler coulometric system.

EXPERIMENTAL

THE THORN T.E. 110 CONSTANT-CURRENT COULOMETER—

For the determination of total sulphur content, a constant-current coulometer should be just as suitable as the variable-current Dohrmann type. It is not necessary to examine the concentration profile of the sulphur dioxide as it enters the cell, and only the total amount of sulphur dioxide produced by the combustion of the sample need be determined. A suitable coulometer, the Thorn T.E. 110,† the cost of which is about £400, was already available.

The T.E. 110 instrument is basically a constant-current generator with facilities for automatically switching off the generator current when the titration reaches a pre-set end-point. The titration is carried out by electrically generating the required titrant *in situ* and detecting the end-point by a potentiometric or amperometric method.

The constant-current circuit incorporates a selector, which, when set to the equivalent mass of the reacting species, directly converts the titration time into micrograms of reacting species, *i.e.*, the selector varies the current output according to the equivalent mass of the reacting species. The titration time itself is measured with a built-in digital chronometer. Hence, when the equivalent mass selector is set to "*x*" the chronometer reads directly in micrograms of a substance of equivalent mass "*x*."

The end-point detector is a high-input resistance potentiometer, which trips the generator and chronometer circuits when its potential reaches a pre-set value. The potentiometer circuit has facilities for both potentiometric and amperometric systems.

In order to plot titration graphs (plots of mass of reagent generated against potential of the indicator electrode), the instrument has a "hold" control, which, when activated, switches off the generator current. A titration graph is plotted as for a normal potentiometric titration, *i.e.*, incremental amounts of reagent are added and the potential of the indicator electrode is measured after each addition.

* Present address: Mid-Cheshire Water Board, Hurleston Treatment Works, Chester Road, Nantwich, Cheshire.

† Analytical Measurements Ltd., Ensoleille Works, Feltham, Middlesex.

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By examining the titration graph, the end-point (in fact, the point of maximum slope) can be found. The potential at which the maximum slope occurs is then set on the potentiometer and further titrations can be carried out automatically by simply starting the current. When the pre-set potential is reached, the current will be switched off. The number of micrograms of titrant can be read directly from the chronometer.

A suitable furnace and a coulometric cell were constructed in order to assess the capabilities of the T.E. 110 instrument with regard to the determination of sulphur in oils, mainly of the middle distillate type. The system described is shown diagrammatically in Fig. 1, and a photograph of the whole set-up is shown in Fig. 2.

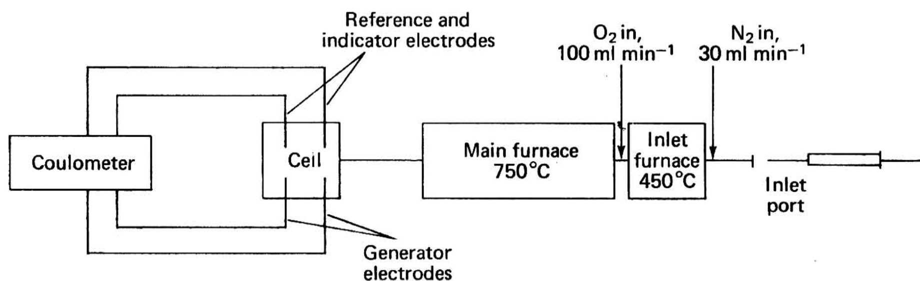

Fig. 1. Block diagram of T.E. 110 system

COMBUSTION FURNACE—

The combustion furnace consists of a Gallenkamp C and H micro-furnace with suitable modifications. These consisted in fixing the movable furnace about ¼ inch from the fixed furnace and constructing a new quartz combustion tube. The combustion tube is shown in Fig. 3. It consists of a 1 cm diameter quartz tube with two gas inlets, one for nitrogen and the other for oxygen. The inlet end of the tube is fitted with a ⅜-inch silicone-rubber septum held in place with a short length of Tygon tubing and a piece of glass capillary tube. The glass tube helps to guide the syringe needle into the septum. The exit end of the tube is fitted with a quartz S13 ball-joint for connection to the coulometric cell. The inlet furnace is maintained at 450 °C and the outlet furnace at 750 to 800 °C.

The combustion is carried out as follows. The sample is injected into the inlet furnace with a 1-μl Hamilton syringe. Pure nitrogen flows through the inlet furnace at about 30 ml min^{-1}, so that the sample is pyrolysed in nitrogen. The pyrolysed sample is mixed with oxygen (100 ml min^{-1}) as it passes from the inlet furnace to the combustion furnace. Passage of the mixture through the combustion furnace causes the oil to be burnt to water and carbon dioxide and the sulphur mainly to sulphur dioxide. The combustion products are then passed into the coulometric cell.

A three-elevation diagram of the coulometric cell is shown in Fig. 4. The cell consists of a main compartment, which contains the platinum indicator and generator electrodes in base electrolyte (0·1 per cent. *m*/*m* potassium iodide and 1·0 per cent. *m*/*m* acetic acid). The reference electrode is contained in a side-arm that is separated from the main compartment by a frit and a salt bridge (2 per cent. of agar in saturated potassium chloride solution). The reference electrode is a silver wire plated with silver chloride and immersed in saturated potassium chloride solution. The counter electrode is a platinum spiral placed in a similar side-arm without a salt bridge. This side-arm is filled with base electrolyte.

The effluent from the combustion furnace enters the cell via a capillary inlet, which terminates in a fine jet. The solution in the main compartment is stirred magnetically with a small PTFE-covered stirring bar. The stirring action combined with the fine jet causes the gas stream to be broken up into small bubbles.

REAGENTS—

Base electrolyte—Dissolve 1·0 g of potassium iodide and 1·0 ml of glacial acetic acid in de-ionised water and dilute to 1 litre with de-ionised water.

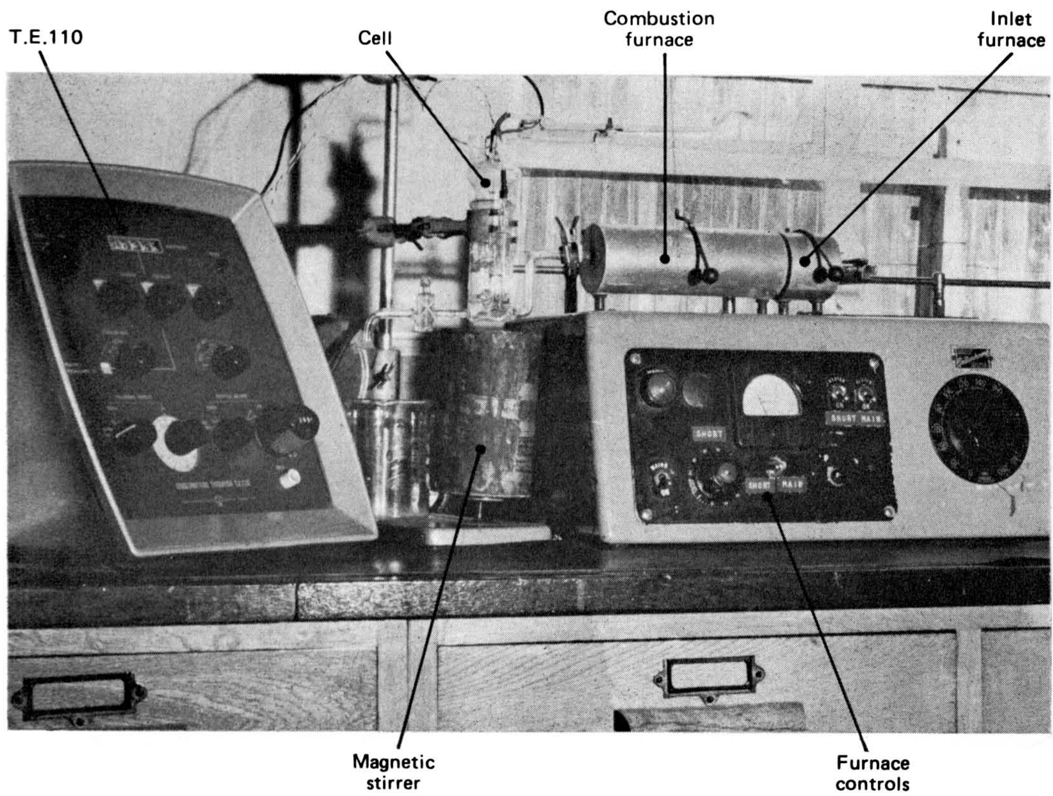

Fig. 2. T.E. 110 system

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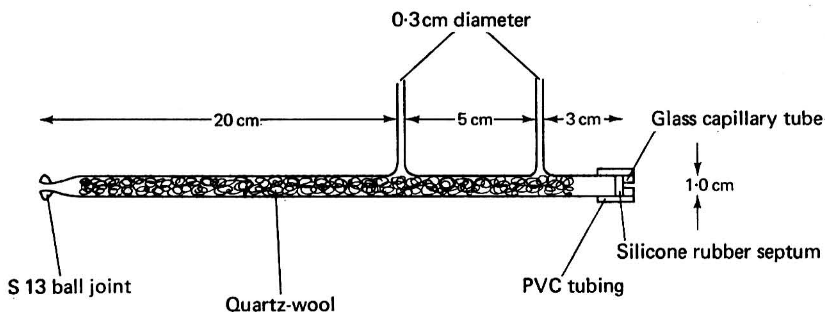

Fig. 3. Furnace tube

Standard sulphur solution—Obtain a typical sample of oil that has a sulphur content at the expected level (0·2 per cent. *m*/*m*). Determine the sulphur content of this sample in triplicate by the quartz-tube method. The individual results should not differ by more than 0·005 per cent. *m*/*m* of sulphur. Take the mean of the triplicate results as the sulphur content of the standard solution. If a wide range of sulphur contents is being dealt with, several standards should be prepared and the most appropriate one used.

Fig. 4. Coulometric cell: (*a*) front elevation with tap and inlet omitted; (*b*) side elevation with side-arms omitted; and (*c*) plan through section as marked on front elevation

PROCEDURE—

Set up the apparatus under the following conditions—

Main furnace temperature	800 °C
Inlet furnace temperature	450 °C
Oxygen flow-rate	100 ml min^{-1}
Nitrogen flow-rate	30 ml min^{-1}

Fill the cell with base electrolyte (main compartment 40 ml, counter electrode compartment 5 ml) and switch on the stirrer. Set the end-point potential on the coulometer to about 280 mV (the exact value is found by preparing a titration graph). Allow 5 minutes for the apparatus to stabilise and warm up.

If the measured potential on the coulometer potentiometer is less than 280 mV, switch on the coulometer and titrate to the end-point. If the measured potential is greater than 280 mV, inject 0·50-μl portions of a sample or standard, containing about 0·2 per cent. *m/m* of sulphur, until the measured potential is less than 280 mV. Switch on the coulometer and titrate to the end-point.

Inject 0·50-μl portions of standard sulphur solution until a consistent titration ($\pm$10 ng) is obtained. Take the mean of the consistent figures as the standard titration (S).

Run each sample in triplicate and take the mean of the three results as the sample titration (T). The apparatus should be checked at frequent intervals by re-running the standard.

The sulphur content (per cent. *m/m*) of the sample is then $\frac{T}{S} \times C$, where C is the concentration of sulphur (per cent. *m/m*) in the standard. When using this formula it is assumed that the density of the sample is constant.

DEVELOPMENT OF METHOD

The T.E. 110 instrumental arrangement was evaluated in three stages. Firstly, the coulometer and cell were checked by titrating nanogram amounts of sulphite ion. Secondly, the absorption of sulphur dioxide in the base electrolyte was investigated by injecting sulphurous acid into the combustion tube. Lastly, the combustion of organosulphur compounds in hydrocarbon solvents was carried out.

Fig. 5 shows a coulometric titration graph obtained by using 3·2 μg of sulphur in the form of sodium sulphite solution. The end-point (the potential at which the graph has the greatest slope) of the titration is at 280 mV. As maximum sensitivity is required, this value is the potential to which the T.E. 110 instrument should be pre-set. The other aspect of interest in Fig. 5 is the value of the slope at the end-point. By drawing tangents to the curve at the end-point, the maximum slope is found to be 10 ng mV^{-1} of sulphur. This value is of importance as it determines the limit of detection of the method.

Automatic titrations of sulphite solution were performed by setting the coulometer to 280 mV and injecting sulphite solution directly into the cell.

The results indicate that down to 60 ng of sulphur can be titrated with a precision of 5 to 10 per cent. at the lowest level. It is not possible to titrate less than 60 ng of sulphur as the coulometer overshoots the set end-point by about 5 mV, and it is therefore necessary to add enough sulphur to the cell to reduce the potential to 280 mV before the generator current can be switched on. From Fig. 5, the amount of sulphur required to change the potential from 285 to 280 mV is 50 ng. This limit can be reduced by manually switching on the coulometer by raising the set potential to 290 mV, starting the generator current and quickly reducing the potential to 280 mV. The technique is very difficult to apply with titrations of less than 50 ng, as these take only 5 s to carry out and hence there is very little time to adjust the potential.

The efficiency of absorption of sulphur dioxide in the base electrolyte was checked by preparing a solution of sulphur dioxide in water and standardising it by injection directly to the cell. A 10-μl volume of the solution was found to contain 1·12 μg of sulphur. The same volume of solution was then injected into the injection port of the furnace under the following conditions—

Main furnace temperature	800 °C
Inlet furnace temperature	300 °C
Oxygen flow-rate	100 ml min^{-1}
Nitrogen flow-rate	30 ml min^{-1}

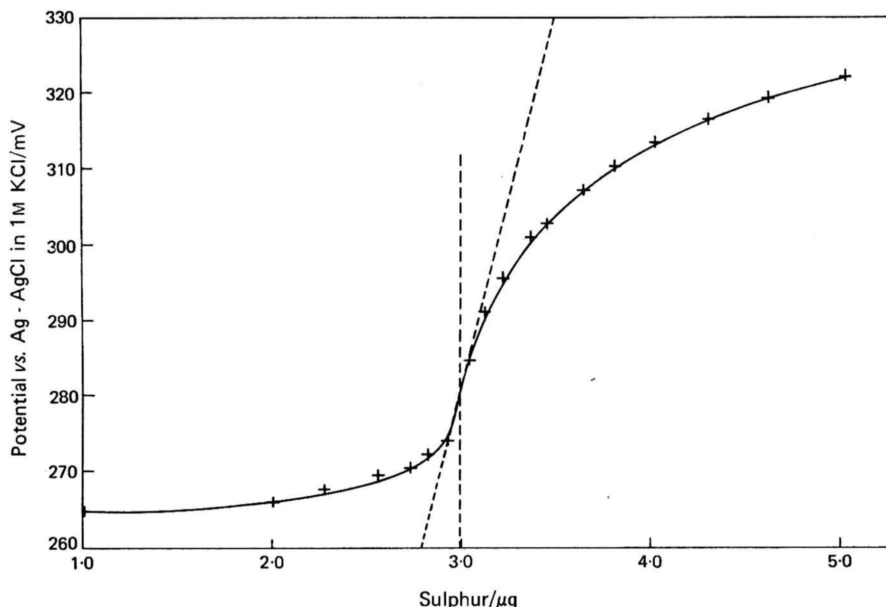

Fig. 5. Titration graph

A mean recovery of 1·10 μg of sulphur was obtained, indicating that the sulphur dioxide is absorbed in the base electrolyte with virtually 100 per cent. efficiency.

The only really pure organosulphur compound available was dibenzyl disulphide, which is normally used as a microchemical standard for sulphur. A solution of dibenzyl disulphide in tetradecene was prepared so as to contain 2000 μg ml^{-1} of sulphur. This solution was diluted with tetradecene to provide sulphur standards of lower concentrations.

The main furnace was set at 800 °C and the inlet furnace at 300 °C. After injection, the inlet furnace temperature was raised to 800 °C so as to ensure complete pyrolysis of the dibenzyl disulphide. Then 1·00-μl injections of various solutions of dibenzyl disulphide were made and a blank on the tetradecene was obtained by injecting five 1-μl portions of the solvent. The blank value was 0·082 μg μl^{-1} of sulphur.

TABLE I

RECOVERY OF SULPHUR FROM A 1·00-μl SAMPLE OF TETRADECENE

Concentration of sulphur/μg ml^{-1}	Sulphur found/μg	Sulphur found, mean/μg	Sulphur found *minus* blank/μg	Recovery, per cent.
2000	1·821, 1·789	1·81	1·73	86·5
1000	0·915, 0·929	0·92	0·84	84
500	0·520, 0·517	0·52	0·44	88
250	0·284, 0·269	0·28	0·20	80
125	0·185	0·18	0·10	80

The results in Table I show that 80 to 88 per cent. of the sulphur in dibenzyl disulphide is converted into sulphur dioxide. As the titration times for the lower recoveries were very short, the recovery of sulphur was assumed to be about 85 per cent. As dibenzyl disulphide is not volatile, it is not a good standard, but no other sulphur compounds of purity greater than 99·5 per cent. could be found. Recovery experiments were carried out with compounds that contained various sulphur groups, but the purities of the sulphur compounds concerned were not known; they were probably greater than 97 per cent. The recoveries obtained for the different compounds are shown in Table II and show clearly that different recoveries are obtained for different forms of sulphur. The results for dimethylsulpholane (2,4-dimethyltetrahydrothiophen 1,1-dioxide), dimethyl sulphoxide and thiophen are remarkably

good. There is obviously an error in the figures for the two thiols. The normal explanation for high results is to assume that incomplete combustion has taken place, which results in the formation of unsaturated compounds that consume iodine in the cell. However, in this instance, incomplete combustion can be ruled out as the recoveries are repeatable ($\nu = 1{\cdot}5$ per cent.). As the hydrocarbon level in the sample is about 500 times greater than the sulphur level, the degree of incomplete combustion must be very low and very reproducible. The only way in which incomplete combustion, which would account for the high results, could occur would be if the base electrolyte became saturated with the iodine-consuming material and the remainder was swept through the apparatus without being absorbed. This possibility was excluded by injecting different amounts of the same solution. In each instance the calculated sulphur concentration was the same. If the base electrolyte were becoming saturated, one would expect the lower volumes to give higher results.

The only other explanation that can be offered for the high results is the formation of hydrogen sulphide. This would seem extremely unlikely in an atmosphere containing 80 per cent. of oxygen.

Hence, at present, no really satisfactory reason can be advanced to explain the recoveries of over 100 per cent.

The effect of varying different parameters was investigated and optimum values were established. Gas flow-rates had very little effect on recovery over the ranges 10 to 50 ml min^{-1} of nitrogen and 50 to 100 ml min^{-1} of oxygen. Optimum flow-rates were 30 ml min^{-1} of nitrogen and 100 ml min^{-1} of oxygen.

TABLE II

RECOVERIES OF DIFFERENT SULPHUR COMPOUNDS

Compound	Sulphur group	Recovery, per cent.
Butanethiol	—SH	113
Dimethyl sulphide	>S	103
Dimethylsulpholane	$>SO_2$	97
Dimethyl sulphoxide	>SO	96
α-Toluenethiol	—SH	115
Thiophen	>S	98

Variation of the inlet furnace temperature had no effect, provided that it remained above about 200 °C (the boiling-point of the oil). The combustion furnace temperature made no significant difference to the recovery, provided that it was in the range 700 to 800 °C. Temperatures of the inlet and combustion furnaces of 450 and 750 °C, respectively, were used.

The amount of sample injected was varied between 0·2 and 1·0 μl. With a 1·0-μl amount there was some slight evidence of incomplete combustion, and for this reason the sample volume should not exceed 0·5 μl.

Variation of the set end-point by ±10 mV about the optimum value had no measurable effect.

COMPARISON OF RESULTS WITH THOSE OBTAINED BY THE QUARTZ-TUBE METHOD—

Six samples of oil were analysed to determine sulphur by the procedure described. One of these samples (418) was used as a standard against which the others could be calculated. The sulphur content of sample 418 as determined by the quartz-tube method was 0·204 per cent. *m*/*m*.

The reproducibility of the technique was first checked by running sample 418 thirty-two times. The mean titration was equivalent to 748 ng of sulphur with a range of 733 to 770 ng, the standard deviation (σ) 10 ng and the coefficient of variation 1·4 per cent. The density of the oil sample was 0·84 g ml^{-1}. Hence, as 0·50 μl of sample was used, the sulphur recovery was 87 per cent.

The other five samples were analysed in triplicate on three different occasions by two different operators. The sulphur contents were calculated by using sample 418 as a standard of sulphur content 0·204 per cent. *m*/*m*. The results are shown in Table III, together with the results for the quartz-tube method.

The results in Table III show that the method developed gives results in reasonable agreement with those obtained by the quartz-tube method and the Dohrmann method.

The mean coefficient of variation for the T.E. 110 method (1·4 per cent.) is that to be expected by considering the coefficient of variation of 1·5 per cent. obtained for single injections.

The coulometric apparatus was transferred to the catalyst-analysis laboratory and run in routine service alongside the quartz-tube apparatus. A comparison of the results obtained shows that the quartz-tube and the coulometric methods give the same results, within the limits of experimental error. A few exceptions have been noted in which the coulometer gives results about 8 per cent. higher than those obtained with the quartz tube.

TABLE III

COMPARISON OF SULPHUR CONCENTRATIONS DETERMINED BY DIFFERENT METHODS

Results are expressed as per cent. *m/m* of sulphur

		Sample No.				
Method		419	292	3	2	1
Quartz tube		0·137	0·156	0·133	0·208	0·301
Dohrmann coulometer		0·157	0·150	0·143	0·208	0·300
T.E. 110	Operator 1	0·153	0·151	0·145	0·207	0·324
		0·151	0·151	0·147	0·208	0·317
		0·153	0·159	0·146	0·214	0·325
	Operator 2	0·154	0·151	0·146	0·208	0·324
		0·150	0·149	0·146	0·208	0·328
		0·150	0·148	0·148	0·209	0·317
	Mean	0·152	0·151	0·146	0·209	0·322
	Range	0·150 to 0·154	0·148 to 0·159	0·145 to 0·148	0·207 to 0·214	0·377 to 0·328
	Standard deviation	0·0013	0·0039	0·0011	0·0025	0·0044
	Coefficient of variation	0·8	2·6	0·8	1·2	1·4

DISCUSSION

The coulometric method developed seems to be very suitable for the determination of 0·01 to 1·0 per cent. *m/m* of sulphur in hydrocarbon oils. The method has the following advantages—

(*i*) It is very rapid. A sample can be analysed in triplicate in 5 minutes.

(*ii*) A low degree of operator skill is required for the operation of the apparatus. However, a senior assistant is required in the laboratory to resolve difficulties if necessary.

(*iii*) The method has good precision. The coefficient of variation for the mean of triplicates is 1·4 per cent.

(*iv*) It should be possible to determine nitrogen in oils at similar levels. This will require hydrogenation of the sample so as to convert the nitrogen into ammonia. The ammonia is then determined by use of a coulometric cell that is sensitive to hydrogen-ion concentration.

(*v*) A very small sample is used (0·5 μl).

(*vi*) The complete set-up costs only about £600.

The disadvantages of the method are—

(*i*) The T.E. 110 instrument will not respond to less than 50 to 100 ng of sulphur. Further, it is not possible to combust conveniently more than 1·0 μl of oil with the present combustion tube, which sets a limit of detection of 0·01 per cent. *m/m* of sulphur. The use of a "repeater" type of syringe may overcome this difficulty.

(*ii*) Various sulphur compounds are combusted to sulphur dioxide with different efficiencies.

(*iii*) About 7 per cent. of the samples tested gave results for sulphur content by coulometry that were about 8 per cent. higher than those given by the quartz-tube method.

FUTURE DEVELOPMENTS—

The method described will not permit the determination of sulphur at levels below 100 $\mu g\ g^{-1}$. Preliminary work with a Vibron electrometer as a detector has shown that it is possible to detect 1 $\mu g\ g^{-1}$ of sulphur in a 1-μl sample by this technique. It appears that this technique could form the basis of a routine method.

The author thanks the Board of Laporte Industries Limited for permission to publish this work, and F. C. A. Killer and K. E. Underhill of Esso Petroleum Company Limited, Abingdon, Berkshire, for obtaining the Dohrmann coulometric results.

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Received *December* 13*th*, 1971
Accepted *August* 15*th*, 1972

Analyst, December, 1972, Vol. 97, *pp.* 937–939

The Determination of Sulphur Dioxide in Meats

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Several methods were compared in determining sulphur dioxide in meat samples that contained no added sulphur dioxide but that had various pH values, in order to determine whether or not false positive results were obtained owing to the presence of hydrogen sulphide in the meat. The recovery of sulphur dioxide that had been added to meat samples was also studied.

An iodimetric method gave false positive results for sulphur dioxide. A modified Monier-Williams method did not give false positive results, but it has limited sensitivity, giving a possible error of ±6·4 p.p.m. with a 10-g sample. The relative merits of each method are discussed.

In some countries, sulphur dioxide is a permitted additive to minced meat and sausages and its presence minimises oxidative colour changes and inhibits microbial spoilage[1] for short periods. Unfortunately, sulphur dioxide inhibits thiamine activity, and for this and other reasons its use in meat is prohibited in many countries. When the presence of sulphur dioxide in meat is in dispute, the method for its determination must be sensitive and reliable and it must not give false positive results.

From time to time it has been reported that meat samples have contained low levels of sulphur dioxide although the addition of this preservative was denied by the packers. In order to determine whether or not currently used methods can give false results for sulphur dioxide, a range of meat samples with various pH values but containing no preservative was analysed by the Monier-Williams method[2] with Shipton's modification.[3] This method is still recognised as a standard procedure for determining sulphur dioxide in foods. The samples were also analysed to determine sulphur dioxide by an iodimetric method and to determine hydrogen sulphide by the colorimetric method of Marbach and Doty.[4] Samples with different pH levels were used because Johnson and Vickery[5] showed that the amount of hydrogen sulphide that is produced during the heating of meat is directly related to the pH of the meat.

Experimental

Samples of gastrocnemius muscle were taken from nineteen beef carcases and stored at −40 °C prior to analysis. Seventeen of the nineteen animals had received adrenaline injections before slaughter in order to produce a wide range of ultimate pH values in the carcases. The high pH values produced in some of the muscles would not occur very frequently in commercially produced beef. The following treatments were applied to each sample in duplicate:

(*i*) The meat was thawed and finely minced.

(*ii*) The pH of a slurry of 10 g of meat and 20 g of water was determined.

(*iii*) A 50-g sample of minced meat was analysed to determine sulphur dioxide by Shipton's method[3] by using 0·3 per cent. *m*/*m* hydrogen peroxide in the trap and titrating with 0·001 N sodium hydroxide solution.

(*iv*) A 10-g sample of minced meat was treated as in (*iii*) but the hydrogen peroxide trap was replaced with one containing cadmium hydroxide solution so as to absorb hydrogen sulphide in accordance with the colorimetric method described by Marbach and Doty.[4]

(*v*) A 50-g sample was boiled with 0·5 N hydrochloric acid but the gas released was passed through a trap containing 20 ml of 0·001 N iodine solution and then through a trap containing 10 ml of 0·001 N sodium thiosulphate solution so as to collect any released iodine. This procedure was used to show whether iodimetric methods might give small false positive results for sulphur dioxide in the analysis of meats.

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To measure the efficiency of the recovery of sulphur dioxide from meat, two samples with pH values near the extremes of the range, 7·0 and 5·85, respectively, were treated with solutions of sodium pyrosulphite so as to give levels of sulphur dioxide of 500, 250 and 100 p.p.m. in the meat; the samples were then analysed by Shipton's method.[3]

RESULTS

The pH of the beef samples ranged from 5·6 to 7·15. None of the samples gave positive results when analysed to determine sulphur dioxide by the modified Monier-Williams method.[3] With the iodimetric procedure, the apparent sulphur dioxide level ranged from 2·43 to 4·04 p.p.m. (with a mean value of 2·95 p.p.m.); when calculated as hydrogen sulphide these levels corresponded to 1·29 to 2·14 p.p.m. with a mean value of 1·57 p.p.m. These latter values are similar to hydrogen sulphide values found by the colorimetric method,[4] which range from 1·28 to 2·15 p.p.m. with a mean of 1·89 p.p.m. The regression coefficients for pH *versus* hydrogen sulphide concentration and for pH *versus* apparent sulphur dioxide concentration were both 0·02, and it is concluded that the pH of the sample had no significant effect on the results.

The recoveries of added sulphur dioxide from meat samples were similar for samples of different pH but the recovery decreased as the level of addition of sulphur dioxide decreased. The results suggest that about 20 p.p.m. of sulphur dioxide is bound to the meat and is not determined by the Monier-Williams method[2]:

Sulphur dioxide added, p.p.m.	500	250	100
Mean recovery (4 determinations), per cent. ..	93·3	91·4	86

DISCUSSION

The quantitative Monier-Williams method[2] or its modifications, such as that of Shipton,[3] did not give false positive results when used to determine sulphur dioxide in meat. As hydrogen sulphide may be evolved from meat that contains no sulphur dioxide under the conditions of the A.O.A.C. qualitative test,[2] "trace" positive results may arise from the darkening of lead(II) acetate paper by this hydrogen sulphide as well as by hydrogen sulphide produced from sulphur dioxide.

The iodimetric procedure gave positive results in the absence of sulphur dioxide and this method should not be used when the presence of the preservative is in dispute.

The limits of the sensitivity of the Monier-Williams method as given by the A.O.A.C.[2] should be fully appreciated. Titration of the acid formed in the hydrogen peroxide trap is normally carried out by using 0·1 N sodium hydroxide solution, of which 1 ml is equivalent to 3·2 mg of sulphur dioxide. Hence, with a 10-g sample, a 1-ml titre is equivalent to 320 p.p.m. of sulphur dioxide. Burettes graduated at 0·01-ml intervals are commonly used and give an absolute limit of $\pm$3·2 p.p.m. with a 10-g sample. Increased sensitivity might be obtained by using larger samples of meat, but there still remains the limitation imposed by the indicator, bromophenol blue, which normally requires more than 0·02 ml of 0·1 N sodium hydroxide solution in order to produce a detectable colour change. There is therefore a possible error of $\pm$6·4 p.p.m. with a 10-g sample. The A.O.A.C. procedure[2] specifically warns that determinations of small amounts of sulphur dioxide that require a titre of less than 0·5 ml are subject to error.

Another method for determining sulphur dioxide, a spectrophotometric procedure in which the malachite green test of Richter and Kny[6] is used, is stated to be subject to interference by hydrogen sulphide under certain conditions. However, the method is generally limited to amounts of 25 p.p.m. or more and these amounts would be determined more conveniently by the standard Monier-Williams quantitative method.

CONCLUSION

A series of meat samples covering a wide range of pH values produced, during refluxing, amounts of hydrogen sulphide, which, on being titrated iodimetrically, could be reported as sulphur dioxide. However, such levels of "apparent sulphur dioxide" did not exceed 4 p.p.m.

The Monier-Williams method, as described by the A.O.A.C., does not give false positive values when meat is free from sulphur dioxide and is recommended when the presence of this preservative in meat is in dispute.

The meat samples used in these experiments were prepared at the Meat Research Laboratories of the Division of Food Research, Cannon Hill, Brisbane, under the supervision of Dr. W. R. Shorthose.

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Received *June 19th*, 1972
Accepted *June 28th*, 1972

Analyst, December, 1972, Vol. 97, *pp*. 940–950

The Determination of Organocarbon in Clay Materials

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A dry-combustion method is described for the determination of organocarbon in clay materials. The essential features are the use of purified oxygen; slow insertion of the sample into the furnace to avoid rapid dehydroxylation of the kaolinite and attendant loss of pyrolysis fragments in the atmosphere of steam that is generated; and the use of traps to remove gaseous fluorine compounds, steam and sulphur dioxide. Extensive tests on the apparatus with calcium carbonate and with mixtures of kaolin with glucose and with tannic acid are described. Tests with a commercial kaolin containing organic matter equivalent to about 550 μg of carbon per gram of material indicated a standard error of 8 μg, or $\pm 1 \cdot 5$ per cent. A published method in which the kaolin is heated in a stream of oxygen containing 2 per cent. V/V of ozone has been re-investigated; the organocarbon was only partially oxidised so that the method has no analytical value. Two wet-combustion methods involving the separate use of potassium dichromate and potassium persulphate, which are used in soil analysis, were evaluated. The organic matter on the clay was again only partially oxidised to carbon dioxide under the experimental conditions.

All naturally occurring clay materials have some organic impurities adsorbed on to their surfaces. Within the Cornish deposits, amounts vary from pit to pit and indeed within a given pit. Typical values for the refined English clays are about 500 μg of carbon per gram of clay. Although we were not specifically concerned with ball clays in this work, the amounts of organic matter involved are relatively large, being about 10 mg of carbon per gram of clay. Such organic matter includes the lignite that is mined together with the ball clay.[1,2]

The organocarbon derives from the decay of vegetable material and is probably chemically similar to the humic and fulvic acids extensively studied by soil chemists. Tannins are found[3] on some clays. The biochemistry of humus formation is reviewed by Kononova,[4] while Steelink[5] gives an excellent introductory survey of the humic acids, which are a complex mixture of compounds based on polymerised polyhydric phenols with various substituent groups including the carboxylic acid group.[6–8]

Organocarbon is inevitably a complicating feature in studies of the surface chemistry of kaolins. Further, it absorbs visible radiation and so reduces the brightness of the clay.[9] Because of this effect, a number of commercial oxidative treatments have been developed[10–13] in which the organic impurities are degraded to less coloured or colourless species. We know of no method other than a destructive one that will completely remove organic impurities from a kaolinite surface. Further, there is evidence for surface attack on clay minerals[14–17] during even mild oxidative treatments.

There is extensive literature on the determination of organocarbon in soils in which amounts are typically a few per cent. m/m with an upper limit of about 30 per cent. m/m for the peats. The subject has been reviewed by Allison, Bollen and Moodie[18] and by Debras-Guédon.[19] The methods generally involve oxidation of the organocarbon to carbon dioxide followed by its determination by one of the available methods; the oxidation can be carried out under "wet" or "dry" conditions. Of the wet-oxidation methods, that developed by Allison and co-workers[18,20] has become accepted as the most reliable. In this method, the sample is refluxed with a solution of potassium dichromate in a mixture of sulphuric and orthophosphoric acids and the carbon dioxide evolved is displaced in a current of air, absorbed and weighed. With calcareous materials it is necessary first to pre-treat the soil, usually with either sulphurous or diluted orthophosphoric acid. Various modifications

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aimed at increasing the speed of analysis have been described.[21–24] Of the dry methods, that involving a high-frequency induction furnace[25,26] is claimed to permit about 150 determinations to be performed in 1 day; combustion accelerators are used and the sample is heated to 1650 °C in a stream of oxygen. A novel method involving a serial oxidation - reduction procedure has been described[27] in which complete oxidation to carbon dioxide is followed by reduction to methane, which is then determined with a hydrogen flame-ionisation detector. A precision of ±5 per cent. is claimed for the determination of microgram amounts of organocarbon contained in milligram amounts of samples. Methods involving oxidation with chromic acid followed by determination of the unused chromic acid are impaired by several serious errors and their use is not advocated.[23]

With clay materials, amounts of organocarbon are generally less than 0·1 per cent. *m*/*m*, so that refined analytical techniques become necessary. As an example, the blank value for organocarbon by the Allison, Bollen and Moodie method[18] is 220 to 330 μg compared with the total of 1500 μg or so determined here. We have found serious errors when applying the conventional dry-combustion method to clay materials rich in kaolinite. Pyrolysis fragments are swept out of the reaction zone in the atmosphere of steam formed by dehydroxylation of the kaolinite. Similar difficulties were experienced in the measurement of organic substances dissolved in sea water.[28] We have modified the dry-combustion procedure and, as an example, about 1500 μg of organocarbon contained in a 3-g sample of kaolin can be determined with a precision of ±4 per cent. and a comparable accuracy. The method suggested by Wiegmann and Horte[29] involving oxidation with a mixture of ozone and oxygen has been evaluated and found unsatisfactory. Two of the wet-combustion methods[18,20,30] have been critically evaluated and found to fail in that the organocarbon on the clay is not completely oxidised.

EXPERIMENTAL

REAGENTS—

Absorption solution—Add 10 ml of thymolphthalein indicator solution to 50 ml of monoethanolamine and dilute the mixture to 1 litre with dimethylformamide.

Benzoic acid—Analytical-reagent grade.

Calcium carbonate—Analytical-reagent grade.

Chromic acid - sulphuric acid solution—Dissolve 10 g of chromium trioxide in 10 to 20 ml of water and make the volume up to 1 litre with concentrated sulphuric acid.

Dimethylformamide—General-purpose reagent grade.

D-*Glucose, anhydrous*—Analytical-reagent grade.

Magnesium perchlorate—Anhydrone, 14 to 22 mesh.

Monoethanolamine—General-purpose reagent grade.

Potassium persulphate—General-purpose reagent grade.

Kaolin—SPS* grade. This clay contains about 95 per cent. *m*/*m* of kaolinite with the balance present largely as mica with trace amounts of feldspar.

Soda-asbestos—Carbosorb brand.

Tannic acid—General-purpose reagent grade.

Tetrabutylammonium hydroxide, 0·1 M *in toluene - methanol*—General-purpose reagent grade. Dilute 200 ml of the reagent to 1 litre with analytical-reagent grade toluene *plus* the minimum volume of analytical-reagent grade methanol to ensure complete miscibility. This solution is allowed to stand for 24 hours before use.

Thymolphthalein indicator solution—Prepare a 0·10 per cent. *m*/*V* solution of thymolphthalein in analytical-reagent grade methanol.

CONTAMINATION OF SAMPLES—

We have observed that a dried clay shows an apparent increase in organocarbon content when stored in a polyethylene container; sorption of a plasticiser or antioxidant is suspected to be the cause. In one experiment, 50 g of kaolin were placed in contact for 24 hours with 10 g of polyethylene in the form of strips cut from a bag and the organocarbon content of the kaolin increased from 540 to 700 μg g^{-1}. Polyethylene bottles are less objectionable than polyethylene bags. The use of both should be avoided and in our work glass jars were used for storage. Gibbs[31,32] has shown that asbestos samples become seriously contaminated when stored in polyethylene bags.

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DRY COMBUSTION

BASIS OF THE METHOD—

The clay material is heated at 900 °C in a stream of purified oxygen, the organocarbon being converted into carbon dioxide, which is removed from the gas stream by passage through a solution of monoethanolamine in dimethylformamide. The dissolved carbon dioxide is then determined by titration with a solution of tetrabutylammonium hydroxide in a mixture of toluene and methanol, with thymolphthalein as indicator—

$$CO_2 + (n\text{-}C_4H_9)_4.NOH \rightarrow (n\text{-}C_4H_9)_4.NHCO_3$$

Evidently, 0·24 mg of carbon as carbon dioxide is equivalent to 1 ml of 0·02 M tetrabutylammonium hydroxide solution. The technique is therefore a modification of Pregl's method, which incorporates some of the procedures used by Jones, Gale, Hopkins and Powell[33,34] to determine carbon in iron and steels.

When, as in the present work, the clay material is rich in kaolinite, several experimental problems emerge. The ignition of 3 g of kaolinite, the amount used here, releases about 0·4 g of water as steam, which has two consequences. Firstly, if the experimental conditions are such that the dehydroxylation is rapid, pyrolysis fragments pass through the hot zone of the furnace in an environment of steam, and combustion is incomplete. Secondly, this steam must be removed from the oxygen stream with a desiccant, otherwise carbon dioxide is lost through dissolution in the water that condenses.

Certain impurities, principally sulphur dioxide (from the trace amounts of sodium sulphate in commercial clays after processing, about 0·05 per cent. *m*/*m*) and silicon tetrafluoride *plus* hydrogen fluoride (from the combined fluorine present in ancillary minerals, about 0·15 per cent. *m*/*m*), which enter the gas stream during calcination, interfere in the titration and must be removed.

APPARATUS—

The apparatus (Fig. 1) consists of two mullite tubes contained in a twin-bore furnace with a central zone at 900 °C (the choice of temperature is discussed below). The mullite tubes are attached to Pyrex tubing through brass collars with an Araldite resin. Oxygen of commercial purity from a cylinder passes through a needle valve and then a rotameter into the first mullite tube, M_1. Hydrocarbon impurities contained in the oxygen are oxidised to carbon dioxide and water, which are removed in turn by passage through trap T_1 containing Carbosorb and then trap T_2 containing Anhydrone. The purified oxygen enters the second mullite tube, M_2, and, after passing over the sample at Z, contains fluorine compounds, water, sulphur dioxide and carbon dioxide.

Preliminary experiments established that, while either sodium fluoride or manganese dioxide satisfactorily removed[35,36] the fluorine compounds from the gas stream, their use involved an unacceptable loss of carbon dioxide. Two silver gauzes (Note 1) in the mullite tube M_2 placed at positions W and X, corresponding to temperatures of 680 and 180 °C, respectively, proved acceptable. No detectable loss of carbon dioxide occurred and the fluorine compounds were satisfactorily removed.

NOTE 1—

The silver gauzes lose their efficiency after extended use, which can be restored by steeping the gauzes in ammonia solution (sp. gr. 0·88) followed by a short immersion in concentrated nitric acid; they are rinsed in water and then in ethanol and heated to dull redness.

The gas stream leaving the mullite tube is bubbled through chromic acid - sulphuric acid (traps T_3 and T_4) to remove sulphur dioxide and water and the line from the apparatus up to and including trap T_3 is heated to about 50 °C so as to prevent condensation of water vapour. Sulphuric acid was chosen instead of other desiccants only after extensive trial experiments; in particular, we experienced significant losses of carbon dioxide with the use of magnesium perchlorate.[26]

The carbon dioxide is removed from the oxygen stream by passing it through the absorption solutions in flasks F_1 and F_2, each flask carrying a 10-ml microburette B fitted with a PTFE stopcock. The microburettes, which are connected through joint-free glass tubing to a reservoir containing the 0·02 M tetrabutylammonium hydroxide solution, are filled by applying positive air pressure to the reservoir. All solutions are protected from atmospheric carbon dioxide and water vapour with guard tubes containing Carbosorb and

then Anhydrone. In test experiments in which calcium carbonate, glucose and tannic acid were used, the titre from flask F_2 was in general less than 0·5 per cent. of that from flask F_1. For practical purposes, all the carbon dioxide (Note 2) was removed from the oxygen stream after passage through flask F_1. Flask F_2 was retained because of a complication (discussed below), which emerged when analysing clay materials.

NOTE 2—
Carbon dioxide is more soluble in a mixture of ethanolamine and dimethylformamide than in dimethylformamide alone. Amine additions, however, tend to give a less sharp end-point[37,38] in the titration with tetrabutylammonium hydroxide. This effect is least evident with ethanolamine and the addition used in the present work was acceptable.

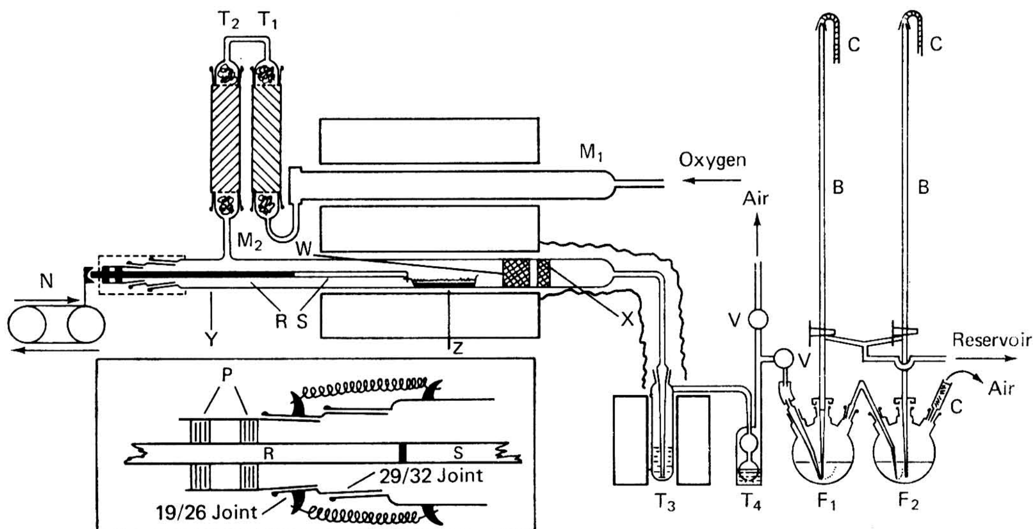
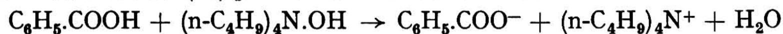


Fig. 1. Apparatus used to determine organocarbon by the dry-combustion method: B, 10-ml micro-burettes fitted with side-arm connected to reservoir containing tetrabutylammonium hydroxide; C, guard tubes filled with Carbosorb and then Anhydrone; F_1 and F_2, 250-ml round-bottomed three-necked flasks (Quickfit 24/29 centre; 19/26 side); M_1 and M_2, mullite tubes each 80 cm long and 2·5 cm i.d.; N, automatic drive; P, rubber plug; R, brass rod, 6·5 mm diameter, attached to silica rod S, 6·5 mm diameter; T_1, tube filled with Carbosorb, 23 cm long and 3·4 cm i.d.; T_2, tube filled with Anhydrone, dimensions as for T_1; T_3, trap containing the chromic acid - sulphuric acid at 50 °C; T_4, trap containing the chromic acid - sulphuric acid at room temperature; V, greaseless taps (Springham); W, silver gauze at 680 °C; X, silver gauze at 180 °C; Y, position of silica boat containing clay material at start of experiment; and Z, 900 °C zone of furnace representing final position of silica boat at completion of experiment

PROCEDURE—

Benzoic acid is used to standardise the tetrabutylammonium hydroxide solution. A known amount (about 15 mg) is added to the absorption solution, which is continuously agitated with a magnetic stirrer. Ten repeat determinations of the molarity of one particular batch of tetrabutylammonium hydroxide gave a mean value of 0·019 84 and a standard error (of the mean) of ±0·000 03. [Mean values and standard errors are expressed below in the form 0·019 84 ± 0·000 03 (10).] The reaction is as follows—



$$C_6H_5.COOH + (n\text{-}C_4H_9)_4N.OH \rightarrow C_6H_5.COO^- + (n\text{-}C_4H_9)_4N^+ + H_2O$$

At the start of a series of determinations, each burette is flushed with the tetrabutylammonium hydroxide solution and then filled. Approximately 50 ml of the absorption solution are added to each of the flasks F_1 and F_2 and the apparatus is flushed with oxygen. The gas blank is next determined for reference purposes as follows. The absorption solution in flask F_1 is titrated to the blue form and oxygen is passed through the apparatus at a rate of 100 ml min^{-1} (S.T.P.) for about 10 minutes. Values were generally in the range 0·5 to 1·0 μg min^{-1} of carbon dioxide (as carbon). The gas blank for the solution in flask F_2 is generally negligible and therefore need not be determined.

A pre-ignited silica boat (Note 3) containing the sample is inserted into the mullite tube, M_2, to position Y and left there for 10 minutes. The absorption solutions in flasks F_1 and F_2 are next titrated to the blue form. The brass rod, R, attached to the silica rod, S, is pushed through the rubber plugs, P, by using a variable-speed electrically operated drive; the boat traverses the 30-cm distance from Y to Z at a low rate (about 3 cm min^{-1}). We term this *slow insertion* in order to distinguish it from a second method called *rapid insertion,* which is used for comparative tests only. In the rapid insertion method the brass rod, R, is pushed manually through the rubber plug so as to bring the boat into the 900 °C zone of the furnace as rapidly as possible. In this method, the oxygen flow-rate is 200 ml min^{-1} (S.T.P.).

NOTE 3—
Provided that each silica boat was ignited to 1000 °C in air and cooled in a carbon dioxide free atmosphere, the correction for the boat was equivalent to only 5 μg of carbon.

The burette reading from flask F_1 is recorded and used to construct a graph of titre *versus* time (graph 1, Fig. 2). The part AB of the graph has a small positive slope that reflects the gas blank, and it is desirable to accumulate sufficient experimental points to permit an extrapolation back to zero time. With correct operating conditions, the magnitude of the gas blank correction is comparable with the value determined at the start. Any discrepancy is usually an indication that fluorine compounds are no longer being removed and that the silver gauzes need to be cleaned.

Close inspection of graph 1 in Fig. 2 reveals a small discontinuity close to X after about 35 minutes when about 90 per cent. of the organocarbon had been converted into carbon dioxide. At about this time the titre from flask F_2 generally increased from zero to follow graph 2 shown on the expanded scale in Fig. 2. The increases in titre coincided with fumes appearing in both flasks and corresponded with the boat entering the 900 °C zone of the furnace. Extensive experiments with deliberate additions of calcium fluoride and topaz proved that the fumes were not due to sulphur dioxide or fluorine compounds. In precise determinations, these increases in titre must be taken into account, otherwise the organocarbon content of the clay will be over-estimated. We assumed that the titre from flask F_1 was increased by the amount recorded from flask F_2. The procedure adopted was to subtract graph 2 from graph 1, allowing for the different scales, and to calculate a corrected graph 3 (not shown in Fig. 2), which is extrapolated back to zero time in the manner described above. In the example shown, the correction reduces the value of the organocarbon content derived by the simpler procedure by about 2·5 per cent.

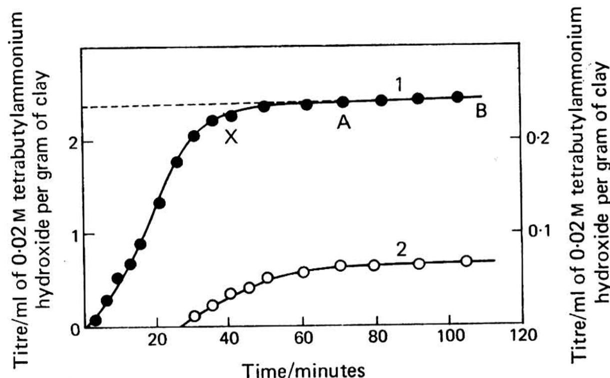


Fig. 2. Determination of organocarbon by the dry-combustion method (slow insertion). Graph 1 (left-hand scale), titre from flask F_1; and graph 2 (right-hand scale), titre from flask F_2 on expanded scale

The absorption solution in flask F_1 was discarded after every sixth determination and the chromic acid - sulphuric acid mixture in traps T_3 and T_4 was replaced after each determination.

TESTS WITH CALCIUM CARBONATE AND WITH GLUCOSE—

Tests were made with calcium carbonate and, by using nominal amounts of sample of 8, 15 and 30 mg, the measured carbon dioxide contents were 42·8 ± 0·1 (8), 42·9 ± 0·1 (7) and 42·9 ± 0·1 per cent. (7), respectively; analysis by dissolution in standard hydrochloric acid followed by back-titration with standard sodium hydroxide solution gave 43·3 per cent.

Fewer tests were made with glucose. With 12-mg samples, the measured carbon content was 39·8 ± 0·1 per cent. (5), thus demonstrating an almost quantitative conversion of combined carbon into carbon dioxide (theoretical value, 40·0 per cent.).

CLAY ANALYSIS—

The organocarbon content of the particular SPS clay used for evaluation was such that a 3-g sample gave a convenient titre and this amount was used in the experiments described below. Extensive preliminary experiments by using the rapid insertion method showed that 900 °C was the lowest suitable operating temperature consistent with an acceptable oxidation rate.

The rapid and the slow insertion methods were compared. Typical graphs of titre against time (flask F_1 only) are shown in Fig. 3, from which it is evident that the rapid insertion method gives the lower value. Repeat determinations gave 380 ± 5 $\mu g\, g^{-1}$ (5) and 548 ± 8 $\mu g\, g^{-1}$ (7) for the rapid and slow methods, respectively.

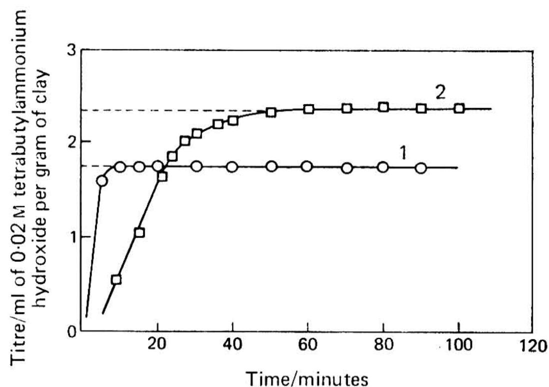


Fig. 3. Determination of organocarbon by the dry-combustion method before correcting for the gas blank. Titre is plotted against time. Graph 1 refers to rapid insertion and graph 2 refers to slow insertion

The lower value is attributed to incomplete combustion of organic matter from loss of pyrolysis products in the 2 litres of steam (at 900 °C and 1 atmosphere), which are generated when the kaolinite dehydroxylates. Such dehydroxylation occurs extremely rapidly at 900 °C. With the slow insertion method, dehydroxylation is slower so that there is always sufficient partial pressure of oxygen around the sample boat to ensure complete oxidation. (Thermogravimetric experiments with a bed thickness similar to that in the silica boat showed that at 450 °C, for example, dehydroxylation was only 20 per cent. complete after 30 minutes.)

The conditions for slow insertion were arbitrarily chosen and it is possible that the rate of insertion could have been increased over that used. That the conditions were adequate was demonstrated by experiments with mixtures of clay and glucose and of clay and tannic acid.

An aqueous solution of glucose (about 0·5 ml) of known concentration was run into a boat from a weighed container and the water evaporated at 80 °C so as to leave a film of glucose along the length of the boat. Next, about 3 g of kaolinite were placed in the boat and the total carbon was determined by the slow insertion method. This procedure was

repeated with different amounts of glucose and an approximately constant mass of clay. For the analysis of a mixture containing a mass x of clay and a mass y of glucose, the measured carbon content of the mixture is given by

$$M = Ax + By,$$

where A and B are the masses of combined carbon in unit mass of clay and in unit mass of glucose, respectively. The experimental results when plotted as M/x *versus* y/x fell on a good straight line (graph 1, Fig. 4); the method of least squares gave $A = 543 \pm 23$ μg of carbon per gram of clay and $B = 0{\cdot}397 \pm 0{\cdot}005$ g of carbon per gram of glucose with the indicated standard errors. The close agreement between B and the theoretical value (0·400) shows that the slow insertion method is adequate to oxidise the combined carbon in the glucose to carbon dioxide in the presence of the steam generated during the kaolinite dehydroxylation. The value of A is in good agreement with that found in the experiments with the clay alone [548 $\pm$ 8 μg g^{-1} (7)].

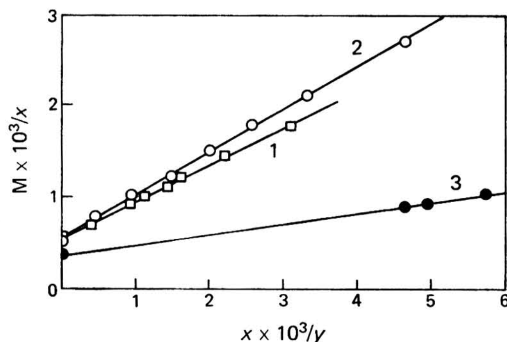


Fig. 4. Determination of organocarbon by the dry-combustion method by using the method of additions. Graph 1, clay *plus* glucose; graph 2, clay *plus* tannic acid; and graph 3, clay *plus* calcium carbonate

Similar experiments were carried out by using mixtures of tannic acid and clay; it was considered that the use of tannic acid, as it is chemically more similar to humic acid than to glucose, would be a more severe test. The results are shown as graph 2 in Fig. 4; the derived values with their standard errors were $A = 550 \pm 16$ μg of carbon per gram of clay and $B = 0{\cdot}463 \pm 0{\cdot}007$ g of carbon per gram of tannic acid. Again there is excellent agreement between the value for A and the values found above. The derived carbon content of the tannic acid is in good agreement with the value $0{\cdot}467 \pm 0{\cdot}003$ g g^{-1} (5) determined in the absence of clay.

When mixtures of calcium carbonate and clay were analysed by the rapid insertion method the plotted results fell on a good straight line (graph 3, Fig. 4) giving a correct value (B) of 0·114 g g^{-1} of carbon for the calcium carbonate but an incorrect value (A) for the clay of 350 μg g^{-1} of carbon. When mixtures of glucose and kaolinite were analysed by the rapid insertion method the value of B was typically 0·28 g g^{-1}, indicating incomplete combustion of the glucose and hence of the organic matter on the clay.

USE OF OZONE—

According to Wiegmann and Horte,[29] it is possible to remove organic matter from clays by heating them as a thin layer (the thickness was not specified in their original paper) to a temperature of 150 °C in a stream of oxygen containing about 3 per cent. V/V of ozone, which has the potential advantage of avoiding dehydroxylation of the kaolinite (and of most clay materials) and could lead to a simple and rapid analytical method.

We investigated the method by using a vertical glass tube containing a column of clay (2 cm in diameter and 10 cm high) supported on a sintered disc. The clay was heated for

4 hours in a stream of oxygen containing 2 per cent. V/V of ozone (Note 4) at a flow-rate of about 200 ml min^{-1} (S.T.P.). The clay was then thoroughly mixed and a 3-g sample used for the determination of carbon by the slow insertion method. Results covering the temperature range 20 to 200 °C are shown in Fig. 5; there is no advantage in heating to temperatures above 100 °C and at most only about 50 per cent. of the organocarbon is removed.

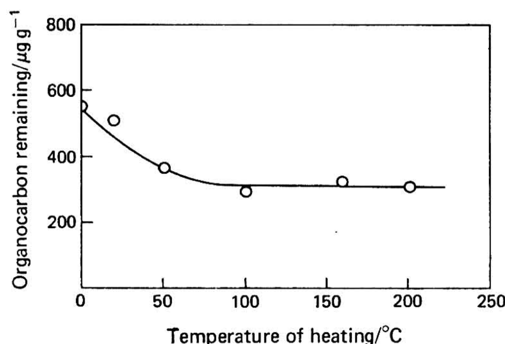


Fig. 5. Oxidation of organocarbon by heating the clay in a stream of oxygen containing 2 per cent. V/V of ozone. The amount of organocarbon remaining is plotted against temperature of heating

The method has no potential as an analytical technique. The discrepancy between our findings and those of Wiegmann and Horte[29] might be explained in terms of their use of differential thermal analysis to determine organocarbon; this technique can only be qualitative and the peak from the partially oxidised organic matter could well overlap that from kaolinite dehydroxylation.

NOTE 4—

There is disagreement between the various titrimetric methods[39–41] for ozone determination. We used neutral potassium iodide solution for absorption, then acidified the solution and finally determined the iodine by titration with sodium thiosulphate solution. A stoicheiometry of $I_2 \equiv O_3$ was assumed.

WET COMBUSTION

Several wet-oxidation methods have been used in soil analysis. We have investigated the applicability of two of these methods to the determination of organocarbon in clay materials and preliminary experiments led us to modify the published experimental methods. The methods and results obtained are given under the reagent system used.

POTASSIUM PERSULPHATE—

Potassium persulphate was used as an oxidant by Menzel and Vaccaro[30] in their work on determining organic impurities in sea-water samples. The experimental method that we developed can be understood by reference to Fig. 6. A known amount of clay (about 1 g) is placed in bulb A followed by about 0·1 g of potassium persulphate; 5 ml of 0·02 M orthophosphoric acid are added to bulb B. Nitrogen, free from carbon dioxide, is passed through the vessel for about 5 minutes to displace carbon dioxide. Tubes T_1 and T_2 are then heat sealed, care being taken to ensure that no combustion gases enter. The vessel is next tilted to allow the phosphoric acid to run on to the clay and the capsule is heated for 1 hour in an autoclave at 130 °C.

The carbon dioxide is determined by attaching the capsule via the ground-glass joints, J_1 and J_2, to the apparatus shown in Fig. 1 at a position between T_2 and M_2. Details of the apparatus are self-evident and are not shown. After chilling the vessel to reduce the internal pressure, the seals are broken in the order S_2 followed by S_1; the carbon dioxide is displaced with the purified oxygen and determined by non-aqueous titration as before. Any halides produced during the persulphate oxidation are removed by the silver gauzes (the gas is passed through aqueous potassium iodide solution in the original method).

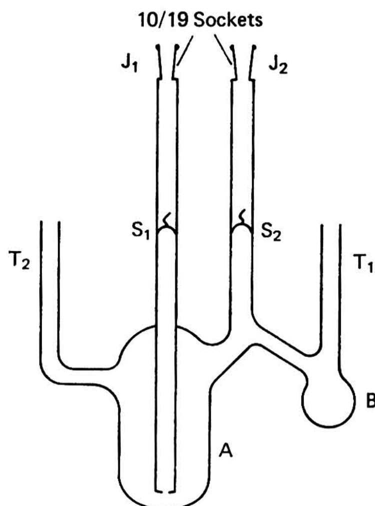


Fig. 6. Apparatus used to determine organocarbon by the wet-combustion method. The letters are referred to in the text

Before use, each vessel was heated in air to 550 °C for at least 1 hour so as to oxidise any organic matter. The combined vessel and reagent blank was equivalent to 9 μg of carbon and eight determinations on the reference clay gave 395 $\pm$ 5 μg of carbon per gram of clay. In other experiments, longer times of heating (4 hours) and the use of increased amounts of potassium persulphate (0·5 g) were investigated. The measured carbon content was essentially unchanged.

POTASSIUM DICHROMATE—

Potassium dichromate was used as an oxidant following the method developed by Allison, Bollen and Moodie,[18] and the experimental procedure was the same as that described above with about 1 g of potassium dichromate placed in bulb A after the addition of the known amount of clay (about 1 g); 6 ml of sulphuric acid (18 M) - orthophosphoric acid (16 M) mixture (3 + 2 *V*/*V*) are placed in bulb B. The capsule is then heated in an autoclave to 170 °C for 30 minutes. The combined reagent and vessel blank was equivalent to 39 μg of carbon and six determinations on the reference clay gave 389 $\pm$ 10 μg of carbon per gram of clay.

ILLUSTRATIVE EXAMPLES—

Two experiments are given to illustrate the application of the dry-combustion method. In the first, 10 g of clay were placed in contact with 100 ml of 100-volume hydrogen peroxide solution at 60 to 70 °C for 24 hours and then washed and dried. The organocarbon content of the clay had been reduced from 543 to 109 $\mu g\ g^{-1}$. In the second, an oxygen stream containing 2 per cent. *V*/*V* of ozone was passed through a 10 per cent. *m*/*m* slurry of the clay in water at room temperature for 24 hours, and the clay was then washed and dried. The organocarbon content was reduced to 305 $\mu g\ g^{-1}$.

It should be emphasised that in neither example was the organocarbon completely removed from the clay.

DISCUSSION

Dry-combustion methods used in soil analysis are inadequate for the determination of organocarbon in kaolins. The experimental difficulty arises from evolution of water from the kaolinite under the conditions of combustion. The organocarbon is not completely oxidised and pyrolysis fragments pass through the reaction zone in an atmosphere of steam. The measured organocarbon content, while highly reproducible, is well below the true value.

This fact was demonstrated by experiments with glucose - kaolin mixtures that gave the carbon content of the glucose as 28 per cent. *m/m* compared with the true value of 40 per cent. *m/m*. This discrepancy can be removed if the organocarbon is oxidised slowly, which is conveniently achieved experimentally by slowly moving the boat containing the sample into the hot zone (here 900 °C) of the furnace. Kaolinite dehydroxylation is now slow and we infer that there is always sufficient oxygen in the hot zone to oxidise any pyrolysis fragments to carbon dioxide. That the experimental conditions are adequate was proved by two series of experiments in which glucose - clay and tannic acid - clay mixtures were analysed. In each series the derived carbon content of the organic compound was close to the value determined in the absence of clay. Mixtures of calcium carbonate and clay should not be used for evaluation.

Wet-combustion methods are used in the analysis of waters and of soils. We first modified a published method[18] in order to reduce the vessel and reagent blanks and then applied the methods of Menzel and Vaccaro[30] and of Allison, Bollen and Moodie[18] to kaolin analysis, the two methods giving results in good agreement, $395 \pm 5\ \mu g\ g^{-1}$ (8) and $389 \pm 10\ \mu g\ g^{-1}$ (6), respectively. Both results are well below the value $548 \pm 8\ \mu g\ g^{-1}$ (8) determined by dry combustion with slow insertion. It is concluded that oxidation of the organocarbon was incomplete. It is interesting to note that the values agree with the $380 \pm 5\ \mu g\ g^{-1}$ (5) determined by dry combustion with rapid insertion but in the light of our experiments with additions of tannic acid and of glucose, such agreement is seen to be coincidental only. However, it illustrates the necessity of using standard additions of organic material in evaluating and comparing methods. We do not suggest that the dry and wet-combustion methods are inapplicable to soil analysis when amounts of organocarbon are relatively large and high accuracy is not required.

Our attention has been drawn to a recent paper by Read[42] in which a non-aqueous titrimetric method, which is closely similar to our own but devised for the determination of carbonate in silicate rocks, is described. In this method the carbonate was decomposed with dilute orthophosphoric acid. Read's use of lauric acid as a standard for the tetrabutylammonium hydroxide would appear to be preferable to our own use of benzoic acid. In the light of our extensive work on evaluating the wet-combustion procedures we would not advocate the extension of Read's method (as he suggests) to the determination of non-carbonate carbon in silicate rocks by wet oxidation with an orthophosphoric acid - chromic acid mixture.

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Received *March 2nd*, 1972
Accepted *July 24th*, 1972

Analyst, December, 1972, Vol. 97, *pp*. 951–957

Derivatives for the Identification and Quantitative Determination of Some Keto and Aldo-carboxylic Acids by Gas - Liquid Chromatography

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Studies on the derivatives of some physiologically and pathologically important keto and aldo-acids (pyruvic, glyoxylic, 2-oxoglutaric, oxaloacetic and 4-hydroxyphenylpyruvic) have shown that the *O*-ethoxime trimethylsilyl esters are the most suitable for quantitative gas - liquid chromatography. The *O*-trimethylsilyl oxime trimethylsilyl esters were thermally unstable under the conditions used. The *O*-methyl and *O*-benzyl oxime trimethylsilyl esters are also suitable for gas - liquid chromatography and would be useful in identification studies. The *O*-benzyl oxime trimethylsilyl derivatives are suggested as being especially suitable when only the keto and aldo-acids are to be studied, and it is not necessary to identify the lower boiling trimethylsilyl esters of other carboxylic acids on the same chromatogram.

Two aldehyde-specific reagents, dimedone and *NN'*-diphenylethylenediamine, were also examined for use with glyoxylic acid. The trimethylsilyl ester of 1,3-diphenylimidazolidine-2-carboxylic acid (the derivative of glyoxylic acid with *NN'*-diphenylethylenediamine) has a high boiling-point and is suitable for quantitative gas - liquid chromatography. This derivative should be especially useful in studies that are particularly concerned with the metabolism of glyoxylic acid or possibly other aldehydes.

The retention times of the derivatives relative to the retention times of n-tetracosane and n-hexacosane are compared with the corresponding values for the trimethylsilyl derivatives of some physiologically related carboxylic acids that do not contain keto or aldo groups.

AMINO-ACIDS have proved to be of great value as biochemical markers of inherited metabolic diseases, and it is likely that more of these disorders could be identified if other classes of chemical compounds could be used for this purpose. Some carboxylic acids are important intermediary metabolites, and isolated examples of their diagnostic use are already available (*e.g.*, propionic acidaemia,[1] methylmalonic acidaemia and aciduria[2] and the two types of primary hyperoxaluria[3]). Further advances in this area require the development of methods for the separation, identification and quantitative determination of closely related members of this group of compounds after they have been extracted from urine and blood. Gas - liquid chromatography is potentially the most useful basic method for this purpose.

Many of the physiologically important carboxylic acids contain other functional groups, such as keto, hydroxyl and aldo groups, and derivatives of these groups must be prepared in order to reduce the polarity and enhance the thermal stability and volatility required for gas - liquid chromatography. Trimethylsilyl derivatives are the most suitable for the gas - liquid chromatographic separation of carboxyl and hydroxyl compounds as their trimethylsilyl esters and trimethylsilyl ethers, respectively.[4] However, some of the keto compounds present special problems because of keto - enol tautomerism, as the two tautomers form trimethylsilyl derivatives that behave differently on gas - liquid chromatography. This effect makes qualitative work difficult and quantitative work impossible, but can be overcome by preparing a derivative of the keto group before silylating the other functional group. The aldo-acids also react with carbonyl-active reagents, but aldehyde-specific reagents are available and have therefore been considered in this work.

In this paper, an investigation into the relative merits of oximes, *O*-substituted (methyl, ethyl and benzyl) oximes and two specific aldehyde derivatives (the methone and 1,3-diphenylimidazolidine) for use as derivatives of some physiologically important keto and aldo-carboxylic acids prior to trimethylsilylation and quantitative gas - liquid chromatography is reported. Pyruvic, 2-oxoglutaric, oxaloacetic, 4-hydroxyphenylpyruvic and glyoxylic acids

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were selected as being representative of this group of compounds. The retention times of the derivatives relative to n-tetracosane ($C_{24}H_{50}$) and n-hexacosane ($C_{26}H_{54}$) are compared with the corresponding relative retention times for the trimethylsilyl derivatives of some physiologically related carboxylic acids that do not contain keto or aldo groups. The response factors of the keto and aldo-acid derivatives relative to an internal standard have been determined.

EXPERIMENTAL

APPARATUS—

Gas - liquid chromatography was carried out with a Hewlett-Packard F&M 402 gas chromatograph equipped with dual U-shaped columns and dual flame-ionisation detectors. Separations were carried out by using glass columns, 1·83 m long × 0·003 m i.d., packed with 10 per cent. of OV-101 on High Performance Chromosorb W (acid washed and dimethylchlorosilane treated) of 80 to 100 mesh that had been previously conditioned for 48 hours ("no flow") and with Silyl-8 column conditioner (Pierce Chemical Co. Inc.) as required.

MATERIALS—

Acids—2-Oxoglutaric, oxaloacetic and 4-hydroxyphenylpyruvic acids were obtained from Fluka A.G. through Fluorochem Ltd., pyruvic acid (free from dimer) from Sigma (London) Chemical Co. Ltd. and glyoxylic acid monohydrate from Koch-Light Ltd.

Silylating reagents—*NO*-Bis(trimethylsilyl)acetamide (BSA) and TriSil BSA (a formulation of BSA in pyridine) were obtained from Pierce Chemical Co. through Phase Separations Ltd.

Carbonyl-active reagents—*O*-Methylhydroxylammonium chloride and *O*-ethylhydroxylammonium chloride were obtained from Kodak Ltd., *O*-benzylhydroxylammonium chloride from Aldrich Chemical Co. through Ralph N. Emmanuel Ltd., and hydroxylammonium chloride from BDH Ltd.

Aldehyde-specific reagents—*NN'*-Diphenylethylenediamine (dianilinoethane) was obtained from Kodak Ltd. and 5,5'-dimethylcyclohexane-1,3-dione (dimedone) from BDH Ltd.

All other reagents were of analytical-reagent grade when possible. n-Tetracosane and n-hexacosane for use as internal standards in gas chromatography were obtained from Ralph N. Emmanuel Ltd. and Fluka A.G., respectively.

PREPARATION OF DERIVATIVES—

The derivatives were prepared in air-tight, screw-capped glass septum vials fitted with silicone-rubber septa.

Oximes—Hydroxylammonium chloride or its *O*-substituted derivative (10 to 20 mg) was added to the dry free acid to give a minimum five-fold molar excess of reagent, together with pyridine (0·4 ml), mixed and the mixture allowed to stand for 2 hours. BSA (0·5 ml) was then added together with 50 μl of tetracosane solution (0·100 g per 5·0 ml in n-heptane) and 50 μl of hexacosane solution (0·100 g per 5·0 ml in n-heptane), the solution was well mixed and the vial recapped and allowed to stand for at least 2 hours before gas - liquid chromatography. Typical reactions are shown in Fig. 1.

Dimedone derivative—The methone of glyoxylic acid was prepared and silylated as described for the oximes, by using dimedone (10 mg, 0·07 mmol) and glyoxylic acid monohydrate (1 mg, 0·01 mmol). The reaction is shown in Fig. 2.

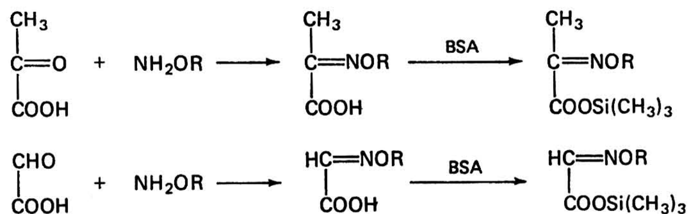

Fig. 1. Formation of *O*-substituted oxime trimethylsilyl esters. R = H, CH_3, C_2H_5 . . . $CH_2C_6H_5$ . . .; BSA is *NO*-bis(trimethylsilyl)acetamide. When R = H, further silylation occurs so that $>C=NOH \rightarrow >C=NOSi(CH_3)_3$

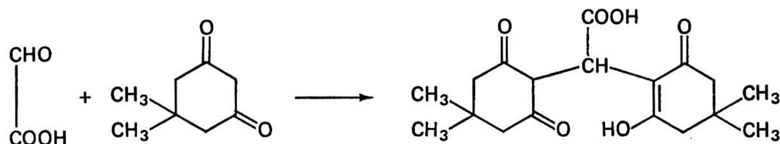

Fig. 2. Formation of glyoxylic acid methone [carboxymethylene bis(5,5-dimethylcyclohexane-1,3-dione)], which forms oximes on the oxo groups and trimethylsilylates on the hydroxyl and carboxylic acid groups

NN′-Diphenylethylenediamine derivative of glyoxylic acid—This derivative (1,3-diphenylimidazolidine-2-carboxylic acid) was prepared and silylated in a similar manner to the oximes, by using 10 mg (0·047 mmol) of *NN′*-diphenylethylenediamine per 2 mg (0·02 mmol) of free acid (as its monohydrate). The reaction is shown in Fig. 3.

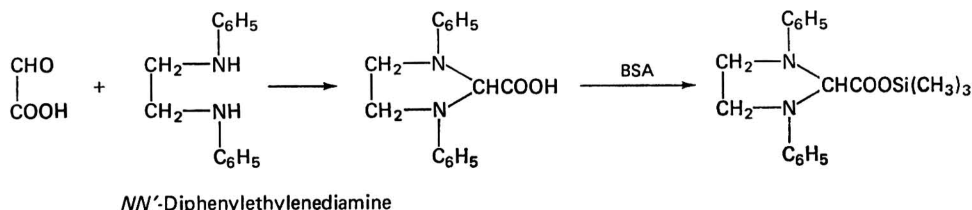

Fig. 3. Formation of 1,3-diphenylimidazolidine-2-carboxylic acid trimethylsilyl ester. BSA is *NO*-bis(trimethylsilyl)acetamide

Gas - liquid chromatography—

The derivatives were separated by using temperature programming from 110 to 280 °C at 5 °C min^{-1} with a 5-minute initial isothermal delay. Between 0·5 and 5 μl of each sample was injected as required. This procedure is described in detail elsewhere.[4]

In analyses involving only the diphenylethylenediamine derivative of glyoxylic acid, temperature programming from 210 to 280 °C at 5 °C min^{-1} was used, and n-hexacosane only was used as an internal standard.

Quantitative determinations were made by measurement of peak areas (peak height × width at half-height) with reference to the internal standard. When necessary, retention times relative to both n-tetracosane and n-hexacosane can be used for identification purposes.

Results

No sample peaks were observed in gas - liquid chromatography when hydroxylammonium chloride was used as reagent. Trimethylsilylated *O*-methyl, *O*-ethyl and *O*-benzyl oximes were all suitable for the determination of keto-acids. The peak of the trimethylsilyl-*O*-methyl oxime of glyoxylic acid overlapped completely the last peak in the BSA - pyridine reagent - solvent region, but the peaks of both the trimethylsilyl-*O*-ethyl and trimethylsilyl-*O*-benzyl oximes of this acid were well separated from other peaks.

The relative retention times of the trimethylsilyl esters of the *O*-substituted oxime derivatives of pyruvic, 2-oxoglutaric, oxaloacetic, 4-hydroxyphenylpyruvic and glyoxylic acids, that of 1,3-diphenylimidazolidine-2-carboxylic acid and those of the trimethylsilyl derivatives of some other physiologically important carboxylic acids that do not contain keto or aldo groups are presented in Table I.

Satisfactory separations of the individual peaks on the gas chromatogram were obtained by using the trimethylsilyl derivatives of either the methoxime, ethoxime or *O*-benzyl oximes of the keto and aldo-acids. The different *O*-substituents change the positions of the peaks relative to those of the simple trimethylsilyl esters and ethers; this effect, together with the use of the *NN′*-diphenylethylenediamine derivative, gives the method great flexibility. Trimethylsilyl esters and ethers and ethoximes are at present used as the combination of derivatives of first choice in our laboratory and a specimen gas chromatogram is published elsewhere.[4]

Linear relative response graphs were obtained for each type of derivative with all the acids studied. The regression equations for the linear graphs are summarised in Table II.

The dimedone reagent and its glyoxylic acid derivative reacted both with *O*-methylhydroxylamine and with the trimethylsilylating reagents. The final product from glyoxylic acid was insufficiently volatile for use in the present gas - liquid chromatographic system.

TABLE I

RETENTION TIMES OF DERIVATIVES OF SOME ORGANIC ACIDS AND OXO-ACIDS

Separation conditions: 1·83 × 0·003-m column of 10 per cent. of OV-101 on HP Chromosorb W (AW-DMCS) with temperature programming from 110 to 280 °C at 5 °C min^{-1} with an initial isothermal delay of 5 minutes; flow-rate of nitrogen carrier gas, 30 to 40 ml min^{-1}

Acid or compound*	Derivative*	Retention time/minutes	Retention temperature/°C		Retention time relative to n-tetracosane
Solvent - reagent tail peak	—	3·0	110	Isothermal	0·083
Glyoxylic (oxoethanoic)	TMS-MO	3·0	110	Isothermal	0·083
Pyruvic (2-oxopropanoic)	TMS-MO	4·3	110	Isothermal	0·119
Glyoxylic	TMS-EO	4·4	110	Isothermal	0·122
L-Lactic (2-hydroxypropanoic)	TMS	4·7	110	Isothermal	0·130
Glycollic (hydroxyethanoic)	TMS	5·0	110	Isothermal	0·138
Pyruvic	TMS	5·2	111		0·144
Pyruvic	TMS-EO	5·8	114		0·160
Oxalic (ethanedioic)	TMS	6·6	118		0·182
Methylmalonic (2-methylpropanedioic)	TMS	10·0	135		0·276
Glyoxylic	TMS	10·3	137		0·284
Benzoic	TMS	10·7	138		0·296
Succinic (butanedioic)	TMS	12·9	150		0·356
Fumaric (*trans*-butenedioic)	TMS	14·1	156		0·390
Oxaloacetic (2-oxobutanedioic)	TMS-MO	17·1	170		0·472
Oxaloacetic	TMS-EO	18·0	175		0·497
L-Malic (2-hydroxybutanedioic)	TMS	18·1	176		0·500
BO reagent	—	18·6	178		0·514
Glyoxylic	TMS-BO	20·4	187		0·564
2-Oxoglutaric (2-oxopentanedioic)	TMS-EO	20·8	189		0·575
2-Oxoglutaric	TMS (of enol form)	21·0	190		0·580
Hippuric (2-benzoylaminoethanoic)	TMS	25·2	211		0·696
Citric (2-hydroxypropane-1,2,3-tricarboxylic)	TMS	26·0	215		0·718
2-Oxoglutaric	TMS (of keto form)	26·3	217		0·727
4-Hydroxyphenylpyruvic [3-(4-hydroxyphenyl)-2-oxopropanoic]	TMS-EO	27·7	224		0·765
Undecanedioic	TMS	28·8	229		0·796
Oxaloacetic	TMS-BO	29·4	232		0·812
DIPED reagent	—	30·0	235		0·829
2-Oxoglutaric	TMS-BO	31·6	243		0·873
Glyoxylic	TMS-DIPED	35·7	264		0·986
n-Tetracosane	—	36·2	266		1·000
4-Hydroxyphenylpyruvic	TMS-BO	37·2	271		1·028
n-Hexacosane	—	38·2	280		1·055

* TMS = trimethylsilyl; MO = methoxime; EO = ethoxime; BO = *O*-benzyloxime; DIPED = *NN'*-diphenylethylenediamine derivative.

The *NN'*-diphenylethylenediamine derivative of glyoxylic acid proved to be thermally stable and is formed quantitatively in pyridine. The reagent did not react with either *O*-substituted oximes or with BSA under the conditions used, and the derivative was trimethylsilylated only on the carboxylic acid group introduced from the glyoxylic acid. The derivative was eluted at a high temperature (Table I) and tended to overlap with tetracosane so that hexacosane was more suitable for use as an internal standard. Hexacosane was essential when the high-temperature programming (210 to 280 °C) was used because the derivative and tetracosane peaks then overlapped completely. A linear relative response graph was obtained for the derivative by using hexacosane as internal standard (Table II).

TABLE II

RELATIVE RESPONSE OF KETO AND ALDO-ACID DERIVATIVES TO AN INTERNAL STANDARD EXPRESSED AS THEIR REGRESSION EQUATIONS

Acid	Derivative*	Internal standard	Linear concentration range studied/ µg	Number of determinations (N)	Least-square regression equation constants† a	b	Standard error of of the estimate‡
Oxaloacetic (2-oxobutanedioic)	TMS-methoxime	n-Tetracosane	0·5 to 7	10	−0·43	0·588	0·09
2-Oxoglutaric (2-oxopentanedioic)	TMS-methoxime	n-Tetracosane	0·6 to 8	12	−0·75	0·702	0·13
Glyoxylic (oxoethanoic)	TMS-ethoxime	n-Tetracosane	1 to 14	12	+0·21	0·376	0·36
Pyruvic (2-oxopropanoic)	TMS-ethoxime	n-Tetracosane	1 to 12	12	+0·11	0·441	$0{\cdot}26_5$
Oxaloacetic	TMS-ethoxime	n-Tetracosane	1 to 13	12	−0·500	0·487	$0{\cdot}23_5$
2-Oxoglutaric	TMS-ethoxime	n-Tetracosane	1 to 10	12	−0·72	0·672	0·12
4-Hydroxyphenylpyruvic [3-(4-hydroxyphenyl)-2-oxopropanoic]	TMS-ethoxime	n-Tetracosane	1 to 10	12	−1·36	0·653	0·15
Glyoxylic	TMS-*O*-benzyl-oxime	n-Tetracosane	0·5 to 10	12	−0·23	1·270	0·15
Pyruvic	TMS-*O*-benzyl-oxime	n-Tetracosane	0·4 to 8	12	−0·31	1·204	0·16
Oxaloacetic	TMS-*O*-benzyl-oxime	n-Tetracosane	0·4 to 9	12	−0·37	0·723	0·15
2-Oxoglutaric	TMS-*O*-benzyl-oxime	n-Tetracosane	0·4 to 9	12	$-0{\cdot}73_5$	1·110	0·35
4-Hydroxyphenylpyruvic	TMS-*O*-benzyl-oxime	n-Tetracosane	0·4 to 10	12	−1·64	0·725	0·37
Glyoxylic	*NN'*-Diphenyl-ethylenediamine	n-Hexacosane	0·4 to 10	38	−0·48	1·963	0·70

* TMS = Trimethylsilyl.

† The regression equation is given by $y = a + bx$ where $a = \frac{\Sigma y - b\Sigma x}{N}$ and $b = \frac{N\Sigma xy - (\Sigma x)(\Sigma y)}{N(\Sigma x^2) - (\Sigma x)^2}$; x = amount of acid (µg) and y = response relative to internal standard.

‡ The standard error of the estimate of y on x is $S_{yx} = \sqrt{\frac{\Sigma y^2 - a\Sigma y - b\Sigma xy}{N}}$.

Discussion

We required a single reaction that could be used to prepare derivatives of the keto and aldo-acids before trimethylsilylating the carboxyl and hydroxyl groups. Simple oximes proved to be unsatisfactory, as no sample peaks were obtained on gas - liquid chromatography of the *O*-trimethylsilyloxime derivatives, although these should have volatilities similar to *O*-t-butyl derivatives. This lack of peaks is contrary to the findings of Horii, Makita and Tamura[5] and may be attributed to the different experimental conditions used. The problem of double derivative formation owing to the tautomerism of 2-oxoglutaric and oxaloacetic acids was overcome by forming an *O*-substituted oxime before trimethylsilylation, and it is suggested that the group >C=N–O–C, which is unaffected by trimethylsilylation, confers greater thermal stability on the molecule than the >C=N–O–Si group, which is formed from the simple oximes. The *O*-methoxime trimethylsilyl derivative was initially abandoned because the glyoxylic acid derivative did not give a sample peak, which was attributed to conversion of the aldoxime into the corresponding nitrile.[6] However, further study showed that a thermally stable trimethylsilylated glyoxylic acid *O*-methoxime was formed but that it was eluted with the last peak of the solvent reagent front obtained from the BSA - pyridine mixture. This result agrees with earlier studies, which showed that more vigorous conditions than those which were used in this work are needed to convert aldoximes into nitriles.[6–9]

Devaux, Horning, Hill and Horning[10] used *O*-benzyl oximes as derivatives of the oxo-steroids, as the greater increment in the relative molecular mass offered some advantages over the methoxime derivatives for gas - liquid chromatographic characterisation. The present work shows that the trimethylsilylated *O*-benzyl oximes are also suitable derivatives for gas - liquid chromatography of the keto and aldo-acids, especially if other carboxylic acids are not of interest and a relatively high-temperature analysis is required. The formation of a thermally stable *O*-benzyl oxime of glyoxylic acid with a relatively low volatility was of particular interest and stimulated a search for another *O*-substituted oxime with a volatility intermediate between that of the *O*-methyl and *O*-benzyl derivatives in order to move the glyoxylic acid derivative peak away from the solvent - reagent region while retaining the over-all separations achieved when using the *O*-methoxime derivatives. The *O*-ethyl derivatives were selected as being potentially the most useful as the low increment in the relative molecular mass would be expected to achieve the required movement of the acids of low relative molecular mass such as glyoxylic acid without gross changes in the relative position of the oxo-acids of higher relative molecular mass, which were separated satisfactorily from the other carboxylic acids studied. This was shown to be the case in practice, the *O*-ethyl oximes being formed quantitatively in pyridine and giving linear response graphs with all the acids studied. The glyoxylic acid derivative peak is moved away from the reagent - solvent region and pyruvic acid, another oxo-acid of low relative molecular mass in the same region of the chromatogram, is also subjected to less interference from neighbouring peaks.

The formation of a derivative with an aldehyde-specific reagent sequentially before *O*-substituted oxime formation and trimethylsilylation makes the over-all method more flexible in special circumstances. Thus, it permits the use of the methoximes when this is advantageous from the point of view of the retention times of other acids that have to be studied, for example L-malic and oxaloacetic acids. It also allows for a situation in which the peaks of the trimethylsilylated glyoxylic acid *O*-ethoxime or *O*-benzyl oximes are not resolved from other interfering peaks, as may occur in the analysis of biological materials of unknown composition. Dimedone and its glyoxylic acid derivative react with both *O*-methylhydroxylammonium chloride and the trimethylsilylating reagent to form a product that is too involatile for use in the present gas-chromatographic system. *NN′*-Diphenylethylenediamine[11] reacted quantitatively with glyoxylic acid in pyridine solution and the final derivative, 1,3-diphenylimidazolidine-2-carboxylic acid trimethylsilyl ester, was suitable for gas - liquid chromatography and was less volatile than any of the other derivatives with which it was compared (Table I). The methanol used as solvent by previous workers[11] cannot be used with acids because of the partial formation of methyl esters.

Ethoxime formation followed by trimethylsilylation offers the most satisfactory procedure for the general study of physiologically important acids by gas - liquid chromatography based on the results obtained with the present range of organic acids. The methoximes

and *O*-benzyloximes would also be of use for identification purposes, and the use of the latter derivatives permits a high-temperature analysis in which the peaks of most of the other acids are in the solvent - reagent region of the chromatogram. These studies have also shown that the trimethylsilylated diphenylethylenediamine derivative of glyoxylic acid is suitable for quantitative gas - liquid chromatography and this derivative would be of use when only this acid or other aldehydes are to be studied.

This work will be submitted by R.A.C. in partial fulfilment of the requirements for the degree of Ph.D. of the Council for National Academic Awards.

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Received *February 29th*, 1972
Accepted *August 7th*, 1972

Analyst, December, 1972, Vol. 97, *pp*. 958–967

The Quantitative Extraction and Gas - Liquid Chromatographic Determination of Organic Acids in Urine

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A method for the quantitative extraction and gas - liquid chromatographic determination of organic acids in aqueous solution and biological fluids has been developed. The acids are extracted by a DEAE-Sephadex anion-exchange column, neutral and basic compounds are removed by washing with water and the acids are eluted with a pyridinium acetate buffer. The ethoximes of oxo-acids are formed in the eluate to stabilise these compounds and the eluate is then freeze-dried under carefully controlled conditions. The residue of dry free acids and their pyridinium salts is trimethylsilylated and the trimethylsilyl derivatives are separated and quantitatively determined by gas - liquid chromatography with the use of internal standards. The method would not be suitable for the determination of formic, acetic, propionic or butyric acid because of their volatility.

The accuracy and precision of the method, which can be applied to other protein-free biological fluids as well as urine, are discussed.

THE carboxylic acids present in urine and plasma have been little studied although the importance of these acids as indicators of diseases of metabolism, including some which present with mental retardation, is possibly as great as that of the amino-acids.[1] One of the major reasons for the deficiency of previous studies in this field is the lack of suitable methods and instrumentation for the qualitative and quantitative analysis of a wide range of acidic metabolites simultaneously. The use of gas - liquid chromatography enables such analyses to be carried out and this technique, combined with high-resolution mass spectrometry for absolute identification of the metabolites, should be an extremely powerful tool for metabolic investigations of this type. Before detailed quantitative studies on organic acids in man can be undertaken, however, sufficiently reliable and precise extraction and analytical methods must be available for their determination. In this paper, methods that have been developed for the quantitative extraction and determination of organic acids in biological fluids are described. The development of these methods and their application is discussed and reference is made to methods that have been used previously by other workers.

The organic acids that have been studied in the present work are representative of the members of this class of compounds, which are already known to be of either physiological or pathological importance.

EXPERIMENTAL

The organic acids are extracted from aqueous solution or urine by using an A25 DEAE-Sephadex anion-exchange column. The neutral and basic components are removed by washing with water and the acids are eluted with an aqueous pyridinium acetate buffer. The *O*-ethyl oxime derivatives of the oxo-acids present are prepared in the aqueous pyridinium acetate eluate so as to stabilise these compounds[2] and the extract is freeze-dried under carefully controlled conditions.[3] The dry residue of free acids is trimethylsilylated to give relatively non-polar trimethylsilyl esters of simple acids and trimethylsilyl ethers and esters of hydroxy-acids. The silyl derivatives are then separated and quantitatively determined by temperature-programmed gas - liquid chromatography.

APPARATUS—

Chromatographic columns—The columns were 9 cm long × 0·7 cm i.d. expanding to a reservoir 8 cm long × 2 cm i.d. at the top and fitted with a PTFE tap (2 mm bore) at the base (fabricated by D. Gill, Glass Engineering Section, C.R.C. Division of Bioengineering). The lower end of the column was plugged with a small amount of glass-wool.

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MATERIALS—

Glycollic, methylmalonic, hippuric and undecanedioic acids were obtained from Fluka A.G. through Fluorochem Ltd.; pyruvic (free from dimer), fumaric, succinic and L-malic acids from Sigma (London) Chemical Co. Ltd.; and oxalic acid dihydrate, citric acid monohydrate and benzoic acid from BDH Ltd. All of the compounds were of the highest purity available. Glyoxylic acid monohydrate was obtained from Koch-Light Ltd. *NO*-Bis(trimethylsilyl)-acetamide (BSA) and TriSil BSA (a formulation of BSA in pyridine) were obtained from Pierce Chemical Co. through Phase Separations Ltd. *O*-Ethylhydroxylammonium chloride for the preparation of oxo-acid derivatives was obtained from Kodak Ltd. A25 DEAE-Sephadex anion-exchange gel was obtained from Pharmacia Ltd. [^{14}C]oxalic acid and sodium [1-^{14}C]-glycollate were purchased from the Radiochemical Centre, Amersham, Buckinghamshire.

REAGENTS—

All reagents used were of analytical-reagent grade when possible and of the highest grade available in other instances. De-ionised water was used throughout, when appropriate.

Pyridinium acetate buffer, 0·5 M—This was prepared by diluting 40·3 ml of pyridine and 28·6 ml of glacial acetic acid to 1 litre with water. This buffer must be freshly prepared each week.

Pyridinium acetate buffer, 1·5 M—This was prepared by diluting 119 ml of pyridine and 90 ml of glacial acetic acid to 1 litre with water. This buffer must be freshly prepared each week.

Undecanedioic acid solution—This solution was prepared by dissolving 0·100 g of undecanedioic acid in the minimum volume of 0·1 N sodium hydroxide solution and diluting the solution to 100·0 ml with water.

Standard solutions of the acids—Standard solutions were prepared by dissolving 0·020 g of each acid in water, using the minimum volume of 0·1 N sodium hydroxide solution when necessary, and diluting to 100·0 ml with water.

n-Tetracosane and n-hexacosane solutions—These solutions were prepared by dissolving 0·100 g of the hydrocarbon in 5·0 ml of heptane [conforming to the I.P. specification for heptane (BDH Ltd.)].

PROCEDURE—

A 4·5 × 0·7-cm column of A25 DEAE-Sephadex was prepared by pouring an aqueous slurry of gel, previously swollen in water, into the chromatographic columns and allowing it to settle. The columns were washed with 3 ml of water and 20 ml of 0·5 M aqueous pyridinium acetate buffer prior to use.

An aliquot of the standard acid solution (0·25 to 10·0 ml) or of neutralised urine *plus* 1·0 ml of the standard undecanedioic acid solution (≡ 1·00 mg) was applied to the top of the column and allowed to drain in. The column was then washed with two 5-ml volumes of water and the acidic components were eluted with 15 ml of 1·5 M aqueous pyridinium acetate buffer into a 25-ml round-bottomed flask containing 50 mg of *O*-ethylhydroxylammonium chloride.

The eluate and *O*-ethylhydroxylammonium chloride were mixed and allowed to stand for 4 hours or longer. The eluate was frozen as a film by rotating the flask in a solid carbon dioxide - methanol bath and freeze-dried overnight at 0·5 torr and −10 °C as described below.

A 900-µl volume of TriSil BSA was added to the dry residue together with 50 µl of each hydrocarbon internal standard solution, mixed and the mixture was allowed to stand for at least 4 hours. Additional BSA should be added as necessary so as to give a clear solution before analysis.

The trimethylsilyl derivatives were then separated and quantitatively determined by gas - liquid chromatography, as described below, by using 5 µl or an appropriate volume for injection.

FREEZE-DRYING[3]—

An Edwards EF1 freeze-drier fitted with individual closures to each of the eight ports was used. This freeze-drier is equipped with an Edwards ED100 rotary vacuum pump and a Pirani gauge for measuring the vacuum produced, and an air inlet controlled by a needle valve for maintaining the required pressure.

Freeze-drying was carried out with the sample maintained at −10 °C and 0·5 torr; the receiver was maintained at −70 °C with solid carbon dioxide - methanol and the temperature of the sample was controlled by means of a eutectic mixture of potassium chloride and ice, contained in Dewar flasks; the latter method has been described previously.[3]

GAS - LIQUID CHROMATOGRAPHY—

A Hewlett-Packard F&M 402 gas chromatograph equipped with dual glass columns and dual flame-ionisation detectors was used. The columns used were U-shaped, 1·83 m long × 0·003 m i.d., packed with 10 per cent. of OV-101 on High Performance Chromosorb W (acid washed and dimethylchlorosilane treated) of 80 to 100 mesh (Hewlett-Packard Ltd.), and previously conditioned by "no flow" conditioning for 48 hours prior to use. Subsequent conditioning with Silyl-8 (Pierce Chemical Co. Inc.) was also carried out.

Separations were achieved by using linear temperature programming from 110 to 280 °C at 5 °C min^{-1} with a 5-minute initial isothermal delay. On-column injection was used with the injection port heater blocks at 190 °C (in the instrument used, these blocks are housed inside the oven and are heated during programming to about 300 °C; when the injection blocks are outside the oven, as in the Hewlett-Packard 5700 gas chromatograph, the blocks can be maintained at 250 °C); the detectors were maintained at 280 to 300 °C. Nitrogen (Air Products Ltd.) was used as the carrier gas at a flow-rate of 30 to 40 ml min^{-1} (cylinder output 40 p.s.i.g., two-stage regulator); hydrogen and air (Air Products Ltd.) were maintained at flow-rates of 20 ml min^{-1} (cylinder output at 10 p.s.i.g., two-stage regulator) and 200 ml min^{-1} (cylinder output at 40 p.s.i.g., two-stage regulator), respectively. Flow-rates were controlled by use of independent rotameters for each column and detector.

Sample volumes of the trimethylsilylated solution of 5 μl were generally used for injection. Quantitative determinations were made by measurements of peak areas (height × width at half-height) with reference to n-tetracosane as the internal standard. The response factors were derived from the regression equations in each instance. In routine urine analyses, reference is also made to undecanedioic acid as internal standard so as to correct for mechanical losses and to check for complete silylation. Peak identification, when necessary, was made by reference to the retention times of n-tetracosane and n-hexacosane.

RESULTS

COLUMN EXTRACTION—

The extraction procedure on the DEAE-Sephadex column was tested by adding acids labelled with carbon-14 to a carrier solution containing unlabelled acid. Oxalic and glycollic acids were selected for these studies because their pKa_1 values are 1·27 and 3·83, respectively, and represent the range of p*Ka* values expected with most of the anticipated naturally occurring organic acids.

A 10-μl volume of a solution containing 50 μCi of [^{14}C]oxalic acid per 10·0 ml was added to 1·0 ml of a solution containing 3·43 mg of oxalic acid dihydrate per 100 ml on top of the ion-exchange column and washed in with two 5-ml volumes of water. The elution of the acid with 15 ml of 1·5 M pyridinium acetate buffer was checked by collecting fifteen 1-ml fractions and counting 200 μl of each fraction in 10 ml of Bray's phosphor solution.[4] The efficiency of counting was checked by internal standardisation by using [^{14}C]toluene. This procedure was duplicated for oxalic acid, and repeated with a solution containing 100 μCi of sodium [1-^{14}C]glycollate per 10·0 ml and a carrier solution containing 3·20 mg of glycollic acid per 100 ml.

The recoveries of [^{14}C]oxalic acid and of sodium [1-^{14}C]glycollate were 94·5 and 98·5 per cent. and 107 and 119 per cent., respectively, based on the amount of carbon-14 added. The elution patterns are shown in Fig. 1.

GAS - LIQUID CHROMATOGRAPHY—

The relative response graphs for the trimethylsilyl derivatives of all the acids studied were linear, and are given in Table I in terms of their regression equations. Those for the trimethylsilyl ethoxime derivatives of the oxo-acids examined are also linear, as reported previously.[2] Typical gas - liquid chromatographic separations of the derivatives of the acids studied are shown for aqueous solutions and urine in Figs. 2 and 3, respectively.

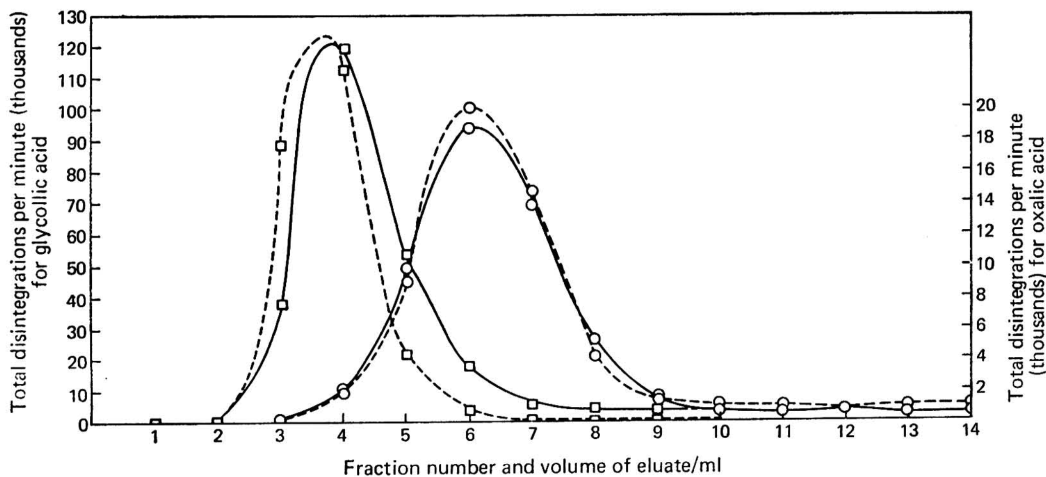

Fig. 1. Elution pattern of [^{14}C]oxalic acid and of [^{14}C]glycollic acid from a column (4·5 × 0·7 cm) of DEAE-Sephadex: □, glycollic acid; and O, oxalic acid

FREEZE-DRYING—

The freeze-drying procedure, together with the gas - liquid chromatographic analysis, have previously been shown to give good recoveries of all acids except free glyoxylic acid.[3] In the procedure described here, this latter acid, and other oxo-acids, are present as ethoximes prior to freeze-drying and are therefore less volatile than the free acids. This effect is discussed below.

TABLE I

RESPONSE OF TRIMETHYLSILYL DERIVATIVES OF SOME CARBOXYLIC ACIDS RELATIVE TO n-TETRACOSANE, EXPRESSED AS THEIR REGRESSION EQUATIONS*

Acid†	Number of points (N)	Regression equation constants* a	b	Standard error of the estimate‡
Glycollic	10	0·061	1·087	0·110
Oxalic, dihydrate	9	−0·054	0·505	0·080
Methylmalonic	11	−0·006	0·723	0·127
Benzoic	12	−0·226	1·189	0·433
Succinic	11	−0·018	0·894	0·144
Fumaric	7	−0·231	0·858	0·168
L-Malic	9	−0·098	0·970	0·101
Hippuric	6	−0·404	0·419	0·072
Citric, monohydrate	9	−0·064	0·826	0·223
Undecanedioic	10	−0·096	0·736	0·141

* The regression equation is given by $y = a + bx$, where $a = \dfrac{\Sigma y - b\Sigma x}{N}$ and $b = \dfrac{N\Sigma xy - (\Sigma x)(\Sigma y)}{N(\Sigma x^2) - (\Sigma x)^2}$; x = amount of acid (µg); and y = response relative to n-tetracosane.

† For I.U.P.A.C. nomenclature, see preceding paper.[2]

‡ The standard error of the estimate of y on x is $S_{yx} = \sqrt{\dfrac{\Sigma y^2 - a\Sigma y - b\Sigma xy}{N}}$.

OVER-ALL PROCEDURE—

The over-all procedure, including freeze-drying and gas - liquid chromatography, was checked by using aliquots of the standard aqueous solution ranging from 0·25 to 10·0 ml both alone and added to a urine sample. The urine specimen was an early morning specimen taken from a fasting normal subject, after rejection of the overnight specimen, and was collected without preservative. When analysed without the addition of known acids, it showed no peaks in the regions of the chromatogram occupied by the added compounds. Aliquots containing 3·18 mg of creatinine were used in each determination.

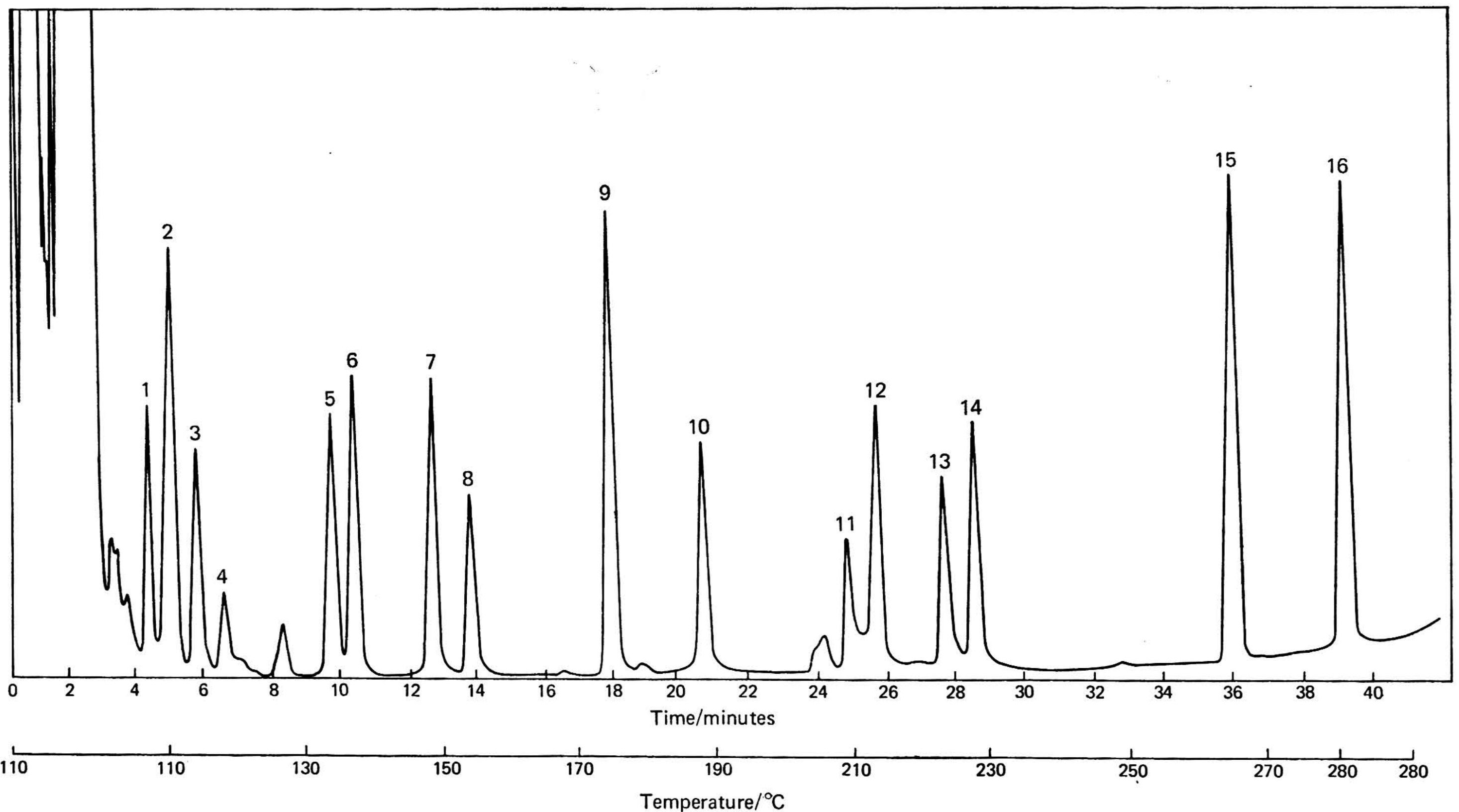

Fig. 2. Gas - liquid chromatographic separation of the trimethylsilyl derivatives of some organic acids extracted from an aqueous solution by DEAE-Sephadex. 1, EO glyoxylate; 2, glycollate; 3, EO pyruvate; 4, oxalate; 5, methylmalonate; 6, benzoate; 7, succinate; 8, fumarate; 9, L-malate; 10, EO 2-oxoglutarate; 11, hippurate; 12, citrate; 13, EO 4-hydroxyphenylpyruvate; 14, undecanedioate; 15, n-tetracosane; and 16, n-hexacosane. EO = ethoxime derivative. Amplification = 10 × 32

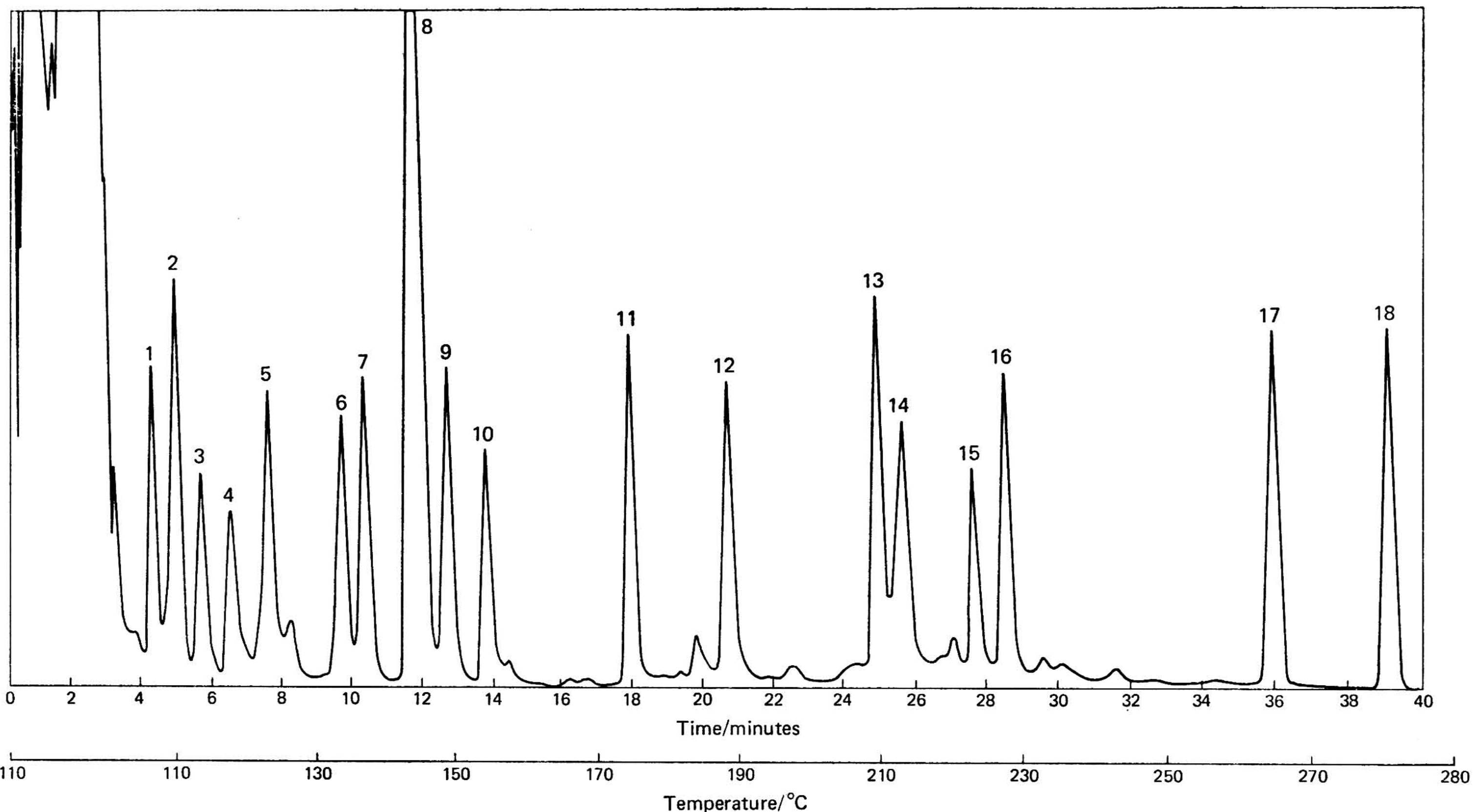

Fig. 3. Gas - liquid chromatographic separation of the trimethylsilyl derivatives of some added organic acids extracted from urine by DEAE-Sephadex. 1, EO glyoxylate; 2, glycollate; 3, EO pyruvate; 4, oxalate; 5, urinary peaks; 6, methylmalonate; 7, benzoate; 8, urinary phosphate; 9, succinate; 10, fumarate; 11, L-malate; 12, EO 2-oxoglutarate; 13, urinary hippurate; 14, urinary citrate; 15, EO 4-hydroxyphenylpyruvate; 16, undecanedioate; 17, n-tetracosane; and 18, n-hexacosane. EO = ethoxime derivative. Amplification = 10 × 64

Recoveries from aqueous solution varied, depending on the acid concerned and the concentrations used, and are shown in Tables II, III and IV.

TABLE II

RECOVERIES OF ORGANIC ACIDS FROM AQUEOUS SOLUTION WITH BETWEEN 0·05 AND 2·0 mg OF EACH ACID USED FOR EXTRACTION

Acid*	Number of determinations (N)	Mean recovery, per cent. m/m (μ)	Standard deviation† (σ)	Standard error of the mean†
Glyoxylic, monohydrate ..	22	92·5	15·5	3·3
Glycollic	20	110·1	14·7	3·3
Pyruvic	23	69·8	13·3	2·8
Oxalic, dihydrate	22	65·9	18·1	3·85
Methylmalonic	24	107·5	14·0	2·9
Benzoic	23	73·4	5·4	1·1
Succinic	22	97·2	8·2	1·8
Fumaric	23	53·9	15·4	3·2
L-Malic	11‡	86·9	12·0	3·6
2-Oxoglutaric	21	66·2	25·4	5·55
Hippuric	17	52·0	25·1	6·1
Citric, monohydrate	22	80·6	17·8	3·8
4-Hydroxyphenylpyruvic ..	20	58·7	25·7	5·7
Undecanedioic	23	95·9	22·8	4·75

* For I.U.P.A.C. nomenclature, see preceding paper.[2]

† Standard deviation, σ, is given by the equation $\sigma = \sqrt{\frac{\Sigma d^2}{N-1}}$ where $d = Xn - \mu$ (Xn = actual result recorded). The standard error of the mean is given by $\sigma/\sqrt{N}$. Results differing from the mean by more than 2σ in the first calculation of standard deviation are rejected in the calculation of the final standard deviation.

‡ Only eleven determinations, including L-malic acid in the mixture, were carried out.

Recoveries from urine are shown in Table V. The reproducibility of recovery with different urine samples is demonstrated by the recovery of undecanedioic acid from fourteen determinations on ten urine specimens: the mean recovery was 116·5 per cent. and the standard error of the mean was 3·1 per cent.

TABLE III

RECOVERIES OF ORGANIC ACIDS FROM AQUEOUS SOLUTION WITH BETWEEN 0·05 and 0·20 mg OF EACH ACID USED FOR EXTRACTION

Acid*	Number of determinations (N)	Mean recovery, per cent. m/m (μ)	Standard deviation† (σ)	Standard error of the mean†
Glyoxylic, monohydrate ..	9	104·6	(16·2)‡	(6·4)‡
Glycollic	7	114·8	(11·4)‡	(4·3)‡
Pyruvic	10	62·9	12·7	4·0
Oxalic, dihydrate	10	52·6	25·8	8·2
Methylmalonic	11	99·5	16·3	4·9
Benzoic	11	68·8	9·1	2·7
Succinic	11	86·3	11·9	3·6
Fumaric	11	47·5	15·1	4·6
L-Malic	5§	68·6	—	—
2-Oxoglutaric	8	37·1	(11·0)‡	(3·3)‡
Hippuric	4	14·6	—	—
Citric, monohydrate	11	55·3	25·0	7·5
4-Hydroxyphenylpyruvic ..	7	27·7	(7·3)‡	(2·8)‡
Undecanedioic	11	69·9	16·5	5·0

* For I.U.P.A.C. nomenclature, see preceding paper.[2]
† See footnotes to Table II.
‡ Figures in parentheses are derived from fewer than ten results.
§ Only five determinations, including L-malic acid in the mixture, were carried out.

Discussion

Previous methods used for studying organic acids in biological materials have included partition chromatography on silicic acid columns[5–7] and anion-exchange chromatography on polystyrene-based resins.[8–12] Poor resolution and incomplete elution of some of the acids, together with the necessity to use non-specific methods to detect as well as to determine the acids, has limited the application of these methods to urine, blood and other biological fluids. These fluids may contain a complex and variable mixture of organic acids at low concentrations with relatively large amounts of other compounds. Provided that the acids can be separated from these gross constituents, it should be possible to overcome the analytical problems by advanced gas-chromatographic methods in which flexible programming and methods of derivative formation to improve resolution and quantitative determination are combined with mass spectrometry for absolute identification.

Table IV

Recoveries of organic acids from aqueous solution with between 0·6 and 2·0 mg of each acid used for extraction

Acid*	Number of determinations (N)	Mean recovery, per cent. m/m (μ)	Standard deviation† (σ)	Standard error of the mean†
Glyoxylic, monohydrate ..	13	84·1	7·8	2·2
Glycollic	13	107·5	16·0	4·4
Pyruvic	13	75·1	11·5	3·2
Oxalic, dihydrate	13	72·1	10·7	3·0
Methylmalonic	13	114·3	6·9	1·9
Benzoic	13	75·3	3·8	1·1
Succinic	13	101·5	6·9	1·9
Fumaric	12	59·7	13·7	3·95
L-Malic	7‡	94·2	(4·8)§	(1·8)§
2-Oxoglutaric	13	84·1	10·0	2·8
Hippuric	13	63·5	15·0	4·2
Citric, monohydrate	12	93·2	7·0	2·0
4-Hydroxyphenylpyruvic ..	13	75·3	12·5	3·5
Undecanedioic	13	113·5	6·7	1·9

* For I.U.P.A.C. nomenclature, see preceding paper.[2]
† See footnotes to Table II.
‡ Only seven determinations, including L-malic acid in the mixture, were carried out.
§ Figures in parentheses are derived from fewer than ten results.

Many workers, when using gas - liquid chromatographic methods to separate and determine carboxylic acids, have converted the acids into methyl esters so as to reduce the polarity and to enhance the thermal stability and volatility of the parent compound.[11,13–15] Multiple products are formed with the hydroxy and oxo-acids, and losses occur owing to the volatility of the derivatives.[16] These problems also arise with other esters, *e.g.*, n-butyl,[15] that are prepared by similar reactions. The use of the trimethylsilyl ether - methyl esters of hydroxy-acids introduced even more problems of multiple product formation, and most recent workers have used the fully trimethylsilylated derivatives for the gas - liquid chromatography of organic acids.[16–21] Carboxylic acids form trimethylsilyl esters, and hydroxy-acids form trimethylsilyl ethers and esters, by simple addition of the reagent to the free acid. Oxo-acids are stabilised prior to silylation to prevent keto - enol tautomerism,[2] and the introduction of this stage before freeze-drying in the present study also reduces the volatility of the acids, particularly of glyoxylic and pyruvic acids. The ethoxime derivatives have been used in the present work for reasons that are discussed elsewhere.[2]

The use of trimethylsilyl derivatives dictates the choice of the extraction procedure that can be applied. Dry free acids are required for trimethylsilylation and the extraction process must be designed so as to yield the acids in this form. Most workers who have been interested in extracting organic acids from biological materials have used solvents such as ether,[14,17,20,21] ethyl acetate,[14,17–19,21] acetonitrile[17] and tri-n-butyl phosphate[22] to extract the acids from the acidified solution. Solvent extraction gives only incomplete recovery, particularly of the hydrophilic aliphatic acids. These methods should not be used in quantitative

studies on such acids, and have only limited application in qualitative work, and in addition they often complicate the over-all procedure by extracting neutral and basic constituents in addition to the acids. Partition chromatography, and anion-exchange chromatography on polystyrene-based resins, are also unsuitable because fractionation of the acids occurs and because of the difficulty in obtaining complete elution with the systems used. Decomposition of some acids on strongly basic resins has also been reported.

The present extraction system in which the weak anion exchanger A25 DEAE-Sephadex gel is used is based on the method used by Jaakonmaki, Knox, Horning and Horning[23] to extract a glucosiduronic acid from rat urine and has been shown in the present studies with compounds labelled with carbon-14 to give good recoveries of acids having p*Ka* values in the range expected with other naturally occurring acids. The acids are finally obtained as their pyridinium salts, which cause no interference in the trimethylsilylation reactions that are used subsequently. The use of pyridine as a buffering counter-ion is important in the extraction because weak acids such as acetic acid will not permit most organic acids to be eluted. Carefully controlled freeze-drying conditions have been shown to give full recoveries of all but the most volatile of the acids. Glyoxylic and pyruvic acids, which are the most volatile[3] of the acids studied, are stabilised, together with all the other oxo-acids present, as their ethoximes in the aqueous pyridinium acetate column eluate before freeze-drying. These ethoxime derivatives are considerably less volatile than the parent acids. This procedure will not allow the most volatile fatty acids such as propionic acid to be studied and other methods must be used for their determination.[24]

The trimethylsilylation reactions and gas - liquid chromatography have also been shown to be quantitative, linear response graphs being obtained for all the derivatives studied. The gas - liquid chromatographic separation of the present acids is good and there is scope for achieving further resolution by making alterations in the gas - liquid chromatographic system or by using other derivatives of the oxo-acids.[2] The use of the methyl silicone OV-101 in a high loading as the stationary phase allows very efficient separations of the low-boiling derivatives of acids such as glycollic, glyoxylic, pyruvic and oxalic acid to be achieved; this methylsilicone is liquid at low temperatures, while all the other OV and SE silicones used as stationary phases are solid gums at low temperatures, and it allows the partition of the low-boiling derivatives to commence immediately after injection during the initial isothermal stage.

TABLE V

RECOVERIES OF ORGANIC ACIDS FROM URINE* WITH BETWEEN 1·0 AND 2·0 mg OF EACH ACID USED FOR EXTRACTION

Acid†	Number of determinations (N)	Mean recovery, per cent. *m/m* (μ)	Standard deviation‡ (σ)	Standard error of the mean‡
Glyoxylic, monohydrate	16	86·8	7·8	2·0
Glycollic	15	98·9	12·1	3·1
Pyruvic	15	128·3	8·3	2·2
Oxalic, dihydrate	16	98·2	8·3	2·1
Methylmalonic	15	116·0	6·4	1·7
Benzoic	16	79·6	4·4	1·1
Succinic	15	104·5	4·7	1·2
Fumaric	16	80·6	8·5	2·1
L-Malic	16	98·0	3·7	0·9
2-Oxoglutaric	16	117·3	6·6	1·7
4-Hydroxyphenylpyruvic	15	82·1	11·1	2·9
Undecanedioic	15	117·1	7·1	1·8

* An early morning specimen, taken from a fasting normal subject, after rejection of the overnight specimen, and containing 3·18 mg of creatinine, was used in each determination.
† For I.U.P.A.C. nomenclature, see preceding paper.[3]
‡ See footnotes to Table II.

Recoveries of acids from aqueous solution have been shown in these studies to be reasonably high when larger amounts of acids are used in the extraction process, and the precision tends also to be greater (Tables II, III and IV). The losses observed at lower concentrations (Table III) may be due to incomplete desorption of some of the acids from

the DEAE-Sephadex by the pyridinium acetate buffer. Greater losses appear to be obtained with acids having lower p*Ka* values, which indicates that the pyridinium counter-ion is unable to remove completely acids with low p*Ka* values from the diethylaminoethyl group on the Sephadex. The losses are most marked in the low concentration range, as would be expected when a finite degree of retention is occurring, and these studies indicate that larger amounts of material are necessary for good quantitative determinations (Table IV). The high losses generally observed with hippuric acid may be due to partial thermal decomposition of the trimethylsilyl ester of this acid during the gas - liquid chromatography and flame-ionisation detection.

Recoveries from urine have been found to be consistently higher than those from aqueous solution, which might indicate that a buffering effect is exercised by other urinary constituents retained with the anions. This effect may assist in the subsequent removal of the anions by the pyridinium acetate buffer. The urine specimen selected showed no peaks in the regions of the chromatogram occupied by the added acid derivatives. Thus, unidentified urinary constituents with the same retention times as the added acids did not contribute to the recovery experiments, which were aimed to test the quantitative nature of the extraction, derivative formation, freeze-drying and gas chromatography overall.

In conclusion, the present method has shown reasonably good recoveries from urine of all the acids studied except hippuric acid, within the limits expected for the analysis of related minor components of a complex mixture by a method that includes several stages. It is felt that the method is sufficiently accurate for trial as a screening method for abnormal organic acidurias in man. It can also be applied to the analysis of deproteinised blood plasma (Chalmers, R. A., and Watts, R. W. E., unpublished results).

This work will be submitted by R.A.C. in partial fulfilment of the requirements for the degree of Ph.D. of the Council for National Academic Awards.

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Received *May 17th*, 1972
Accepted *August 7th*, 1972

Analyst, December, 1972, Vol. 97, *pp*. 968–972

A Simplified Method of Extraction of Diosgenin from *Dioscorea* Tubers and its Determination by Gas - Liquid Chromatography

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A simple method for determining diosgenin in *Dioscorea* tubers involving extraction and gas - liquid chromatography has been developed and used in our laboratory for the last 6 years. A small amount (600 mg) of the ground, dried tuber is hydrolysed by refluxing it for 4 hours with a 1 + 1 mixture of 3 N aqueous hydrochloric acid solution and xylene, after adding a suitable internal standard. After centrifuging the mixture, a portion of the xylene layer is injected on to the gas-chromatographic column, without any further manipulations. Hydrolysis conditions were examined to ensure that the release of diosgenin was complete and its degradation at a minimum. The proposed method enables pennogenin to be separated from diosgenin, but yamogenin is poorly resolved. The relative standard deviation for the determination of diosgenin was about 1·5 per cent. Good agreement with a gravimetric method was found.

DIOSGENIN, 25R-spirost-5-en-3β-ol, an important starting material for the commercial synthesis of steroids, is determined in *Dioscorea* tubers by acidic hydrolysis of the glycosides and extraction with an organic solvent, followed by the application of a widely used gravimetric method,[1] an infrared technique[2] or a densitometric thin-layer[3] or gas - liquid chromatographic[4] method. The first three of these methods have several disadvantages, the chief of which being that they are very laborious; the gravimetric and infrared methods are non-specific or subject to interferences.

A simplified method involving gas - liquid chromatography has been developed and used in these laboratories on about two thousand samples of *D. composita* and *D. floribunda* tubers in the last 6 years. As it offers several advantages over a recently published gas - liquid chromatographic method[4] and does not have the disadvantages of the other methods mentioned above, it should be of interest.

EXPERIMENTAL

APPARATUS—

In this work a Hewlett-Packard, Model 700, gas chromatograph with a hydrogen flame-ionisation detector was used. The glass column was 240 cm long × 3 mm i.d., packed with 2 per cent. SE-30 on Gas-Chrom Q, 100 to 120 mesh (packing purchased from Applied Science Laboratories Inc., State College, Pa., U.S.A.).

An electronic digital integrator (Infotronics, Model CRS-11 HSB-42) was used for peak area measurements.

The hydrolysis apparatus consisted of Pyrex test-tubes, each 12 cm long and of 2·5 cm o.d. with standard taper ground-glass sockets; air condensers, consisting of Pyrex tubing about 38 cm long and 2·2 cm o.d., with a ground-glass cone at one end to fit the test-tubes; and a magnetic stirrer - hot-plate, with an aluminium block for seven test-tubes of 2·5 cm diameter (purchased from Cole-Parmer Instrument Co., Chicago, Ill., U.S.A.).

STANDARDS—

Internal standard—5α-Cholestan-3β-ol (Mann Research Laboratories, New York, U.S.A.).

Diosgenin standard—A repeatedly recrystallised sample that showed one peak with gas - liquid chromatography; melting-point 205·5 to 207 °C and $[\alpha]_D^{25} = -121°$ (concentration 1 g per 100 ml in chloroform).

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Method

Sample preparation and hydrolysis - extraction—

Pulverise the coarsely mashed *Dioscorea* tuber, which has been dried at 100 °C under atmospheric pressure, in a blender for several minutes. Accurately weigh about 600 mg of powdered sample and 15 mg of the internal standard into a hydrolysis tube. If the sample has not been dried previously to a constant mass, the loss in mass can be determined directly in the hydrolysis tube by drying overnight at 100 °C and 30 mm pressure of mercury. Otherwise proceed with the hydrolysis - extraction as follows: add 8 ml of xylene and 8 ml of 3 N aqueous hydrochloric acid solution, attach the air condenser and reflux the mixture for 4 hours on the magnetic stirrer - hot-plate, stirring the mixture rapidly with a small magnetic bar encased in PTFE. After the initial foaming the mixture should reflux vigorously (with the temperature of the liquid at about 93 °C) throughout this period. After cooling, centrifuge the test-tubes or allow the liquid phases to separate (remove the stirring bars with a strong magnet so as to prevent the tubes from breaking in the centrifuge). Inject a portion of the clear upper organic layer on to the gas-chromatographic column.

Gas chromatography—

Before packing the column, silanise the glass coil in the usual manner. Condition the packed column at 300 °C for 1 hour with no carrier gas flow and at least overnight with a fast helium flow. Further condition the column by repeatedly injecting hexamethyldisilazane and finally a concentrated solution of diosgenin.

The gas-chromatographic conditions used were as follows: column at 270 °C, injection port at 280 °C, detector block at 300 °C, helium flow-rate about 60 ml min^{-1} and hydrogen and air flow-rates optimised for maximum response. Under these conditions, typical retention times in minutes were (relative retention times in parentheses): 25R-spirosta-3,5-diene, 4·3 (0·64); 5α-cholestan-3β-ol, 5·0 (0·75); smilagenin, 6·2 (0·93); diosgenin, 6·7 (1·00); yamogenin, 6·9 (1·03); tigogenin, 7·0 (1·05); and pennogenin, 9·2 (1·37).

Preparation of calibration graph—

Prepare individually five standard solutions containing about 5, 10, 15, 20 and 30 mg of diosgenin and about 10 mg of the internal standard in 5 ml of toluene. Inject each solution (2 μl) in duplicate on to the column and plot the mean peak area ratio (A) of diosgenin to internal standard *versus* the mass ratio (M_R) of diosgenin to internal standard. Calculate the equation of the best straight line ($A = a + bM_R$, where a is the intercept and b the slope) by using the least-squares method. The calibration graph should be determined for each apparatus and each new column. Check the instrument performance daily by chromatographing one standard solution and determining if the result is within permissible limits from the calibration line.

Determination of diosgenin—

Inject in duplicate 2 μl of each xylene extract and calculate the mean peak area ratio (A). Calculate the diosgenin content of the dried tuber as follows—

$$\text{Diosgenin, per cent. } m/m = \frac{A - a}{b} \cdot \frac{m_1}{m_2} \times 100$$

where m_1 is the mass of internal standard and m_2 the mass of dried tuber sample.

Results and discussion

The idea of combining the hydrolysis of the glycoside and extraction of the sapogenins in a single step, by refluxing the tuber with a mixture of aqueous solution of acid and an immiscible solvent, considerably simplifies the assay. The conditions with respect to time of refluxing, acid concentration and type of immiscible solvent were carefully determined to ensure the maximum release of diosgenin from the tuber and to minimise the often neglected degradation of diosgenin under acidic conditions. This degradation was noticeable on account of the low yields obtained and the formation of the previously described[5] dehydration product, 25R-spirosta-3,5-diene, which was determined by the same gas - liquid chromatographic method and its identity confirmed by ultraviolet spectrophotometry. Some of the yields of diosgenin and the 3,5-diene obtained under different hydrolysis - extraction

conditions are shown in Table I. Refluxing for 4 hours with 3 N aqueous hydrochloric acid solution *plus* xylene was chosen as the best procedure.

Hydrolysis in the presence of an immiscible solvent has the additional advantage of reducing the rate of dehydration of diosgenin. Thus when diosgenin was refluxed for 4 hours in 3 N aqueous hydrochloric acid solution *plus* an equal volume of an organic solvent, an 80 per cent. yield of the 3,5-diene was obtained when the organic solvent used was ethanol (refluxing temperature 86 °C), but the yield was only 3 per cent. when the solvent used was toluene (refluxing temperature 80 °C).

TABLE I

YIELD OF DIOSGENIN FROM A GROUND *D. composita* SAMPLE

Immiscible solvent	Aqueous HCl solution/N	Refluxing time/hours*	Yield of diosgenin, per cent. *m/m*	Yield of 3,5-diene, per cent. *m/m*
Toluene	3	3	2·96	0·04
	3	5	3·15	0·06
	4	5	3·01	0·14
Xylene	2	4	3·19	0·04
	3	3	3·11	0·09
	3	4	3·23	0·10
	4	3	3·19	0·14
p-Cymene	3	3	2·03	0·12
	4	2	2·16	0·17

* Temperature of refluxing liquid mixture: toluene 84 °C, xylene 93 °C, *p*-cymene 98 °C.

It is of interest that some dehydration of diosgenin was also found to occur under the conditions of the gravimetric method[1]; the crystalline material isolated by the gravimetric procedure contained the 3,5-diene (as shown by gas - liquid chromatography and ultraviolet spectrophotometry) in approximately the same proportion as occurred in the proposed method. It should be added that the latter compound was not formed during gas chromatography as it was completely absent in the chromatograms of purified diosgenin.

It is generally accepted that for an accurate quantitative determination by gas - liquid chromatography the use of an internal standard is required. In this work 5α-cholestan-3β-ol was chosen because its retention time does not overlap with any of the peaks found in *D. composita* and *D. floribunda*, and because it was found to be unchanged by the hydrolysis - extraction procedure used for *Dioscorea* tubers. The calibration of peak areas *versus* masses gave a straight line in the range of injections tested (about 2 to 12 μg), which covered the range of concentrations of diosgenin normally found in *Dioscorea* tubers. A typical calibration line showed the equation $A = 0{\cdot}810\ M_R$, but the use of different columns or different techniques of integration results in different constants in this equation; with some earlier column packings a significant intercept was observed.

The advantage of using gas - liquid chromatography for the determination of diosgenin in *Dioscorea* tubers is that other sapogenins can also be determined. *D. composita* and *D. floribunda* were found to contain, besides diosgenin, small amounts of pennogenin and occasionally yamogenin. The former is well separated from diosgenin; the latter appears as a shoulder on the diosgenin peak (from the practical aspect, yamogenin is as useful as diosgenin for the synthesis of steroids). Although no clear indication of the presence of other sapogenins was obtained, the 5,6-dihydro analogues, smilagenin and tigogenin, if present, should be apparent from the chromatogram. The retention times relative to diosgenin are given under Method. Fig. 1 shows a typical chromatogram of an extract of hydrolysed *D. composita* tuber. It is clear that there is no interference from oils, pigments and other constituents of the extract. Fig. 2 shows the separation of smilagenin, diosgenin and yamogenin (or tigogenin) in a mixture of these standards.

If the quantitative determination of the other sapogenins is required, their calibration lines should be determined. For instance, it was found that the slope of the calibration line for pennogenin was about 65 per cent. of that for diosgenin, while that for yamogenin was essentially the same as for diosgenin.

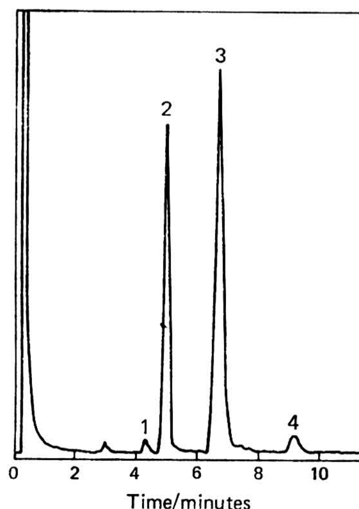

Fig. 1. Gas chromatogram of xylene extract of hydrolysed *Dioscorea composita* tuber: 1, 25R-spirosta-3,5-diene; 2, 5α-cholestan-3β-ol (internal standard); 3, diosgenin; and 4, pennogenin

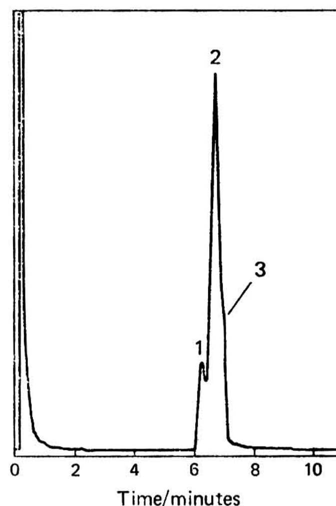

Fig. 2. Gas chromatogram of a mixture of (1) smilagenin, (2) diosgenin and (3) yamogenin. The retention time of tigogenin is almost identical with that of yamogenin

Accuracy and precision—

The accuracy of the proposed gas - liquid chromatographic method was determined by comparing it with the generally accepted and reproducible gravimetric method.[1] A series of samples of *Dioscorea* tubers of different diosgenin content was analysed in duplicate by the two methods. The mean results are shown in Table II. The results by the gravimetric method (which measures the total steroids) are very similar to those for the total sapogenins found by the gas - liquid chromatographic method, the mean difference being insignificant (0·05), which shows that the proposed hydrolysis and extraction are as efficient as in the gravimetric procedure.

Table II

Comparison of results by the proposed method with those by the gravimetric method

Dioscorea sample	Gas - liquid chromatographic method: Diosgenin, per cent.	3,5-Diene, per cent.	Pennogenin, per cent.	Sum of sapogenins, per cent.	Gravimetric method: sapogenins, per cent.	Difference between methods, per cent.
A	8·01	0·12	0·53	8·66	8·49	0·17
B	7·50	0·24	0·49	8·23	7·80	0·43
C	4·82	0·11	0·45	5·38	5·50	−0·12
D	3·17	0·12	0·44	3·73	4·10	−0·37
E	3·20	0·09	0·27	3·56	3·74	−0·18
F	1·97	0·11	0·60	2·68	2·94	−0·26
G	0·93	0·05	0·26	1·24	1·27	−0·03
					Mean	−0·05

All values are means of duplicate determinations, except sample G.

The precision of the proposed method was determined by analysing one sample repeatedly on the same day and the results obtained for the percentage of diosgenin were as follows: 3·10, 3·17, 3·15, 3·21, 3·24 and 3·17, with a mean value of 3·17. The standard deviation was

found to be 0·048 (relative standard deviation 1·53 per cent.). Incidentally, the standard deviation was about the same when peak areas were measured by triangulation instead of electronic integration, probably because of the relatively large peak widths; the saving in time and increase in convenience effected by using the latter method are, however, appreciable.

Conclusion

The proposed method shows precision and accuracy comparable with the often used gravimetric method,[1] but it is much simpler and faster. It differs from a recently described gas - liquid chromatographic method[4] as follows: the hydrolysis and extraction are carried out simultaneously in one simple vessel, by using a two-phase system, and no separate extraction step is needed; an internal standard is used in the gas - liquid chromatographic determination so as to increase the reliability of the method; sample size is considerably smaller (0·6 instead of 5 g); other sapogenins (yamogenin and pennogenin) present have been identified; and the important question of the degradation of the diosgenin when refluxed with acid was investigated.

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Received *February 7th*, 1972
Accepted *June 21st*, 1972

Analyst, December, 1972, Vol. 97, *pp.* 973–976

Spectrophotometric Determination of Diosgenin in *Dioscorea composita* Following Thin-layer Chromatography*

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A rapid and accurate method is described for the determination of diosgenin in *Dioscorea composita* roots. After acid hydrolysis and solvent extraction of the root, pure diosgenin is isolated by thin-layer chromatography, treated with sulphuric acid - methanol, and the resultant chromophore is measured by spectrophotometry.

Similarly, gas - liquid chromatography is used to evaluate statistically the accuracy of the method.

BECAUSE of the importance of diosgenin as a raw material in the synthesis of steroid hormones, various methods for its quantitative determination in several *Dioscorea* roots (*D. mexicana, D. composita* and *D. deltoïdea*), including gravimetric,[1] column-chromatographic and gas-liquid chromatographic methods,[2–4] have been developed.

In this paper an analytical method for the determination of diosgenin in "barbasco" root (*D. composita*) by thin-layer chromatography is described.

APPARATUS AND MATERIALS—

Shandon thin-layer chromatographic equipment with 20 × 20-cm glass plates coated with a layer of silica gel (HR purest grade, Merck), 0·25 mm thick, and a Unicam SP800 automatic spectrophotometer with glass cells of 1-cm path length, were used.

A Hewlett-Packard gas chromatograph, Model 402, with flame-ionisation detector, and a U-shaped glass column 1·2 m long and of 6 mm i.d., packed with 3 per cent. *m*/*m* QF-1 on 100 to 120-mesh Gas-Chrom P (Applied Science Laboratories Inc.), were used.

Operating temperatures were: column, 240 °C; injector block, 310 °C; and detector, 270 °C. Gas flow-rates were nitrogen, 100 ml min^{-1}; hydrogen, 60 ml min^{-1}; and air, 400 ml min^{-1}.

HYDROLYSIS—

Weigh a 2-g amount of ground barbasco root that has previously been dried to constant mass in a vacuum oven at 60 °C, and hydrolyse it by refluxing it with 25 ml of 30 per cent. *V*/*V* hydrochloric acid on a steam-bath for 4 hours. Filter off the solid matter and wash it with water until the washings are neutral (pH 6·8 to 7·0).

EXTRACTION—

Dry the solid matter at 60 °C for 2 hours in a vacuum oven and quantitatively transfer it to a Soxhlet extractor, then extract it with about 80 ml of chloroform for 2 hours. Transfer the extract into a 100-ml calibrated flask and make the volume up to the mark with chloroform (unknown solution P).

SEPARATION BY THIN-LAYER CHROMATOGRAPHY—

Divide the silica gel plate into five lanes. Apply 100 µl of solution P to each of two lanes. To each of two other lanes apply 100 µl of a 1 mg ml^{-1} standard solution of diosgenin in chloroform. Leave the fifth lane free to be used as a blank.

* A summary of this paper was presented at the VIIIth Pan-American Congress on Pharmacy and Biochemistry in Caracas, Venezuela, in December, 1969.

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Develop the chromatogram in a tank saturated with a chloroform - acetone (80 + 20) solvent system. Dry the plate and place it in a chamber saturated with iodine vapour. Mark horizontally the zones corresponding to diosgenin (R_F 0·57) and the area corresponding to the blank, which must be adjacent to the diosgenin spots and have an equivalent R_F value. Place the plate in an oven at 100 °C for 15 minutes to eliminate the iodine. Scrape off the diosgenin and blank zones with a spatula and extract the powder in each zone by stirring it with 5 ml of methanol. Filter the solutions into test-tubes through medium porosity sintered glass funnels. Take 4-ml aliquots of the unknown and standard extracts from the individual test-tubes and evaporate them to dryness on a steam-bath. Cool and add to each residue 4 ml of concentrated sulphuric acid - methanol (80 + 20). Determine the amount of the resulting chromophore spectrophotometrically at $\lambda_{max.}$ 405 ± 1 nm against the blank after allowing the reaction to proceed for 2 hours,[5,6] which is the optimum time required for the chromophore to develop a stable optical density.

GAS - LIQUID CHROMATOGRAPHY—

By following the methods previously described,[3,4] inject an aliquot of solution P into the gas chromatograph. Determine the amounts of diosgenin by comparing the peak areas of the unknown against that of a standard solution (Fig. 1).

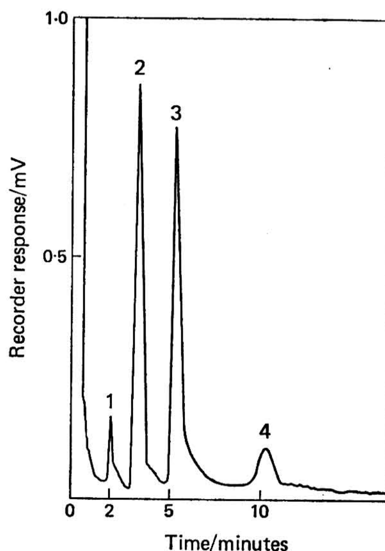

Fig. 1. A typical gas - liquid chromatographic separation with chloroform extract of barbasco root, with cholesterol as internal standard: 1, diosgenin-3,5-diene (0·61); 2, cholesterol (internal standard); 3, diosgenin (1·5); and 4, pennogenin (2·9). Retention times relative to cholesterol are given in parentheses

SEPARATION BY THIN-LAYER CHROMATOGRAPHY—

Fig. 2 indicates the separation obtained with a chloroform extract of barbasco root by thin-layer chromatography. In addition to diosgenin (R_F 0·57), two other products, pennogenin (R_F 0·47) and diosgenin-3,5-diene (R_F 0·85), were identified.

In Fig. 3, it can be observed that the chromophore produced by the reaction of diosgenin with sulphuric acid - methanol obeys Beer's law in the concentration range studied (40 to 120 μg).

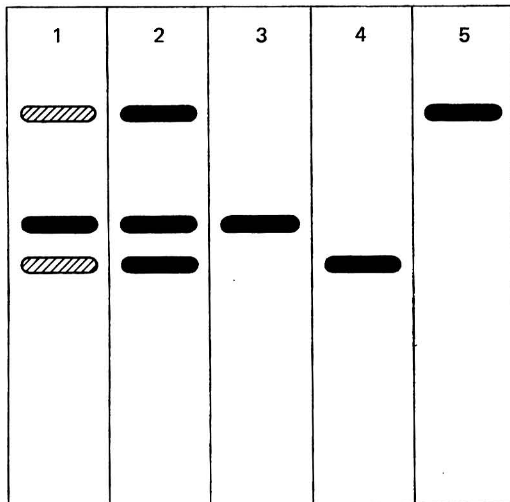

Fig. 2. Separation by thin-layer chromatography with solvent system chloroform - acetone (80 + 20). 1, Chloroform extract of barbasco root; 2, synthetic mixture of pennogenin (R_F 0·47), diosgenin (R_F 0·57) and diosgenin-3,5-diene (R_F 0·85); 3, diosgenin reference sample (R_F 0·57); 4, pennogenin reference sample (R_F 0·47); and 5, diosgenin-3,5-diene reference sample (R_F 0·85)

The accuracy of the chromatographic method was evaluated according to the amount of standard sample recovered when added to diosgenin-free barbasco root, and the precision was based on repeated analyses of a homogeneous sample of barbasco root.[7,8] The results are summarised in Tables I and II.

CONCLUSIONS

Crude diosgenin was obtained from barbasco root by hydrolysis followed by extraction with chloroform. By using thin-layer chromatography, pure diosgenin was separated, eluted and allowed to react with sulphuric acid - methanol (80 + 20). The resulting chromophore was measured spectrophotometrically at $\lambda_{max.}$ 405 ± 1 nm and the diosgenin content was determined quantitatively against a standard. Following this procedure, results by the proposed method were compared statistically with those by the gas - liquid chromatographic method.[3]

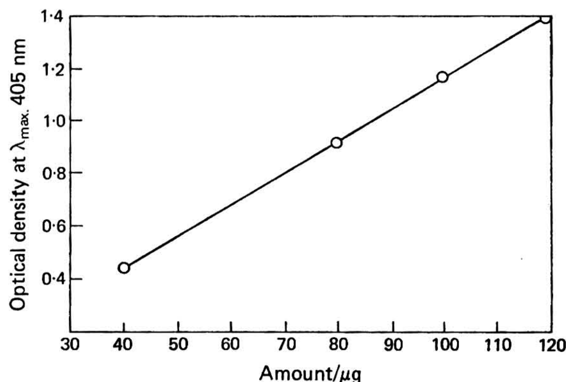

Fig. 3. Linearity of the optical density of the chromophore after reaction for 2 hours

TABLE I

RESULTS FOR ACCURACY BY THE THIN-LAYER CHROMATOGRAPHIC METHOD

Sample	Diosgenin added/mg	Diosgenin found/mg	Recovery, per cent.
1	0·00	0·00	—
2	50·20	49·25	98·0
3	50·20	50·20	100·0
4	95·38	97·20	102·0
5	95·38	100·00	105·2
6	150·65	154·00	102·5
7	150·65	145·30	96·6
		Mean ..	100·7

Consequently, the thin-layer chromatographic method can be recommended for the routine analysis of barbasco root to determine diosgenin because of its accuracy and precision (Tables I and II), economy in cost of equipment used and its ease of operation.

TABLE II

RESULTS FOR PRECISION AND COMPARISON OF STANDARD DEVIATIONS

Determinations	Diosgenin, per cent., by gas - liquid chromatography	Diosgenin, per cent., by thin-layer chromatography
1	5·84	6·17
2	5·95	5·77
3	5·86	5·43
4	5·99	5·60
5	6·05	6·14
6	5·95	6·20
7	5·93	5·95
8	5·38	5·45
9	5·28	5·20
10	5·89	5·48
Mean ..	5·82	Mean .. 5·74
Standard deviation	0·250	0·336
F (found)	1·82	
F (tabulated) at a 95 per cent. confidence limit	3·18	

We appreciate the co-operation of Mr. James Carroll of Syntex Internacional de Asistencia Técnica, S.A., in the preparation of this paper.

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Received *March 15th*, 1971
Amended *January 20th*, 1972
Accepted *June 21st*, 1972

Analyst, December, 1972, Vol. 97, *pp*. 977–980

Thin-layer Chromatographic Separation and Colorimetric Determination of Rhamnose, Quinovose, Fucose and Glucose

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A thin-layer chromatographic method has been developed for the separation and subsequent determination of rhamnose, quinovose, fucose and glucose: 50 to 200 nmol of each of the four sugars can be adequately separated in a single ruled lane 20 mm wide on the plate; after elution the individual sugars are determined colorimetrically with the phenol - sulphuric acid reagent. The method requires the use of only simple apparatus and should have wide application. It has advantages over the gas - liquid and paper chromatographic methods and is less time consuming.

Although several methods[1–8] are available for the separation of rhamnose from fucose and glucose, there appears to be no satisfactory method for the quantitative separation of quinovose and rhamnose even though these sugars often occur together in nature.[9–12] In this laboratory structural studies are being carried out on some convolvulaceous resins, particularly on convolvulin, the active purgative constituent of jalap root. Conflicting reports[9,13] (and J. K. N. Jones and B. E. Stacey, unpublished work) have appeared in the literature concerning the presence of quinovose (in addition to rhamnose, fucose and glucose) in convolvulin, and for this reason a reliable method for the determination of these sugars was required. Of the large number of chromatographic methods examined by us only the paper-chromatographic - borate buffer method of Krauss, Jager, Schindler and Reichstein[14] gave good separation of quinovose from rhamnose. Even this method did not enable the above four sugars to be separated well enough for quantitative purposes. An ion-exchange chromatographic method for the separation of rhamnose, quinovose and glucose has recently been reported.[15] The drawback of this method is that the column has to be operated at 75 °C and rather sophisticated equipment is required.

The gas - liquid chromatographic methods of determining sugars suffer from the serious disadvantage that for each sugar two or more peaks (which result from the various anomers) are obtained. The usual, though time-consuming, method of overcoming this difficulty is to reduce the free sugars to the corresponding alditols with sodium borohydride before forming derivatives (acetates, methyl or trimethylsilyl ethers, etc.) and subjecting them to chromatography.

The present method is an extension of the work of Lato, Brunelli, Ciuffini and Mezzetti[6] and involves continuous development on a thin layer of silica gel buffered with disodium hydrogen orthophosphate. The sugar-containing zones of the adsorbent are scraped off and treated with the well established phenol - sulphuric acid reagent.[16] This reagent is inexpensive, gives stable, coloured species and, unlike the anthrone and 1-naphtholsulphonate reagents, is not affected by small amounts of residual developing solvent arising from the chromatographic procedure.

Experimental

Reagents—

All reagents used were of analytical-reagent grade. Standard sugars were obtained commercially. The developing solvent for thin-layer chromatography was prepared by mixing laboratory-grade materials.

Thin-layer chromatography—

Preparation of plates—Glass plates (25 × 20 cm) are coated to a thickness of 400 µm with a slurry of Merck silica gel H in 0·3 M disodium hydrogen orthophosphate solution and

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the coated plates are allowed to dry at room temperature overnight. Parallel grooves, 2 mm wide and 20 mm apart, are then ruled along the length of the prepared plates. The plates are activated at 110 °C for 2 hours before being stored in a desiccator over silica gel.

Developing solvent—This consisted of the mixture acetone - methanol - chloroform - water (10 + 4 + 2 + 1).

Spray reagent—A 0·5 per cent. *m*/*V* solution of potassium permanganate in 1 M sodium hydroxide was used. This reagent should not be kept for more than 1 or 2 days.

CHROMATOGRAPHIC PROCEDURE—

A glass chromatographic tank (approximately 220 × 70 × 220 mm) is fitted with a special lid made from 6-mm thick Perspex. A 205 × 4-mm section is cut out as shown in Fig. 1 to allow the upper portion of the thin-layer chromatographic plate to protrude through it.

A 1-μl Hamilton syringe is used to apply accurate volumes of sugar solutions to the mid-points of the 20-mm lanes 1 to 8 at a distance of 25 mm from the lower edge of the plate. The developing solvent is prepared immediately before use. The tank is lined with chromatographic paper but prior saturation with vapour is unnecessary. The plate is allowed to develop for 3 hours and is then either allowed to dry at room temperature or placed in a vacuum oven at 40 °C for a few minutes. A clean glass plate, 250 × 130 mm, is placed over the chromatographic plate in a horizontal position so that lanes 2 to 7 are completely covered and the outer lanes 1 and 8 are gently sprayed with the alkaline permanganate solution. After a few seconds distinct yellow spots appear against the purple background. The cover plate is removed and, by using the yellow spots as guides, thin straight lines are drawn across the width of the plate to indicate the various sugar-containing zones. All unwanted silica gel around the perimeter of the plate, including the sprayed area, is removed and discarded. The sugar zones are carefully scraped off into separate small centrifuge tubes of approximately 3 ml capacity. Determinations are carried out in triplicate, that is, zones corresponding to at least three lanes are taken separately for the determination of a particular sugar. Blanks are usually low and can be determined either by keeping some lanes free on the test plate or by using another plate.

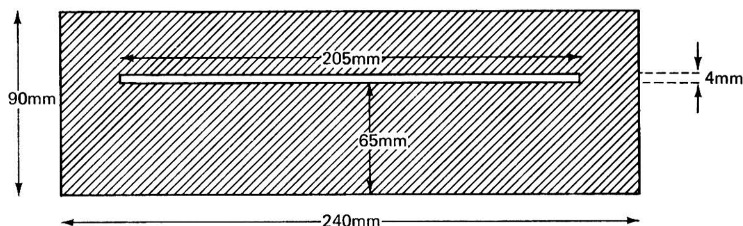

Fig. 1. Special Perspex lid for chromatographic tank

COLORIMETRIC DETERMINATION—

To each centrifuge tube is added 1·0 ml of water and the contents are agitated by gently tapping and rotating the tube. After a few minutes the silica gel is compacted by centrifuging the mixture and the clear liquid is carefully poured into a test-tube (150 × 18 mm) containing 0·2 ml of an aqueous solution of phenol of appropriate concentration (see Table I) prepared from an 80 per cent. stock solution. The washing procedure is repeated with a further 1·0 ml of water (a very thin glass rod being used, if necessary, to stir the silica gel) and the clear liquid combined with the original extract. To each test-tube, 5·0 ml of concentrated sulphuric acid are added from a fast-flow pipette,[16] the contents of the tube being agitated continuously during the addition of the sulphuric acid. After standing for 10 minutes at room temperature the tubes are placed in a water-bath at 25 to 30 °C for 15 minutes and the absorbances measured against the appropriate blanks by using 10-mm cells and a Pye-Unicam SP600 or SP800 spectrophotometer. The $\lambda_{max.}$ values are 481 nm for rhamnose and quinovose, 482 nm for fucose and 486 nm for glucose.

CALIBRATION GRAPHS—

A standard solution of a mixture of the four sugars, which is 0·100 M with respect to each sugar, is prepared and 0·6 to 2·0 μl of solution are applied per lane. For good separation

of rhamnose and quinovose not more than 200 nmol of either sugar is spotted on to the plate. Each determination is carried out in triplicate as in the general procedure.

RESULTS AND DISCUSSION

Fig. 2 shows the typical calibration graphs obtained for rhamnose, quinovose, fucose and glucose by the thin-layer chromatographic - colorimetric method. Variations in colour intensity obtained with 250 nmol of quinovose and various amounts of phenol were determined (Fig. 3). It can be seen that the optimum amount of phenol lies between 50 and 100 mg, or 0·2 and 0·4 mg of phenol per nmol of quinovose. By using this result and similar information given in reference 16 for the other sugars, Table I was constructed.

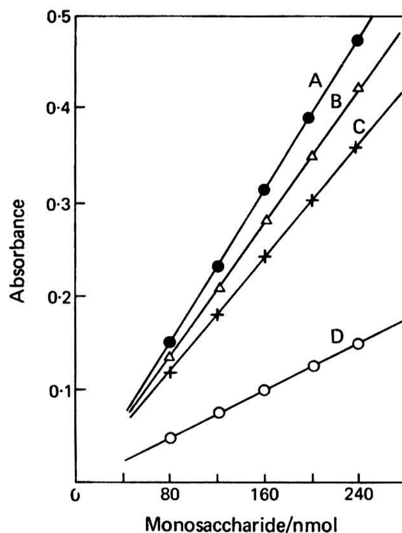

Fig. 2. Calibration graphs for thin-layer chromatographic - colorimetric determination of monosaccharides: graph A, glucose; graph B, quinovose; graph C, rhamnose; and graph D, fucose

When determinations were carried out in triplicate the results fell within ±4 per cent. of the theoretical value as illustrated in Table II. It became apparent early in the work that for successful quantitative application it was essential to divide the plate into lanes

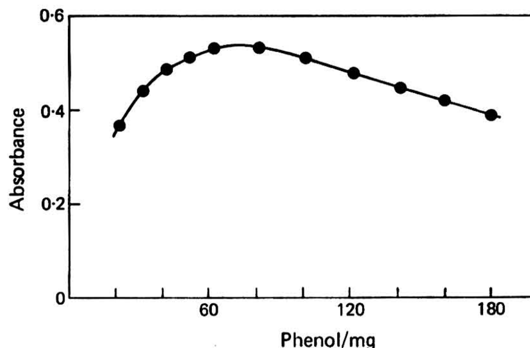

Fig. 3. Effect of phenol concentration on colour development (250 nmol of quinovose)

of equal width. This procedure subjects all parts of the plate to identical "edge effects" and leads to reproducible R_F values. The use of 20 × 20-cm inverted, grooved* plates in closed tanks was also investigated. Satisfactory separations occurred under these conditions but the continuous development technique was marginally preferred.

TABLE I

OPTIMUM AMOUNTS OF PHENOL FOR COLORIMETRIC DETERMINATION OF MONOSACCHARIDE (100 nmol LEVEL)

Monosaccharide	Phenol (80 per cent. aqueous solution) in 0·2 ml of aqueous solution/ml
Rhamnose	0·012†
Quinovose	0·045
Fucose	0·070†
Glucose	0·045†

† Calculated by reference to published information (Fig. 9 in Ref. 16).

Less accurate determinations could be made by directly treating the adsorbed sugars on the silica gel. For this purpose the adsorbent was scraped off, transferred to 50-ml centrifuge tubes and the colour development carried out. The silica gel was compacted before making spectrophotometric measurements on the clear liquid. Under the best conditions, *i.e.*, when the silica gel was pre-washed, etc., the spread of absorbance readings was less than 10 per cent. However, blanks tended to be higher under these conditions, but in spite of this drawback, the shortened method is preferable to the corresponding paper-chromatographic procedures in which even trace amounts of filter-paper fibre can make colorimetric determination very unreliable.

TABLE II

RESULTS OF A TYPICAL CHROMATOGRAM OF 160 nmol OF EACH MONOSACCHARIDE PER LANE

		Monosaccharide found in individual lanes/nmol							
		Lane							
Monosaccharide	$R_{Rhamnose}$	1	2	3	4	5	6	Mean of lanes 1 to 3	Mean of lanes 4 to 6
Rhamnose	1·00	167	161	164	161	155	155	164	157
Quinovose	0·80	158	153	160	163	164	160	157	162
Fucose	0·35	167	152	162	153	164	160	160	159
Glucose	0·10	159	159	164	155	161	162	161	159

We are grateful to Mr. W. H. C. Shaw of Glaxo Laboratories Ltd. for valuable suggestions regarding the preparation of this paper, and thank the Governors of the City of London Polytechnic for the award of a Research Assistantship to one of us (S.S.).

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Received *March 29th*, 1972
Accepted *June 7th*, 1972

* Marketed by May & Baker Ltd., Dagenham, Essex.

Analyst, December, 1972, Vol. 97, *pp*. 981–985

Determination of 5-Hydroxymethyl-2-furaldehyde in Honey

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Because existing methods for the determination of 5-hydroxymethyl-2-furaldehyde in honey were found to be unsatisfactory, a method that involves the use of column adsorption with activated charcoal for clean-up and determination by either a visual or spectrophotometric colorimetric method has been devised. It has been found to be adequate in respect of precision and accuracy.

MATERIALS that contain sugar produce 5-hydroxymethyl-2-furaldehyde on heating, treatment with acids or storage[1–4]; hence, it has been found in caramel[5] and commercial invert sugar.[1–3] Recently, the concentration of 5-hydroxymethyl-2-furaldehyde in honey has been limited to less than 40 p.p.m. to prevent the sale of old or badly stored, although genuine, samples, and also those containing an acid-hydrolysed sugar as an adulterant.[6]

Various methods for the determination of 5-hydroxymethyl-2-furaldehyde in honey have been reported in the literature.[7–11] Four of the methods were examined for precision and accuracy but all were found to be unsatisfactory, especially because of the undesirable formation of chromogens by fructose, which is invariably present and extracted, and because of the high solubility of 5-hydroxymethyl-2-furaldehyde in water, which prevents its complete extraction. A further method is therefore described in this paper.

EXPERIMENTAL

The diphenylamine,[10] *p*-toluidine - barbituric acid,[8] resorcinol[7] and benzidine[11] methods were carried out as recommended and the results are discussed below. 5-Hydroxymethyl-2-furaldehyde (obtained from Fluka), checked for potency by confirming that the molar absorptivity was 16 500 at 285 nm, was used in aqueous solution as a standard for the determination of an unknown concentration and also for recovery experiments. As this solution does not keep well, a freshly prepared solution, or one checked against known standards or a standard calibration graph, was always used.

PREPARATION OF COLUMN—

A column 200 mm long and 11·5 mm i.d. was used. A plug of cotton-wool or glass-wool was placed at the bottom of the column and about 1 g of dry activated charcoal (obtained from E. Merck) was inserted into the dry column. The charcoal was then compressed to a height of about 25 mm under water suction and covered at the top with another plug of cotton-wool or glass-wool. This adsorbent was made wet with water from below and the column was allowed to stand overnight. Excess of charcoal was next removed from the walls of the column, which was then washed with 25 ml of water at a flow-rate of 1ml min^{-1}.

PROCEDURE—

Two grams of honey were dissolved in 5 ml of water and the solution was added to the top of the column, followed by three 5-ml amounts of water, which were drawn into the column by suction, ensuring that the column did not become dry. The column was then washed with 100 ml of water to remove any adsorbed fructose and sucrose. The adsorbed 5-hydroxymethyl-2-furaldehyde was eluted with 150 ml of acetone (aldehyde free) and the eluate evaporated to dryness, after filtration if necessary, at room temperature in a current of air from a fan. Next, the residue was dissolved in distilled water and the volume made up to 10 ml. The amount of 5-hydroxymethyl-2-furaldehyde present was determined colorimetrically by the chromogenic procedure, the *p*-toluidine - barbituric acid method or spectrophotometrically by measuring the absorption at 285 nm. The whole operation should be completed within 1 day and the determination should be carried out immediately after the evaporation of the acetone.

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Results and discussion

The diphenylamine method[10] was found to be very sensitive to changes in the heating temperature used and did not give reproducible results, the results varying with slight variations in temperature.

Table I

Effect of filtration of honey solution on the determination of 5-hydroxymethyl-2-furaldehyde by Winkler's method

Before filtration, p.p.m.	After filtration, p.p.m.	Difference, p.p.m.
23·9	22·8	1·1
27·5	25·7	1·8
98·0	96·0	2·0
209·6	206·4	3·2

According to White, Kushnir and Subers,[4] the Winkler method[8] is superior to that of Schade, March and Eckert.[7] The former method is popular and has gained recognition by the Codex Alimentarius Commission of F.A.O./W.H.O.[6] It is rapid and simple and does not require any solvent extraction. However, it is apparent from Table I that an unfiltered solution has greater absorbance than a filtered solution, even when corrected for unfiltered and filtered blanks, respectively; the reason for this difference is not clear. This phenomenon has also been noted by Winkler. The results given in Table II show that his method gives consistently higher results, presumably for the above reason and because fructose is itself a chromogen (Table III). It has also been found that Winkler's method gives low values in the presence of free sulphur dioxide because of the formation of a hydrogen sulphite addition compound; at levels of sulphur dioxide greater than 10 mg l^{-1} the interference is serious.[12]

Table II

Comparison of Winkler's method and the proposed method for the determination of 5-hydroxymethyl-2-furaldehyde

By Winkler's method, p.p.m.	By proposed method, p.p.m.	Difference, p.p.m.
14·7	11·8	2·9
15·8	12·0	3·8
16·0	12·0	4·0
16·4	13·6	2·8
16·7	13·2	3·5
25·0	21·2	3·8
27·2	24·1	3·1
28·0	25·5	2·5

Schade, March and Eckert[7] advocated a method in which 5-hydroxymethyl-2-furaldehyde was extracted by mixing the sample with diethyl ether in a mortar and the extract was made to react with resorcinol; the results were found to be variable because of variability in the completeness of extraction of 5-hydroxymethyl-2-furaldehyde. These authors reported low results with honey of high moisture content, which they ascribed to difficulties in mixing the sample in the mortar with ether and to poor mixing because of the large amount of moisture present. However, in the course of these studies a different reason was found and this is discussed later. In Table IV it is shown that higher results are obtained by decreasing the moisture content of the honey. It can also be seen, from Table V, that results obtained by use of the Schade *et al.* and Winkler methods on the same sample differ, and from Table VI that the same sample subjected to the Schade *et al.* method at different times gives different results.

Table III

Tendency of fructose to form chromogens in Winkler's method

(5-Hydroxymethyl-2-furaldehyde equivalent of fructose)

Concentration of fructose solution, per cent.	20·0	10·0	8·0	5·0
Concentration of 5-hydroxymethyl-2-furaldehyde, p.p.m.	2·1	1·5	1·0	0·7

TABLE IV

EFFECT OF MOISTURE IN THE HONEY SAMPLE ON THE SCHADE *et al.* METHOD

(Honey with and without 40 per cent. of anhydrous sodium sulphate)

	5-Hydroxymethyl-2-furaldehyde, p.p.m.			
Honey alone	7·0	13·0	22·0	30·0
Honey with anhydrous Na_2SO_4	14·0	20·0	33·0	50·0

Rao and Taiwade[11] adopted a method with benzidine in which a 1 + 1 honey solution was extracted with diethyl ether and the extract made to react with benzidine. Results obtained by use of this procedure were very low and inconsistent, as shown by the recoveries in Table VII. Further, Table VIII shows that larger volumes of extractant will recover correspondingly larger amounts of 5-hydroxymethyl-2-furaldehyde.

TABLE V

COMPARISON OF THE SCHADE *et al.* AND WINKLER METHODS FOR THE DETERMINATION OF 5-HYDROXYMETHYL-2-FURALDEHYDE

By Schade's method, p.p.m. ..	23·8	27·5	44·0	96·0	180·0	209·6	385·0
By Winkler's method, p.p.m. ..	20·0	32·0	30·0	100·0	110·0	190·0	332·0

The method suggested for the extraction of 5-hydroxymethyl-2-furaldehyde in a continuous extractor[13] was also tried but found to be unsuitable.

As the extraction of 5-hydroxymethyl-2-furaldehyde from honey or honey solution with diethyl ether was found to be unsuitable, other solvents, *e.g.*, butanol, chloroform and ethyl acetate, were tried. These solvents also did not give the required recovery of added 5-hydroxymethyl-2-furaldehyde (results not shown).

TABLE VI

DIVERGENT RESULTS FOR 5-HYDROXYMETHYL-2-FURALDEHYDE IN THE SAME SAMPLE BY THE SCHADE *et al.* METHOD

Two values determined at different times

Value 1, p.p.m.	28·0	36·0	39·5	45·0	70·0
Value 2, p.p.m.	32·0	39·0	43·0	48·0	76·0

Good recoveries of 5-hydroxymethyl-2-furaldehyde from aqueous solutions (Table IX) and from honey containing only added 5-hydroxymethyl-2-furaldehyde (Table X) were obtained by using the proposed method. There was no significant difference at either the 1 or 5 per cent. level between the amount of 5-hydroxymethyl-2-furaldehyde added and that found (Tables IX and X) in the χ^2 test, the precision being 99 per cent. and the standard deviation being 1·39. A bias towards the lower values was noted; this effect was not investigated but was probably caused by a handling loss (not unusual with 5-hydroxymethyl-2-furaldehyde). It was found that the solution emerging from the column before elution contained fructose and sucrose but no 5-hydroxymethyl-2-furaldehyde and that the aqueous washings after adsorption removed all of the fructose and almost all of the sucrose, but none of the 5-hydroxymethyl-2-furaldehyde (confirmed by paper and thin-layer chromatography). The acetone eluate showed the presence of 5-hydroxymethyl-2-furaldehyde and the absence of fructose and sucrose (also confirmed by thin-layer chromatography); with samples of honey containing higher concentrations of sucrose, the eluate, while apparently containing no sucrose, showed trace amounts of it when evaporated to a small volume. Sucrose, however,

TABLE VII

RECOVERY OF 5-HYDROXYMETHYL-2-FURALDEHYDE BY RAO'S METHOD

5-Hydroxymethyl-2-furaldehyde added, p.p.m.	22·0	44·0	47·0	50·0
5-Hydroxymethyl-2-furaldehyde found, p.p.m.	3·0	15·0	18·0	20·0

TABLE VIII

RECOVERY OF 5-HYDROXYMETHYL-2-FURALDEHYDE WITH DIFFERENT VOLUMES OF EXTRACTANT BY RAO'S METHOD

5-Hydroxymethyl-2-furaldehyde added, p.p.m.	5-Hydroxymethyl-2-furaldehyde found in extractant, p.p.m.		
	3 × 10 ml	3 × 20 ml	3 × 30 ml
22·0	3·0	5·0	11·0
44·0	15·0	22·0	34·0
47·0	18·0	28·0	39·0
50·0	20·0	31·0	42·0

does not produce a colour with Winkler's reagent. The published methods of paper chromatography[14] and thin-layer chromatography[15] recommended for sugars have been successfully applied here to sugars and 5-hydroxymethyl-2-furaldehyde.

TABLE IX

RECOVERY OF 5-HYDROXYMETHYL-2-FURALDEHYDE FROM STANDARD SOLUTIONS BY THE PROPOSED METHOD

5-Hydroxymethyl-2-furaldehyde—		
in standard, p.p.m.	found, p.p.m.	recovered, per cent.
20·0	18·6	93·0
20·8	19·7	94·7
28·4	28·0	98·6
30·0	29·4	98·0
33·5	31·2	93·2
54·6	54·3	99·4
71·6	69·5	97·1

CONCLUSION

It can be concluded from the results that the four existing methods tried failed for the following reasons.

(*i*) The extractant, diethyl ether, presumably because of its water content and miscibility with water, or both, extracted fructose at the same time as 5-hydroxymethyl-2-furaldehyde.

(*ii*) 5-Hydroxymethyl-2-furaldehyde is moderately soluble in water. Therefore, complete extraction with diethyl ether from honey with a high moisture content or a diluted sample of honey was not possible.

(*iii*) Fructose has been found to react with all of the four chromogenic reagents used in the four methods. It has been shown (Table III) that fructose gives the same colour with the chromogenic reagent used in Winkler's method as that given by 5-hydroxymethyl-2-furaldehyde. Results on the reaction of fructose with the other three reagents have not been given.

The present method, which involves separation and clean-up of the 5-hydroxymethyl-2-furaldehyde from fructose by column adsorption, has been devised in order to overcome these difficulties.

TABLE X

RECOVERY OF 5-HYDROXYMETHYL-2-FURALDEHYDE FROM HONEY BY THE PROPOSED METHOD

5-Hydroxymethyl-2-furaldehyde added, p.p.m.	5-Hydroxymethyl-2-furaldehyde found, p.p.m.	Recovery, per cent.
34·7	33·2	95·7
31·5	30·4	96·5

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Received *September 8th*, 1971
Accepted *June 29th*, 1972

Analyst, December, 1972, Vol. 97, *pp*. 986–992

Solvent Extraction of Trace Amounts of Cobalt from Pure Reagent Chemicals and its Spectrophotometric Determination with 2-Nitroso-5-dimethylaminophenol

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Trace amounts of cobalt in pure reagent chemicals have been determined spectrophotometrically with 2-nitroso-5-dimethylaminophenol. Cobalt in amounts below 0·6 μg in the aqueous phase is extracted into 1,2-dichloroethane (5 ml) at pH 4 to 8. The excess of reagent and metal complexes other than the cobalt complex that are extracted into the organic phase are removed by extraction of the latter with 1 N potassium hydroxide solution followed by hydrochloric acid solution (1 + 2). The organic phase is then filtered through a dry filter-paper, and the absorbance of the organic phase measured in a cell of 50-mm path length at 456 nm. By using the above procedure, trace amounts of cobalt (10^{-8} to 10^{-4} per cent.) in sodium, potassium and iron(III) salts, etc., were determined by the standard addition method.

NITROSO derivatives of phenols and naphthols are well known to be specific reagents for cobalt. Of these compounds, 2-nitroso-1-naphthol[1–3] is widely used as a reagent for the extraction and spectrophotometric determination of micro-amounts of cobalt. Recently, the author reported on the usefulness of 2-nitroso-5-dimethylaminophenol as a reagent for the extraction and determination of micro-amounts of cobalt[4] following a detailed investigation of this reagent and the complex it forms with cobalt.[5] The main advantages in its use are as follows: excess of the reagent in the organic phase is removed by shaking the latter with either an acid or a base; and the molar absorptivity of the cobalt complex is $6{\cdot}0 \times 10^4$ at 456 nm in 1,2-dichloroethane. The author accordingly determined micro-amounts of cobalt in iron and steel[6] and in commercial nickel salts[7] with 2-nitroso-5-dimethylaminophenol.

Previously, Marczenko[8] had determined trace amounts of cobalt (10^{-6} to 10^{-5} per cent.) in very pure alkali hydroxides by extracting their solutions with a solution of dithizone (diphenylthiocarbazone) in carbon tetrachloride and then determining the cobalt spectrophotometrically with 1-nitroso-2-naphthol. Babko, Kuz'min, Listskaya, Ourutskii and Freger[9] determined trace amounts of cobalt (10^{-8} to 10^{-6} per cent.) in alkali hydroxides and sodium and potassium nitrates by emission spectroscopy after extracting the cobalt as the 8-hydroxyquinolinate and diethyldithiocarbamate. More recently, Anderson[10] determined cobalt in sodium by lanthanum hydroxide carrier precipitation and spectrographic analysis.

In the present work, the cobalt in the aqueous phase is concentrated in the organic phase by solvent extraction and the colour developed is measured in a cell of 50-mm path length. By using this procedure trace amounts of cobalt (10^{-8} to 10^{-4} per cent.) in pure reagent chemicals were rapidly and accurately determined.

EXPERIMENTAL

APPARATUS—

An Iwaki, Model KM, shaker was used to shake the separating funnels, and a Hitachi Perkin-Elmer, Model 139, spectrophotometer and Hitachi, Model EPS-3T, recording spectrophotometer were used to measure absorbances.

REAGENTS—

2-Nitroso-5-dimethylaminophenol—The solid reagent was prepared by nitrosation of *m*-dimethylaminophenol with sodium nitrite in hydrochloric acid solution, the crude product

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obtained being recrystallised from hydrochloric acid solution.[7] Dissolve the hydrochloride salt in 0·01 N hydrochloric acid solution to give a 10^{-2} M solution.

Standard cobalt solution—Dissolve 1·19 g of cobalt chloride hexahydrate in 1 litre of distilled water and standardise the solution by titration with EDTA solution. Use this solution after suitable dilution.

Buffer solution, pH 7—Dissolve 40 g of sodium citrate ($Na_3C_6H_5O_7.2H_2O$) in water and dilute the solution to 100 ml with water. Add 5 ml of 2-nitroso-5-dimethylaminophenol solution and allow the mixture to stand for about 15 minutes, then shake it with 5 ml of 1,2-dichloroethane for about 2 minutes. Discard the organic phase. Repeat the extraction until the aqueous phase is colourless. If any 2-nitroso-5-dimethylaminophenol remains in the buffer solution, it will decompose gradually and the absorbance of the reagent blank will increase slightly. Then filter the aqueous solution through a filter-paper and use the filtrate as the cobalt-free buffer solution.

PREPARATION OF SAMPLE SOLUTIONS—

Sodium and potassium salt solutions, 10 *to* 30 *g per* 100 *ml*—The required amounts of pure reagent sodium and potassium salts were weighed and dissolved in distilled water. These solutions were diluted to the required volume with water.

Ammonia, sodium hydroxide and hydrochloric acid solutions—Ammonia solution (sp. gr. about 0·9) and solid sodium hydroxide were diluted with or dissolved in distilled water, and the solutions were neutralised with hydrogen chloride (from a cylinder) which had been passed through a Woulff's scrubbing bottle containing distilled water before being led into the sample solution. Hydrochloric acid (sp. gr. 1·18) was diluted with distilled water and neutralised with the ammonia solution (sp. gr. about 0·9) or sodium hydroxide solution (about 10 N), the cobalt content of which was known. The neutral solutions were diluted to the required volume with water.

Iron(III) salts—The required amounts of pure reagent iron(III) salts were weighed into a beaker, and the citric acid buffer solution (20 ml per 100 ml of sample solution) and distilled water were added. The mixtures were stirred until the salts were dissolved and then diluted to the required volume with water.

PROCEDURE—

Three 100-ml portions of the sample solution (or smaller portions when a large amount of cobalt was present) were transferred by pipette into separating funnels; 5 ml of distilled water were then introduced by pipette into one of the funnels and 5 ml of the solutions containing known amounts of cobalt, by pipette, into the other two funnels. Finally, 5 ml of the buffer and the 2-nitroso-5-dimethylaminophenol solutions were transferred by pipette into each funnel [for iron(III) salts and sodium citrate solution, however, no buffer solution was necessary, and for iron(III) salts, 10 ml of the reagent solution were added], and the solutions were mixed and allowed to stand (see below). To each funnel, 5 ml of 1,2-dichloroethane were added by pipette and the mixtures were shaken in a shaker at the rate of 300 oscillations per minute (see below). After the two phases had separated, the organic phase was run off into a 20-ml stoppered test-tube. The organic phase was washed twice with 5 ml of 1 N potassium hydroxide solution and once with hydrochloric acid solution (1 + 2) and filtered through a dry filter-paper. The absorbance was then measured in a cell of 50-mm path length at 456 nm against the reagent blank. The concentration of cobalt was determined by the standard addition method.

RESULTS

Cobalt is extracted quantitatively into the organic phase at pH 3 to 8.[7] In this work, the extraction of cobalt was carried out at pH 4 to 7 so as to ensure greater accuracy.

SHAKING TIME—

In following the above procedure, the shaking time was examined by using two series of samples of the aqueous phase, one prepared with distilled water containing 0·3 μg of cobalt and the other with a solution of ammonium iron(III) sulphate [$Fe_2(SO_4)_3(NH_4)_2SO_4.24H_2O$]. In both instances, shaking was started 45 minutes after the addition of the 2-nitroso-5-dimethylaminophenol solution. From these results (Fig. 1), it is clear that a shaking time of 2 minutes was sufficient. In this work, the shaking time was fixed at 5 minutes so as to ensure complete extraction.

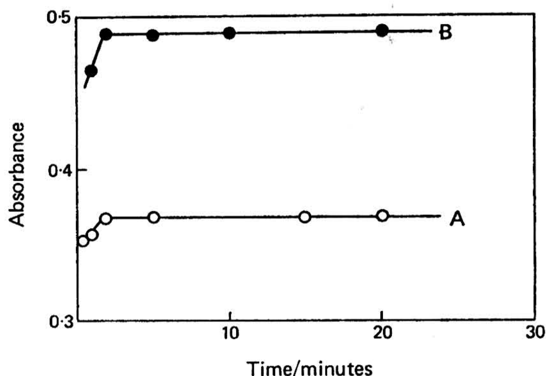

Fig. 1. Absorbance *versus* shaking time: A, cobalt 0·30 μg; and B, ammonium iron(III) sulphate [*a* (*ii*), see Table III] 5·18 g per 100 ml. Standing time 45 minutes

STANDING TIME AFTER ADDITION OF 2-NITROSO-5-DIMETHYLAMINOPHENOL SOLUTION AND BEFORE SHAKING—

It had been shown in previous work[6,7] that when large amounts of metal ions that react with this reagent, such as iron(III) and nickel ions, co-exist, a longer standing time is necessary for the reaction with cobalt to take place than when these ions are absent. When iron(III) ions were present at 0·1 M concentration and 1 ml each of citric acid buffer solution and 2-nitroso-5-dimethylaminophenol solution (5×10^{-3} M) were added to 5 ml of the sample solution, a standing time of 2½ minutes was sufficient. In the present work, the standing time for two series was examined (Fig. 2). When iron(III) ions (at about 0·1 M concentration) were present, a standing time of 5 minutes was found to be sufficient. The standing time was, however, fixed at 10 minutes so as to ensure that the reaction reached completion.

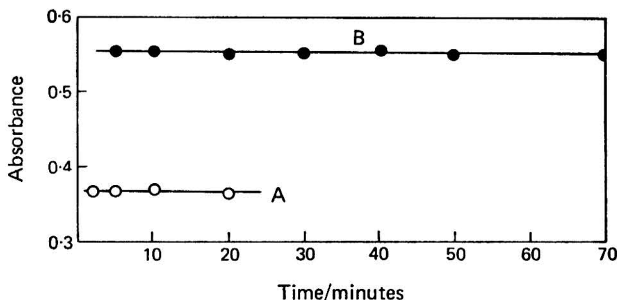

Fig. 2. Absorbance *versus* standing time: A, cobalt 0·30 μg; and B, ammonium iron(III) sulphate [*a* (*ii*), see Table III] 5·66 g per 100 ml. Shaking time 5 minutes

REMOVAL OF THE 2-NITROSO-5-DIMETHYLAMINOPHENOL FROM THE ORGANIC PHASE—

The 2-nitroso-5-dimethylaminophenol in the organic phase is removed by shaking the latter with either an alkaline or an acidic solution. The logarithm of the distribution ratio of 2-nitroso-5-dimethylaminophenol, log q_R, is −2·0 at pH values of −0·3 and 12,[5] so that when the organic phase is washed with hydrochloric acid solution (1 + 2) or 1 N potassium hydroxide solution, the amount of the reagent remaining in the organic phase will be less than 1 per cent. In this work, the organic phase was washed three times so as to ensure as low an absorbance of the reagent blank as possible. Further washing produced no improvement (Table I). The small difference in absorbance between the second and third washings was due to a slight decrease in volume of the organic phase. With samples such as nitrate

and citrate, the first washing must not be carried out with hydrochloric acid (1 + 2), otherwise the organic phase becomes greenish in colour, which is caused by oxidation of the 2-nitroso-5-dimethylaminophenol. Accordingly, the washing was carried out twice with 0·1 N potassium hydroxide solution and once with hydrochloric acid solution (1 + 2), which enabled the excess of reagent and the other metal complexes, except cobalt, to be removed almost completely.

TABLE I

WASHING OF THE ORGANIC PHASE WITH 1 N POTASSIUM HYDROXIDE AND 1 + 2 HYDROCHLORIC ACID SOLUTIONS

Reagent added/ml	Absorbance		
	One washing	Two washings	Three washings
2·5*	0·028 (HCl)	0·022 (HCl)	0·021 (HCl)
5·0*	0·052 (HCl)	0·029 (HCl)	0·027 (HCl)
7·5*	0·058 (HCl)	0·031 (HCl)	0·029 (HCl)
5·0	(HCl)	(HCl)	0·018 (HCl)
	(KOH)	(KOH)	0·013 (HCl)
10·0	(KOH)	(KOH)	0·025 (HCl)
	(KOH)	(KOH)	0·023 (HCl)
	(KOH)	(KOH)	0·019 (HCl)

* Five millilitres of phosphoric acid buffer solution (pH 6·2) were added. Cobalt in this buffer solution was not removed by solvent extraction.

ABSORPTION SPECTRA—

The absorption spectra of the cobalt complex and the reagent blank in 1,2-dichloroethane are shown in Fig. 3. The maximum absorption of the cobalt complex occurs at about 456 nm, at which wavelength the absorbance of the reagent blank is about 0·02; the absorbance was therefore measured at 456 nm.

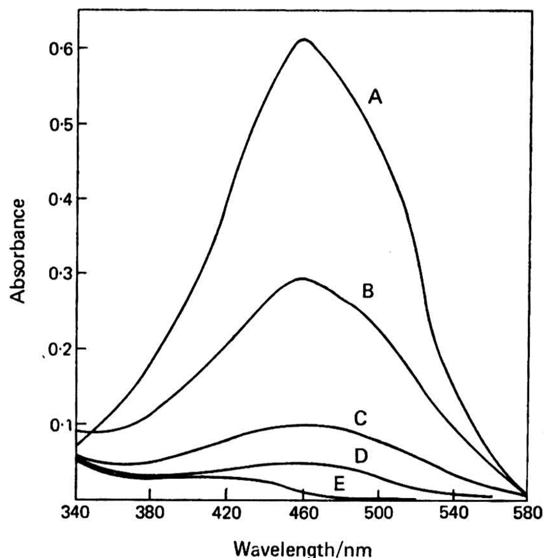

Fig. 3. Absorption spectra: graph A, cobalt 2·9 μg; graph B, cobalt 0·41 μg; graph C, KCl 26·7 g per 100 ml; graph D, NaCl 28·6 g per 100 ml; and graph E, reagent blank. Graph A, ratio of aqueous phase to organic phase 7:5 (cell of 10-mm path length). Graphs B to E, ratio of aqueous phase to organic phase 110:5 (cell of 50-mm path length). Reference 1,2-dichloroethane

DETERMINATION OF COBALT IN SODIUM AND POTASSIUM SALTS—

Trace amounts of cobalt in sodium and potassium salts were determined by the above procedure (Table II). All of the results obtained by the standard addition method gave linear graphs, the slopes of which were, however, different in each instance as a result of the salt effect. In Table II the absorbances obtained by using the sample solutions to which no cobalt had been added are also shown. The absorbances of the reagent blank obtained by using the sample solution from which the cobalt had been removed in the same way as in the preparation of the buffer solution are also shown. From the facts that the wavelengths of maximum absorption of the different samples are identical and that the absorbances of the reagent blank are almost identical, it is concluded that the absorbance measured at 456 nm against the reagent blank is due to the cobalt complex.

TABLE II

DETERMINATION OF COBALT IN SODIUM AND POTASSIUM SALTS AND IN MIXTURES INVOLVING AMMONIA SOLUTION, HYDROCHLORIC ACID SOLUTION AND HYDROGEN CHLORIDE GAS

Sample				Cobalt	
Compound	Grade	Amount taken per 100 ml	Absorbance*	µg per 100 ml	Per cent. *m*/*m*
NaCl	*a*	31·7 g	0·083 (0·022)	0·098$_2$	3·1 × 10^{-7}
	e	28·6 g	0·031 (0·022)	0·048$_7$	1·7 × 10^{-7}
Na_2SO_4	*a*	30·2 g	0·067 (0·017)	0·084$_7$	2·8 × 10^{-7}
	e	27·9 g	0·004 (0·025)	—	—
$NaNO_3$	*a*	28·9 g	0·037 (0·016)	0·048$_7$	1·7 × 10^{-7}
	e	28·9 g	0·012 (0·017)	0·021$_0$	7·3 × 10^{-8}
KCl	*a*	26·6 g	0·070 (0·029)	0·083$_2$	3·1 × 10^{-7}
	e	28·4 g	0·054 (0·022)	0·066$_0$	2·3 × 10^{-7}
K_2SO_4	*a*	14·8 g	0·050 (0·023)	0·067$_5$	4·6 × 10^{-7}
	e	14·6 g	0·269 (0·023)	0·30$_8$	2·1 × 10^{-6}
KNO_3	*a*	25·3 g	0·002 (0·022)	—	—
	e	27·6 g	0·021 (0·025)	0·024$_0$	8·7 × 10^{-8}
Sodium citrate	*a*	38·5 g	0·349 (0·026)	0·43$_1$	1·1$_1$ × 10^{-6}
	e	35·8 g	0·270 (0·017)	0·35$_7$	9·9$_7$ × 10^{-7}
§{ NH_3 + HCl (gas)	*a*	43·3 ml	0·279 (0·022)	0·30$_9$	7·1$_4$ × 10^{-7}‡
§{ NH_3 + HCl (gas)	*e*	44·5 ml	0·036 (0·022)	0·040$_0$	9·0 × 10^{-8}‡
§{ HCl	*e*	7·3 ml	0·133	0·150 (0·107)†	1·4$_7$ × 10^{-6}‡
+ NH_3	*a*	6·0 ml			
§{ NaOH + HCl (gas)	*a*	20·1 g	0·149	0·155	7·7 × 10^{-7}‡
§{ HCl	*a*	41·3 ml	0·177	0·182 (0·033)†	8·0 × 10^{-8}‡
+ NaOH	*a*	19·3 g			

a and *e* denote analytical-reagent grade and extra pure reagent, respectively.

* Obtained by using the sample solution to which cobalt was not added (values in parentheses are the absorbances of the reagent blank).

† The values in parentheses were obtained by subtracting the amounts of cobalt present in ammonia solution or sodium hydroxide solution.

‡ Per cent. *m*/*V*.

§ In each instance, the first compound mentioned was neutralised with the second, as described under Preparation of sample solutions.

DETERMINATION OF COBALT IN AMMONIA, SODIUM HYDROXIDE AND HYDROCHLORIC ACID SOLUTIONS—

Cobalt present in the neutralised solutions was determined in the same way as in the sample solutions of sodium and potassium salts (Table II), it being assumed that no cobalt was present in the hydrogen chloride. The cobalt content of the hydrochloric acid was determined by subtracting the amounts of cobalt found in the ammonia or sodium hydroxide solution.

DETERMINATION OF COBALT IN IRON(III) SALTS—

Cobalt in iron(III) salts was determined by the above procedure (Table III). In each instance the results obtained by the standard addition method gave linear graphs with slopes that were identical with the slope of the calibration graph obtained by using the same volume of distilled water. Consequently, it was found that cobalt in iron(III) salts could be determined conveniently by using the calibration graph. The slope of each graph, however, will be different when different volumes of the aqueous phase are used, owing to the solubility of 1,2-dichloroethane in the aqueous phase. When the sample solution contains relatively large amounts of cobalt, less than 100 ml of solution can be used. The calibration graph must, however, be constructed by using the same volume of distilled water.

TABLE III

DETERMINATION OF COBALT IN IRON(III) SALTS

Sample: Salt	Sample: Grade	Sample: Amount taken/ g per 100 ml	Absorbance*	Cobalt, per cent. *m/m*
$Fe_2(SO_4)_3(NH_4)_2SO_4.24H_2O$ ..	*a* (*i*)	4·062	0·149	$3·5 \times 10^{-6}$
		4·835	0·172	$3·4 \times 10^{-6}$
		10·30†	0·340	$3·1 \times 10^{-6}$
	(*ii*)	4·709	0·489	$9·2 \times 10^{-6}$
		2·502	0·247	$9·0 \times 10^{-6}$
	(*iii*)	1·022	0·297	$2·6 \times 10^{-5}$
	e	1·660	0·105‡	$3·9 \times 10^{-5}$
$Fe(NO_3)_3.9H_2O$	*a*	2·115	0·233‡	$5·9 \times 10^{-5}$
$FeCl_3.6H_2O$	*a*	0·340	0·323‡	$4·5 \times 10^{-4}$
	e	0·090	0·245‡	$1·3 \times 10^{-3}$

a and *e* denote analytical-reagent grade and extra pure reagent, respectively, and (*i*), (*ii*) and (*iii*) different suppliers.

* Obtained by using the sample solution to which cobalt was not added.

† Twenty millilitres of the 2-nitroso-5-dimethylaminophenol solution were added.

‡ Twenty millilitres of sample solutions were used and 2 ml of the 2-nitroso-5-dimethylaminophenol solution added.

The limitation of the above procedure is shown in Fig. 4. When the sample solution contains more than about 6 g of ammonium iron(III) sulphate per 100 ml, cobalt cannot be extracted. In such instances, more of the reagent and citric acid buffer solutions must be added.

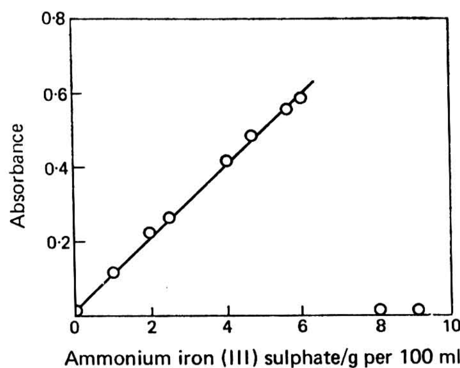

Fig. 4. Effect of iron(III) ions. Sample: ammonium iron(III) sulphate [*a* (*ii*), see Table III]. Absorbances measured against 1,2-dichloroethane

DISCUSSION

To date, many organic analytical reagents have been used for determining cobalt, of which 2-nitroso-5-dimethylaminophenol is the most useful. In previous work,[6,7] 7 ml of the

aqueous phase were shaken with 5 ml of 1,2-dichloroethane, and the absorbance of the cobalt complex was measured in a cell of 10-mm path length.

The value determined for the partition coefficient of the cobalt - 2-nitroso-5-dimethylaminophenol complex, log D_{MR_3} (= log $[MR_3]_o/[MR_3]_a$), was 3·11.[5] From this value, it was found that even when the ratio of the volumes of the aqueous and organic phases is 20:1, most of the cobalt (99·5 per cent.) can be extracted into the organic phase. Consequently, the concentration of trace amounts of cobalt by solvent extraction was examined, and the absorbance of the concentrated cobalt complex in the organic phase was measured in a cell of 50-mm path length. By using this concentration technique the sensitivity of the cobalt determination can be increased by more than 100 times compared with that obtained in the previous work.[6,7] The absorbance is about 0·33 when 100 ml of sample solution with a concentration of cobalt of 5×10^{-8} M is used. The calculated value of the apparent molar absorptivity (absorbance/concentration) is $6{\cdot}6 \times 10^6$, that is, 110 times larger than the true molar absorptivity ($6{\cdot}0 \times 10^4$).

In the spectrophotometric method in which 2-nitroso-5-dimethylaminophenol is used, most cations do not interfere. However, as iron(II) and iron(III), nickel and copper ions react with this reagent and their complexes are partially extracted into the organic phase, large amounts of these cations interfere. Iron(III) and nickel ions were masked with citric acid,[6,7] and the complexes that were co-extracted into the organic phase were easily decomposed with the hydrochloric acid solution (1 + 2).

When many samples of a similar kind are required to be analysed, the calibration graph established as follows can be used to simplify the determination. The calibration graph is obtained by dissolving known amounts of cobalt in the solution from which cobalt has been removed by extraction. For iron(III) salts, the calibration graph obtained by using distilled water can be used.

The proposed method involving solvent extraction with 2-nitroso-5-dimethylaminophenol enables cobalt to be determined with greater sensitivity than is possible with other methods.

The author is indebted to Professor Kyoji Tôei of Okayama University for his valuable advice and discussion.

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Received *April 18th*, 1972
Accepted *July 21st*, 1972

Analyst, December, 1972, Vol. 97, *pp*. 993–997

The Determination of Chloride by Atomic-absorption Spectrophotometry

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A method for the determination of trace amounts of chloride ion in aqueous solution is described. The chloride is converted into phenylmercury(II) chloride, which is extracted into chloroform and subsequently determined in ethyl acetate solution by means of atomic-absorption spectrophotometry. Alternatively, but less conveniently, the phenylmercury(II) chloride can be extracted into isopropyl acetate and this solution, after concentration, is sprayed into the spectrophotometer. As little as 0·015 p.p.m. of chloride can be detected in a 250-ml sample of water, and 0·1 p.p.m. of chloride can be determined with a relative mean error of 4·6 per cent. Interferences are minimal but bromide, iodide, thiocyanate and cyanide must be absent from the solution. The present method compares favourably with a previously described gas - liquid chromatographic method, and is only slightly less sensitive.

FEW procedures for the determination of chloride involving atomic-absorption spectrophotometry[1–6] have been described in the literature. In all of these methods chloride was precipitated with silver ions, and either the excess of silver in solution, or that in the precipitate after dissolution in ammonia solution,[5] was determined by atomic-absorption spectrophotometry.

As described previously,[7] chloride has been determined at low levels by gas chromatography. The chloride ion is converted into phenylmercury(II) chloride by reaction of an aqueous solution of the chloride with an aqueous solution of phenylmercury(II) nitrate in a perchloric acid medium at about pH 1·5. In the present method phenylmercury(II) chloride is quantitatively extracted with chloroform, which is then evaporated off and the residue re-dissolved in ethyl acetate. This solution is then aspirated into the atomic-absorption spectrophotometer. Alternatively, phenylmercury(II) chloride can be quantitatively extracted into isopropyl acetate and this solution can be aspirated, after concentration if necessary, directly into the atomic-absorption spectrophotometer.

EXTRACTION OF PHENYLMERCURY(II) CHLORIDE

CHOICE OF SOLVENT—

Conditions for the reaction of chloride ion with phenylmercury(II) nitrate and extraction into chloroform of the phenylmercury(II) chloride formed have been established previously.[7] However, chloroform is not a suitable solvent for use in atomic-absorption spectrophotometry; two possibilities were therefore examined. The first of these was the evaporation of the solution of phenylmercury(II) chloride in chloroform to dryness and its re-dissolution in a suitable organic solvent, such as isobutyl methyl ketone or ethyl acetate. The well known properties of chloroform that are advantageous in solvent-extraction processes (*e.g.*, high density, low solubility in water) do not extend to atomic-absorption spectrophotometry. Nevertheless, it is possible to remove the chloroform by evaporation and take up the residue in a suitable solvent. The concentration of this solution can then be measured by atomic-absorption spectrophotometry. It was found most convenient to remove the chloroform under a slight vacuum on a water-bath at about 60 °C because at atmospheric pressure the normally white residue of phenylmercury(II) chloride can become brown and cause an error in the results. Ethyl acetate was chosen as the solvent to re-dissolve the residue of phenylmercury(II) chloride because of the considerable enhancement in the sensitivity of mercury absorption thus achieved.

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The second possibility was to find an alternative solvent that is free from the disadvantages of chloroform. Various organic solvents that are useful for atomic-absorption spectrophotometry, including hydrocarbons, ketones and esters, were examined. The main requirements for the present purpose are that the solvent must readily dissolve phenylmercury(II) chloride, it must not dissolve phenylmercury(II) nitrate, the reagent, to any appreciable extent, it must have a low solubility in water and, preferably, it should enhance the mercury signal.

Of several solvents investigated only ethyl acetate and isopropyl acetate were found to meet all of the above conditions. The solubilities of ethyl acetate and isopropyl acetate in water at 20 °C are about 8·6 and 3·0 per cent. *m*/*V*, respectively. The approximate solubilities of phenylmercury(II) chloride and nitrate at 20 °C in ethyl acetate are 0·66 and 0·01 per cent. *m*/*V*, respectively, and in isopropyl acetate, 0·60 and 0·01 per cent. *m*/*V*, respectively.

Good results were obtained with ethyl acetate but it is appreciably soluble in water, so that the less soluble isopropyl acetate was preferred. Preliminary investigations showed that the extraction of chloride as phenylmercury(II) chloride into isopropyl acetate from a large volume of aqueous solution was satisfactory. A two-step extraction with 17 and 5 ml of solvent from a volume of 250 ml of aqueous solution was found to be necessary.

However, the above extraction process was time consuming because of the slow separation of the layers and because the organic phase was now the upper layer. The total time needed to carry out a determination was therefore longer than when chloroform was used. Not only is the extraction step rather long, but also the concentration step (which is necessary in order to increase the sensitivity of the procedure). The boiling-point of isopropyl acetate is about 30 °C higher than that of chloroform. The over-all times for the two procedures were about 45 minutes for chloroform and 75 minutes for isopropyl acetate. This difference arises because chloroform is less soluble than isopropyl acetate in water and separates readily in the lower layer, thus eliminating the step of pouring out the aqueous layer and returning it to the separating funnel with fresh solvent. The chloroform is readily distilled off, leaving the phenylmercury(II) chloride. In fact, the need for the distillation step is the only disadvantage with the chloroform procedure, while the main advantage in the use of isopropyl acetate is that the organic layer is ready for atomic-absorption spectrophotometry.

As the difference in the time required is considerably in favour of the chloroform procedure, several parallel determinations by both procedures were carried out. The results obtained are discussed in the following sections.

CALIBRATION GRAPH—

The calibration graph is linear in the range 0·005 to 0·2 mg ml^{-1} of phenylmercury(II) chloride for both ethyl and isopropyl acetates. Because of the extremely high stability of the 253·65-nm absorption line of mercury, scale expansion has been used for the lower concentrations.[8] The useful range of concentrations for the determination of mercury as phenylmercury(II) chloride in ethyl acetate or isopropyl acetate was found to be between 0·04 and 0·2 mg ml^{-1}, which corresponds to a chloride concentration of 4·4 to 22 μg ml^{-1} in the organic phase.

In the present work, it proved more convenient during the development of the method to base the calibrations on pure phenylmercury(II) chloride, the substance actually being measured. However, there is no reason why the procedure should not be referred to pure sodium chloride as standard; in applications of the method, this would be an appropriate reference material and would avoid the necessity of obtaining or preparing specially pure phenylmercury(II) chloride.

EFFECT OF DIVERSE IONS—

The effect of other species on the formation and extraction of phenylmercury(II) chloride has been studied previously.[7] In this instance nitrite does not interfere, but silver, mercury(I), mercury(II), bromide, iodide, thiocyanate and cyanide interfere and must not be present in the solution. All these ions are, however, readily eliminated from samples of chloride.

EXPERIMENTAL

REAGENTS—

Phenylmercury(II) nitrate—A 0·1 per cent. *m*/*V* aqueous solution was prepared by dissolving the appropriate amount of reagent in warm distilled water. From this solution, a 0·01 per cent. solution was prepared by dilution.

Phenylmercury(II) chloride—A standard solution (1 mg ml^{-1}) of phenylmercury(II) chloride in ethyl acetate or isopropyl acetate was prepared. Other solutions were obtained by diluting this solution. The compound was prepared as previously reported[7] from phenylmercury(II) nitrate and sodium chloride solution.

Standard chloride solution—A standard aqueous solution of chloride (0·1 mg ml^{-1}) was prepared from analytical-reagent grade sodium chloride. Other solutions were prepared by dilution of aliquots of the standard solution.

Perchloric acid, 60 per cent. m/m—Analytical-reagent grade material was used.

The solvents used were of general-purpose reagent quality.

APPARATUS—

Atomic-absorption measurements were obtained with a Varian Techtron AA-5 atomic-absorption spectrophotometer fitted with a 6-inch air - acetylene burner. A mercury hollow-cathode lamp was used with a current of 3 mA and measurements were made at the most suitable absorption line (253·65 nm). The spectral slit width was 100 μm.

PROCEDURE—

With chloroform as solvent—Transfer 250 ml of the sample into a 500-ml separating funnel, add 2 ml of 60 per cent. *m/m* perchloric acid, or enough to provide a final pH of about 1·5, and 1·5 ml of an aqueous 0·01 per cent. solution of phenylmercury(II) nitrate for each 10 μg of chloride (for amounts above 50 μg use a 0·1 per cent. reagent solution). After mixing, leave the solution for approximately 5 minutes and add 10 ml of chloroform. Shake the funnel vigorously for 1 minute and allow the layers to separate. Remove the organic phase and repeat the extraction with 5 ml of chloroform, adding this to the first extract. Evaporate the combined chloroform solutions to dryness under slightly reduced pressure on a water-bath at about 60 °C in a pear-shaped distillation flask, then cool the flask. Dissolve the white residue of phenylmercury(II) chloride in ethyl acetate, transfer the solution to a calibrated flask and rinse the distillation flask twice with small volumes of ethyl acetate. For amounts above 0·1 mg of chloride use a 10-ml calibrated flask, whereas with less than 0·1 mg, use a 5-ml flask. Make up the contents of the flask to the calibration mark with ethyl acetate and determine the amount of mercury by use of the atomic-absorption spectrophotometer.

With isopropyl acetate as solvent—The procedure with isopropyl acetate as solvent is almost the same as that with chloroform. To the chloride solution containing perchloric acid and reagent add 17 ml of isopropyl acetate for the first extraction and 5 ml for the second. Concentrate the combined extracts under reduced pressure on a water-bath and transfer to a calibrated flask as before. On no account should the organic extract be concentrated by direct heating. Not only will this decompose the phenylmercury(II) chloride, but there is a slight risk associated with the heating of an organic solvent recovered from an aqueous perchloric acid solution.

RESULTS

PRECISION AND ACCURACY AND LOWER LIMIT OF DETECTION—

Three test runs with chloroform and ethyl acetate, and two with isopropyl acetate, with different concentrations of chloride were made to establish the precision and accuracy of the method. The results are given in Table I.

Blanks were determined, the values varying according to the amount of reagent used. For amounts of chloride lower than 0·1 p.p.m. the blank is very significant and should be determined for each set of analyses. When the above procedure was applied to a 250-ml sample of water, the limit of detection was found to be 0·015 p.p.m. of chloride in the original aqueous solution.

COMPARISON BETWEEN THE ATOMIC-ABSORPTION SPECTROPHOTOMETRIC AND GAS-CHROMATOGRAPHIC METHODS—

Several samples with different chloride concentrations were analysed by the present method and by the gas-chromatographic method. The results are given in Table II.

From the results in Table II, there appears to be good agreement between the two methods used for the determination of chloride.

TABLE I

PRECISION AND ACCURACY RESULTS FOR THE DETERMINATION OF CHLORIDE BY ATOMIC-ABSORPTION SPECTROPHOTOMETRY

Chloride ion taken/μg ml^{-1}*	Number of deter-minations	Mean chloride ion found/μg ml^{-1}†	s	S_r, per cent.	Mean error	Relative mean error, per cent.
With chloroform and ethyl acetate—						
0·1	9	0·095	0·0069	7·2	0·005	5·0
0·2	9	0·193	0·0065	3·3	0·007	3·5
0·4	8	0·391	0·0192	4·9	0·009	2·2
With isopropyl acetate—						
0·04	6	0·035	0·00593	16·9	0·005	12·5
0·4	6	0·382	0·01728	4·5	0·018	4·5

* These concentrations are equivalent to a final concentration in the ethyl acetate or isopropyl acetate solution of 5, 10, 10, 2 and 10 μg ml^{-1} of chloride ion, respectively.
† Values obtained after deducting the blank.

DETERMINATION OF BROMIDE AND IODIDE—

The present procedure for the determination of chloride ion is based on the determination of the phenylmercury(II) radical by atomic-absorption spectrophotometry and any species that forms an isolable complex with this radical could also be determined. Thus, as indicated in the original gas-chromatographic method, bromide and iodide can be determined in an analogous way. These ions are listed as interferences in the determination of chloride because they react quantitatively with the reagent to form the corresponding phenylmercury(II) halides, which are then extracted together with the chloride complex. This, of course, offers the possibility of a total halide determination followed by step-wise elimination of iodide and bromide by well known chemical reactions[9] to give the individual halide content of a sample.

TABLE II

DETERMINATION OF CHLORIDE BY ATOMIC-ABSORPTION SPECTROPHOTOMETRY AND GAS CHROMATOGRAPHY

The results are given in p.p.m. Each value is an average of four determinations

Method	Sample 1	2	3	4	5	6	7
Gas-liquid chromatography	0·36	0·05	0·19	0·08	0·07	0·14	0·09
Atomic-absorption spectrophotometry	0·31	0·04	0·22	0·085	0·06	0·135	0·06

CONCLUSIONS

The present method for the determination of chloride by atomic-absorption spectrophotometry has several advantages over other atomic-absorption spectrophotometric procedures in that it is much more sensitive, easier to operate, and is less affected by the presence of other ions. It is probable that improved sensitivity would result from the use of flameless atomic-absorption procedures involving carbon rod atomisers, which require only a few microlitres of solution. It should then be possible to concentrate the organic phase containing the phenylmercury(II) chloride to a very small volume. However, care would need to be taken to avoid loss of the volatile phenylmercury(II) chloride from the carbon rod before atomisation of the mercury could be achieved. An enclosed Massman-type furnace would have to be used.

A.N. is grateful to the Arya-Mehr University of Technology, Tehran, for study leave and J.A.R. is grateful to the University of Santiago de Compostela for leave of absence, and to the Consejo Superior de Investigaciones Cientificas and the Ministerio de Education y Ciencia of Spain for financial support. We are grateful to Mr. E. T. Smith of the Chemical Engineering Division, A.E.R.E., Harwell, for the provision of water samples, and to Dr. W. T. Elwell, Imperial Metal Industries Ltd., Birmingham, for tests on the analytical procedure.

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Received *July 6th*, 1972
Accepted *August 15th*, 1972

Analyst, December, 1972, Vol. 97, *pp*. 998–1002

Determination of Organic Hydroxyl Groups by Nuclear Magnetic Resonance Spectroscopy by Using Trimethylsilyl Derivatives

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A nuclear magnetic resonance spectroscopic method is presented for the determination of hydroxyl values in mixtures of organic compounds. A nine-fold enhancement of sensitivity is obtained by the formation of trimethylsilyl derivatives, which makes the method especially useful for the determination of low hydroxyl values. The method can be applied to the rapid determination of the hydroxyl values of fatty acids, esters, polymerised fatty acids, polyesters, tertiary alcohols and certain phenols. *o*-Nitrophenols and highly hindered phenols fail to give correct results, as do hydroxy-compounds of low relative molecular mass, which give volatile trimethylsilyl derivatives. Epoxides and non-enolised ketones do not interfere in the determination. Certain shortcomings of methods for the determination of hydroxyl values based on acetylation and differential titration are discussed.

THE quantitative determination of monomer, dimer and trimer contents in polymerised fatty acids has already been described.[1] When the hydroxyl values of polymerised fatty acid mixtures, and of polyesters derived from them, were required, we first considered a number of standard[2,3] and several rapid[4–7] methods, all of which are based on the esterification of hydroxyl groups followed by differential titration of the esterification reagent used. Among the rapid methods, only Vasilescu's method[6] had been applied to fatty acids and Stetzler and Smullin's method[4] to fatty esters. None of the micro[8,9] or submicro[10] methods had been applied to fatty acids. According to Ulbrich,[11] epoxides interfere in the determination of hydroxyl values with a mixture of acetic anhydride and pyridine, which is the usual reagent in standard methods. Further, ethers, ketones, and aromatic and aliphatic unsaturated compounds interfere[4] in the rapid method catalysed by perchloric acid.[7] None of the methods is applicable when tertiary alcohols are present, except for triphenylmethanol, which can be determined by use of acetic anhydride with perchloric acid as catalyst.[7]

Applewhite[12] and Radin[13] have reviewed a number of methods for the determination of hydroxyl values other than those based on differential titration. O'Connor[14] described a spectrophotometric method at a near-infrared wavelength, while Hopkins[15] used conventional nuclear magnetic resonance spectroscopy for the analysis of hydroxy-substituted fatty acids and Ward[16] used the technique for the determination of the degree of esterification of pentaerythritol, in this instance with the help of an added internal standard.

Reilly[17] has introduced a nuclear magnetic resonance based method that involves the addition of formic acid to the sample, whereby the peaks of the hydroxyl protons are shifted sufficiently to allow their integration to take place free from interference.

Because of the low hydroxyl content in our samples, the methods based on differential titration were inaccurate. This inaccuracy was augmented by the high acid values involved. In addition, we found that considerable errors occurred as a result of the large sample sizes necessitated by the low hydroxyl contents.

We required a method for hydroxyl value determination that would be applicable at levels below 1 g and yet sensitive enough at the low hydroxyl levels found in our samples and it occurred to us that these requirements could be met with nuclear magnetic resonance spectroscopy combined with the nine-fold amplification of the hydroxyl signals by formation of trimethylsilyl derivatives. As reference methods we chose the rapid method described by Stetzler and Smullin[4] and a standard acetylation method.[2]

EXPERIMENTAL

APPARATUS—

A Varian A-60 nuclear magnetic resonance spectrometer (integrator accuracy 2 per cent.) was used. The external standard was tetramethylsilane, for which τ is equal to 10·0.

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STANDARDS—

*High-purity methyl behenate (methyl docosanoate).**

*Methyl 9,10-dihydroxystearate (methyl 9,10-dihydroxyoctadecanoate), high purity.**

2-Hydroxycaproic acid (2-hydroxyhexanoic acid).†

Phthalide (melting-point 74 °C).

Anthrone [9(10H)-*anthracenone*].†

p-*Nitroacetophenone.*†

SAMPLES—

Ensol 1 (*tall oil fatty acid distillate*).‡

Polymerised fatty acids—These were made from Ensol 1 by polymerisation at 220 or 271 °C catalysed by acidic clay (see Table II). Prior to polymerisation, 4 per cent. of water and 4 per cent. *m/m* of clay were added.

γ-Stearolactone (4-*octadecanolide*).[18]

Methyl 9,10-epoxystearate (methyl 9,10-epoxyoctadecanoate).[19]

Methyl 9- and 10-oxostearate (methyl 9- and 10-oxooctadecanoate).[20]

1-*Phenoxy-2-methylpropan-2-ol.*[21]

o-*Nitrophenol.*†

2,4-*Dinitrophenol.*†

Polyester resin 1—This was made from 1·98 mol of ethane-1,2-diol and 0·67 mol each of adipic acid, maleic anhydride and polymerised tall oil fatty acid. In use, 0·27 part of styrene is added to 1 part of resin, which results in an acid value of 27·5.

Polyester resin 2—This was made from 2·2 mol of propane-1,2-diol and 10·67 mol each of phthalic anhydride, maleic anhydride and polymerised tall oil fatty acid. In use, 0·45 part of styrene is added to 1 part of resin, which results in an acid value of 19·0.

Polyester resin 3—This was made in the same way as resin 2 but with a 40 per cent. excess of propane-1,2-diol. In use, 0·45 part of styrene is added to 1 part of resin, which results in an acid value of 11·1.

PROCEDURE—

Weigh 20 to 1000 mg of sample into a 50-ml flask, add 1 to 10 ml of benzene and esterify the free carboxyl groups with an ethereal solution of diazomethane. In the absence of carboxylic acids in the sample, the treatment with diazomethane can be omitted. Allow the mixture to stand for 5 minutes at ambient temperature and evaporate it to dryness in a rotary evaporator at a bath temperature of 70 °C for 10 minutes. Cool the residue to room temperature and add 10 to 60 mg of internal standard (*p*-nitroacetophenone or phthalide). For the silylation, add 1 ml of carbon tetrachloride, 0·5 ml of dried pyridine and 200 μl of trimethylchlorosilane, allow the mixture to stand for 10 minutes at ambient temperature and evaporate it to dryness to remove excess of reagent and any hexamethyldisiloxane (bath temperature 70 °C, immersion time 10 minutes). Dissolve the product in deuterochloroform, carbon tetrachloride or benzene and record the spectrum.

The height (15 to 220 mm) of integrals of the standard and trimethylsilyl ether peaks, at or near 7·4 τ (*p*-nitroacetophenone) and 10·0 τ, respectively, are measured and the mean values of at least triplicate determinations used for the calculation of the hydroxyl value, taking into account the number of protons contained in the peaks (three and nine, respectively). The hydroxyl value is defined[2] as the number of milligrams of potassium hydroxide required to neutralise the amount of acetic acid that combines by acetylation with 1 g of sample.

RESULTS AND DISCUSSION

In order to evaluate the sensitivity of our method a series of dilutions of methyl 9,10-dihydroxystearate *plus* methyl behenate was prepared so as to contain about 2, 5, 10, 12 and 100 per cent. of dihydroxystearate. *p*-Nitroacetophenone was used as the reference standard. The results, each calculated from five spectra, are given in Table I.

It can be seen in Table I that reasonable results are obtained even at quite low hydroxyl values. However, the relative accuracy is then much inferior compared with that at high hydroxyl values.

* Supplied by the Hormel Institute.
† Supplied by Fluka.
‡ Supplied by Enso-Gutzeit O.Y., Finland.

TABLE I

HYDROXYL VALUES OF MIXTURES OF METHYL BEHENATE AND METHYL 9,10-DIHYDROXYSTEARATE

Content of methyl 9,10-dihydroxystearate, per cent.	Total amount of sample/mg	Amount of *p*-nitroacetophenone/mg	Proton ratio, $(CH_3)_3SiO$ to $CH_3COC_6H_4NO_2$	Hydroxyl value by nuclear magnetic resonance spectroscopy	Standard deviation	Theoretical hydroxyl value
1·96	1015·2	44·9	1·307	6·5	± 1·4	6·7
4·81	210·2	20·0	1·505	16·2	± 2·6	16·3
9·10	220·9	31·8	1·89	30·7	± 2·8	30·8
12·34	82·6	20·0	1·597	43·7	± 3·0	41·8
100	20·0	20·0	2·928	330	±16·1	339

Application of the method to similar mixtures of methyl behenate and 2-hydroxycaproic acid (M_r 132) was unsuccessful. Evidently a trimethylsilyl derivative of such molecular size is fairly easily evaporated off, and we found that our results were inversely proportional to the time of evaporation.

In principle, the presence of unevaporated trimethylchlorosilane (τ value about 9·6) in the nuclear magnetic resonance spectroscopic samples should not interfere in the measurement of the $(CH_3)_3SiO$– peak (τ value about 10·0). However, when a large excess of trimethylchlorosilane is present, the appearance of side bands and base-line elevation makes accurate measurement of small $(CH_3)_3SiO$– peaks difficult. In addition, we found that with unevaporated samples another small peak appeared at about 10·0 τ, which almost coincided with the $(CH_3)_3SiO$– peak and led to errors, especially with small samples and low hydroxyl values. This peak is caused by hexamethyldisiloxane, a volatile impurity of trimethylchlorosilane. Re-distilling the reagent twice yielded uncontaminated trimethylchlorosilane, but contact with atmospheric or sample moisture again caused errors. Thus, when the excess of reagent cannot be evaporated off owing to the volatility of the sample, measurements may be unreliable, even when rigorously purified reagents and solvents are used.

When methanol (0·5 ml) was added prior to the final evaporation to destroy the excess of the silylation reagent, hydroxyl values were obtained that were excessively high although the methoxytrimethylsilane formed should have been fairly volatile. Similar results were obtained when a mixture of trimethylchlorosilane and hexamethyldisilazane was used as the silylation reagent, presumably because of the higher boiling-point of the latter.

p-Nitroacetophenone and phthalide were found to be suitable reference compounds for our purposes; a third possibility, anthrone, was eliminated because it enolised under the conditions used. The use of the methoxycarbonyl signals of fatty acid methyl esters was also considered as a reference method. Although these signals could probably normally be used, it was found that the CH–O–$Si(CH_3)_3$ signals are shifted to lower values by the presence of another oxygen function at the vicinal carbon in 9,10-dihydroxystearate derivatives, and very closely coincide with the CH_3OCO– signals at $\tau = 6·3$.

Hydroxyl values for a fatty acid mixture, certain polymerised fatty acids and polyester resins are given in Table II, with *p*-nitroacetophenone as the internal standard. In addition, hydroxyl values for a number of related model compounds are included.

It can be seen in Table II that comparable results for polyester resins are obtained by the rapid titration method[4] and by silylation followed by nuclear magnetic resonance spectroscopy. It is also apparent that epoxides and ketones interfere in the determination of hydroxyl values by acid-catalysed acetylation and titration[4] whereas these functions do not cause errors in the silylation and nuclear magnetic resonance spectroscopic method. Of course, epoxides interfere even in the acetic anhydride - pyridine standard titration.[11]

Widely different hydroxyl values for fatty acids and polymerised fatty acids result from the two titration methods. The rapid titration method used[4] in this work is based on acetylation (by acetic anhydride in ethyl acetate), catalysed by toluene-4-sulphonic acid, with a 15-minute reaction time. Excess of acetic anhydride is hydrolysed by adding water and aqueous pyridine and allowing the mixture to stand for 5 minutes at room temperature. The acetic acid formed in the solution is then titrated with an alcoholic solution of potassium hydroxide, and the hydroxyl value obtained from the difference between this titration and

a blank determination. The result is finally corrected for the effect of any acidic or basic functions in the sample. The low hydroxyl contents of our samples required fairly large amounts (5 to 20 g) to be used for acetylation. The hydroxyl value then obtained for the tall oil fatty acid distillate was about 30. The deviation among different measurements was very great, the hydroxyl values from twenty-three determinations ranging from 17 to 42. Several sources of error are apparent. Any titration method is inherently inaccurate when large acid values and small hydroxyl values are involved. The hydroxyl values obtained by using this method were dependent on the size of the sample actually used for the determination, this effect being more pronounced with fatty acids than with esters. Samples in excess of 2 g dissolve acetic anhydride effectively enough to require a much longer time than 5 minutes for the subsequent hydrolysis at room temperature. The temperature during hydrolysis cannot be increased as the solvent, ethyl acetate, will then be hydrolysed. Further, unsaturated fatty acids react under acidic catalysis, presumably to give lactones, as was indicated by the fact that a sample of pure oleic acid of less than 1 g gave a hydroxyl value of 6·2, whereas pure methyl oleate gave a hydroxyl value of 0·0. A sample of less than 1 g of pure linolic acid gave a hydroxyl value of 9·4, and the tall oil fatty acid mixture used for our polymerisation gave a hydroxyl value of 9·1 or, when esterified, 1·9.

It is widely known[22] that unsaturated carboxylic acids give lactones on treatment with strong acids. The method of Stetzler and Smullin[4] discussed above was originally introduced for the determination of hydroxyl values in polyethers. It can also be used with fatty esters and polyesters, but fatty acids may give erroneous results. On the other hand, hydroxyl values of fatty acids, obtained by use of a standard method,[2] are in agreement with those obtained by silylation and nuclear magnetic resonance spectroscopy.

Schenk and Santiago[9] have found that acetylation catalysed by perchloric acid does not give reliable hydroxyl values with tertiary alcohols and *o*-nitrophenols. Results with such compounds obtained by trimethylsilylation followed by nuclear magnetic resonance spectroscopy are presented in Table III. It can be seen that the tertiary alcohol, which is also

TABLE II

HYDROXYL VALUES OF FATTY ACIDS, POLYESTERS AND RELATED COMPOUNDS

Sample		Amount of sample/ mg	Amount of internal standard/ mg	Proton ratio, $(CH_3)_3SiO$ to $CH_3COC_6H_4NO_2$	Hydroxyl value by nuclear magnetic resonance spectroscopy	Standard deviation	Hydroxyl value by titration[4]	Hydroxyl value by titration[2]
Tall oil fatty acids (Ensol 1)		312·6	43·3	0·11	1·7	±0·5	9·1	0·4
Polymerised tall oil fatty acids of Ensol 1								
Polymerisation temperature/°C	Time/ minutes							
220	15	195·7	36·5	0·24	5·1	±1·6	28·5	—
220	60	231·5	43·0	0·26	5·4	±1·6	22·5	8·9
220	120	197·0	38·4	0·20	4·3	±1·6	24·1	10·4
271	15	306·3	39·0	0·58	8·4	±1·8	19·2	—
271	30	307·3	31·6	0·54	7·8	±1·7	30·8	7·2
271	60	216·8	41·4	0·29	6·9	±1·7	—	12·8
271	120	206·3	41·7	0·32	7·7	±1·8	—	10·4
Polyester resin 1 ..		286·5	40·4	1·60	25·5	±4·0	29·3	—
Polyester resin 2 ..		206·3	43·6	2·44	58·3	±4·5	42·0	—
Polyester resin 3 ..		102·8	40·7	2·27	102	±8·7	107	—
Methyl 9,10-epoxystearate		295·1	41·2	0·65	10·2*	±2·1	59·9	—
Methyl 9- and 10-oxostearate ..		300·0	41·0	0·22	3·4	±1·7	66·2	—
γ-Stearolactone ..		299·6	41·1	0·067	1·04	±0·5	0·0	—

* Probably due to a contaminant (methyl 9,10-dihydroxystearate).

a 1,2-diol monoether, is determined accurately, whereas the *o*-nitrophenols are not. It is known[24] that highly hindered phenols such as 2,6-di-t-butylphenol fail to undergo silylation under these mild conditions.

TABLE III

HYDROXYL VALUES OF A TERTIARY ALCOHOL AND *o*-NITROPHENOLS

Sample	Amount of sample/mg	Amount of internal standard/mg	Proton ratio, $(CH_3)_3SiO$ to reference compound	Hydroxyl value by nuclear magnetic resonance spectroscopy	Theoretical hydroxyl value
1-Phenoxy-2-methyl-propan-2-ol	29·0	52·2*	2·04	347	341·6
o-Nitrophenol	31·2	51·7†	0·788	150	402·6
2,4-Dinitrophenol ..	29·9	56·8†	0·399	85·7	309·4
2,6-Di-t-butylphenol ..	31·2	50·6†	0·0	0·0	271·8

* Phthalide.
† *p*-Nitroacetophenone.

This trimethylsilylation and nuclear magnetic resonance spectroscopic method can be used over a wide range of hydroxyl values and it is especially convenient when small samples with low hydroxyl values are to be analysed. The method is fairly rapid, and few functional groups (C–H acids, primary and secondary amines) interfere. The method should also be useful for the determination of hydroxyl groups, especially in vicinal diols, in the presence of epoxides, when the established methods often seem to fail[4,11,23] or involve the use of special apparatus.[25] The samples on which the hydroxyl value is determined by our method should preferably be dry, although no stringent requirements are necessary in this respect as the small amounts of hexamethyldisiloxane formed in the presence of water are easily removed during evaporation. A major disadvantage of the method is that hydroxylated compounds of low relative molecular mass (about 150, depending on structure) do not respond satisfactorily as the trimethylsilyl ethers of such compounds are too volatile and give hydroxyl values that are too low.

We thank Miss Tuula Tyrväinen for her skilful assistance in the silylations and titrations. We are also grateful to the Alfred Kordelin Foundation for financial support.

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Received *March 6th*, 1972
Accepted *June 2nd*, 1972

The Determination of Tin in Biological Material by Using Neutron-activation Analysis

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A method for the determination of tin in biological material, by using neutron-activation analysis and radiochemical separation of tin-121, is described. Decontamination from sodium and antimony was shown to be satisfactory by tracer techniques, and the method was applied to biological standard materials and other samples. The precision is ± 10 per cent. at the submicrogram level, and the sensitivity (which could be lowered by increasing the neutron flux) was found to be 10 to 20 ng.

TIN is an element that cannot readily be determined in biological material by any method of analysis. For many years the main interest has lain in determining the amount of tin that contaminates canned foodstuffs, as this must be kept below statutory limits and may be as high as 0·2 per cent. Recently Schwarz, Milne and Vinyard[1] have shown that this element is essential for the healthy growth of rats, although the concentration required in the diet is only of the order of 1 mg kg^{-1}. As Schwarz has remarked, experiments of this kind are frequently limited by the analytical sensitivity available.[2] The present work describes a technique involving neutron activation of tin-121 of half-life 27 hours, which is a pure beta-emitter. The method appears to be reasonably precise and sufficiently sensitive to permit the determination of natural concentrations of tin in biological material.

METHOD

ACTIVATION—

Aliquots (0·5 to 1 g) of dry biological materials were weighed on to squares of aluminium foil, which were then folded into packets. Standards were prepared by dissolving Specpure tin in a mixture of sulphuric and nitric acids and diluting the solution so that 1 drop contained 10 to 20 μg of tin. Drops of this stock solution were weighed on to 1×1-cm squares of Whatman 541 filter-paper, after which the squares were dried and enclosed in aluminium-foil packets. For each run, samples, standards and filter-paper blanks were activated for about 24 hours in a flux of about 2×10^{12} neutrons cm^{-2} s^{-1}.

REAGENTS—

All reagents were of recognised analytical grade.
Sulphuric acid, 18 M.
Nitric acid, 16 M.
Perchloric acid, 60 *per cent.* m/m.
Hydrochloric acid, 12 *and* 1 M.
Potassium iodide solution, 5 M.
First washing reagent—Prepare a 1 M solution of potassium iodide in 1·5 M sulphuric acid.
Potassium thiocyanate solution, 3 M.
Second washing reagent—Prepare a 1 M solution of potassium thiocyanate in 0·6 M hydrochloric acid.
Sodium hydroxide solution, 2 M.
Tin carrier—Prepare by dissolving tin in warm 6 M hydrochloric acid and diluting the solution to obtain a concentration of 20 mg cm^{-3} of tin.
Antimony carrier—Dissolve sodium pyroantimonate in warm 6 M hydrochloric acid to give a solution containing 20 mg cm^{-3} of antimony.
Arsenic carrier—Dissolve sodium arsenate in water to give a solution containing 20 mg cm^{-3} of arsenic.

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DESTRUCTION OF ORGANIC MATTER—

For all samples except bone, the activated samples were transferred to 150-cm³ tall-form beakers containing 2.5 cm³ of sulphuric acid, 1·0 cm³ of tin carrier, 10 cm³ of nitric acid and 2 cm³ of perchloric acid. The beakers were covered and heated on a hot-plate behind a screen of lead bricks; if charring occurred, more nitric and perchloric acids were added and the excess of these acids was evaporated off. The beakers were then allowed to cool behind the lead screen.

No completely satisfactory method of ashing was evolved for bone samples. Activated bone (0·5 g) was added to a nickel crucible containing 2 g of an equimolar mixture of sodium and potassium nitrates and 1·0 cm³ of tin carrier. This mixture was heated to about 400 °C over a bunsen burner for a few minutes, cooled and then treated with 4·5 M sulphuric acid that was 0·5 M in potassium iodide so as to obtain tin(IV) iodide.

CHEMICAL SEPARATION OF TIN—

The first step in the separation is based on that described by Byrne,[3] who determined tin-123*m* of half-life 40 minutes. As this procedure gave inadequate decontamination from arsenic and antimony, a further step that involved solvent extraction of the tin as the thiocyanate was added before precipitating the tin as tin(II) sulphide for determining the chemical yield. The extraction step with thiocyanate was recommended by Morrison and Freiser.[4]

The sample, wet ashed as described above, was made 4·5 M in sulphuric acid and 0·5 M in potassium iodide by adding 6·5 cm³ of water, cooling and then adding 1 cm³ of potassium iodide solution to precipitate yellow tin(IV) iodide. This suspension was shaken with 10 cm³ of toluene in a separating funnel and the toluene was poured off into a clean funnel and washed twice with the first washing reagent. The toluene was then shaken with 10 cm³ of 1 M hydrochloric acid and the organic layer was rejected.

The aqueous layer was treated with 1 drop each of arsenic and antimony carriers, followed by 5·0 cm³ of potassium thiocyanate solution. It was then extracted with 15 cm³ of diethyl ether in a separating funnel, and the aqueous layer was rejected while the ethereal layer was washed twice with the second washing reagent. Tin was back-extracted from the ethereal layer by shaking the latter with 10·0 cm³ of sodium hydroxide solution; the addition of a few drops of universal indicator aided in defining the phase boundary at this stage. The sodium hydroxide solution was neutralised with 3·0 cm³ of concentrated hydrochloric acid, and hydrogen sulphide was then passed through the solution to precipitate tin(II) sulphide; this precipitation is rather slow. The tin(II) sulphide was centrifuged, washed three times with water and finally transferred to a weighed aluminium counting tray for weighing and counting. The chemical yield was usually about 75 per cent., and the separation time for eight samples was about 1½ hours. Standards were treated in the same manner as samples.

DETERMINATION OF RADIOACTIVITY—

The tin(II) sulphide samples were counted by using a scintillation counter with a thin crystal of anthracene as a detector. Samples were usually counted for 1000 s and the background count was 0·2 s^{-1}. No corrections were needed for dead time or self-absorption. All samples were checked for gamma-emitting impurities with a 256-channel gamma spectrometer, and a few samples were found to be contaminated with a 0·56-MeV gamma-emitter, probably arsenic-76 or antimony-122. These contaminated samples were rejected. As an additional check on contamination, the decay of samples was compared with that of standards over a period of 5 to 10 days.

TESTING THE SEPARATION PROCEDURE—

The efficiency of the separation procedure was tested by using 0·5-g aliquots of inactive kale powder spiked with tin-113, antimony-124 and sodium-22. The results are shown for each step in Table I.

From the results in Table I it can be calculated that for a chemical yield of 88 per cent. of tin, the decontamination factor from sodium is less than 10^{-12} per cent., while the decontamination from antimony is 0·0018 per cent. Other separation steps that were tested included the solvent extraction of tin(IV) with a solution of 0·5 M 2-thenoyltrifluoroacetone in isobutyl methyl ketone,[5] the precipitation of tin with *N*-benzylphenylhydroxylamine[6,7] and the

TABLE I

EFFICIENCY OF STEPS IN SEPARATION PROCEDURE

Separation step	Tin retained, per cent.	Antimony retained, per cent.	Sodium retained, per cent.
(*i*) Extraction of tin(IV) iodide into toluene	96·01	29·8	0·066
(*ii*) Two washes of toluene layer	96·71	1·56	<0·002
(*iii*) Toluene after back-extraction of tin as $Sn(SCN)_4$ ..	0·24	0·21	—
(*iv*) Extraction of $Sn(SCN)_4$ into diethyl ether	98·99	1·63	8·7
(*v*) Two washes of ether layer	99·46	23·4	0·76
(*vi*) Diethyl ether after back-extraction of tin	0·28	0·03	—
(*vii*) Precipitation of tin(II) sulphide, with three washes of the precipitate	97·54	99·5	0·11

extraction of tin(IV) iodide by using polyurethane foam,[8] but none of these procedures offered any significant advantages. It was not found possible to separate tin and antimony by adding sodium fluoride to the solution prior to the sulphide precipitation step, as the amount of fluoride needed to suppress the precipitation of tin(II) sulphide greatly inhibited the precipitation of antimony(III) sulphide as well.

RESULTS AND DISCUSSION

The results for the analysis of a number of replicate samples with mean values (in parentheses) are given in Table II.

TABLE II

ANALYSIS OF REPLICATE SAMPLES

Material	Tin/ng g^{-1}
Standard kale (dry)	321, 322, 337, 402, 411 (359)
I.A.E.A. standard flour	58, 59, 61, 61 (59·9)
I.A.E.A. dried animal blood	63, 84, 88, 97 (82·9)
Wheat flour	214, 222 (218)
Barley seed	344, 499 (421)
Rice seed	295, 295 (295)
N.B.S. orchard leaves 1571	3570, 4640 (4100)

The accuracy of the technique is not easily assessed, as few workers have determined tin at the low concentrations involved. Values of 280 ng of tin per gram of standard kale have been published by Byrne[3] and agree fairly well with those found by the proposed method.

The precision of the technique is generally better than ± 10 per cent.; for example, for the first three samples in Table II above the probable errors were $\pm 7·3$, $\pm 1·5$ and $\pm 10·1$ per cent. The precision is probably affected by the arsenic and antimony present in the sample, as it was least satisfactory for the N.B.S. orchard leaves 1571, which are known to contain rather large amounts of these elements, both of which are readily activated by thermal neutrons and are difficult to separate from tin. Several of the tin(II) sulphide sources that were separated from N.B.S. 1571 were contaminated with a 0·56-MeV gamma-emitter and were rejected.

The sensitivity of the technique depends on the neutron flux available for activation, but the amount of tin needed to double the background count in this work was 10 to 20 ng, which compares favourably with other methods of determining tin.[3,7,9]

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Received *June 19th*, 1972
Accepted *August 7th*, 1972

Book Reviews

ANALYTICAL GEOCHEMISTRY. By LORD ENERGLYN and L. BREALEY. *Methods in Geochemistry and Geophysics. Volume* 5. Pp. xvi + 426. Amsterdam, London and New York: Elsevier Publishing Company. 1971. Price £9·25.

The publishers must to a very large extent take the blame for the gross inadequacies of this book. This reviewer has criticised an earlier volume in this series, and the lack of a capable authority as Series Editor is even more apparent in this volume. The book is intended to serve those geologists who have not studied chemistry but require guidance rather than instruction (!) in this topic. In my view, geologists, students of geology and others active in this field would do well to avoid this volume.

The choice of material is unfortunate, especially the omission of any description of atomic-absorption techniques. Electron-probe analysis, with less than 1½ pages, deserves a great deal more, whilst the brief sections on solvent-extraction and ion-exchange methods are almost lost at the tail-end of the chapter on flame photometry. The chapters on X-ray diffraction and X-ray fluorescence are adequate and these, together with that on emission spectroscopy, form the best parts of the book. Absorption spectroscopy is sadly neglected.

Even taking the authors' special interests and expertise into consideration, over 60 pages on chromatography, including 46 pages on gas chromatography, is excessive when compared, for example, with none at all on calculations, errors, correlation of data, tests of significance and distribution of results—all topics of great importance to a geologist seeking an insight into the problems of geochemistry.

I wish I could say that, within the material selected, the authors have made a good job of their task. Unfortunately, I cannot. Never have I encountered an analytical text with so many errors. These range from errors of spelling (drogen on p. 111; smalite on p. 65) to errors of formula (CaCl, Tl_2O_2, ThO_3 on p. 230; Al_2O_2 on p. 293) to inconsistencies (feldspar and felspar; caesium and cesium; columbium and niobium; millimicron and angstrom) to lack of clarity (p. 282, Fig. 61, in which the Compton peak is not properly identified), to errors of fact (p. 48, carbonates of tin and titanium; p. 49, oxidation with ammonia; p. 111, titanium dioxide used to prepare sodium hydrogen sulphate). These examples include some of the worst errors, but there are many others. There is a curious duplication of the text on pp. 276 and 277, whilst Table VIII on p. 52 duplicates material given in the text on p. 53, although the authors indicate that it contains a list of the principal characteristics of microchemical tests.

Chapter 4, on the determination of minor elements, leaves much to be desired. The material upon which it is based is sadly out of date and the authors' choice of methods has not always been the best. The detailed criticisms are legion. Why is no mannitol method given for determining boron or Willard and Winter distillation for fluorine? Why is there no phenylfluorone method for germanium? Why are photometric methods for mercury, molybdenum, nickel, titanium and tungsten omitted? Why is sulphur, a commonly determined element, given only eight lines on pp. 200–201, whereas rhenium, a rare and seldom determined element, is given thirty lines on pp. 190–191?

This volume is expensive by present-day standards; for me it would be dear at any price.

P. G. JEFFERY

A TEXTBOOK OF SOIL CHEMICAL ANALYSIS. By P. R. HESSE. Pp. xxvi + 520. London: John Murray. 1971. Price £7·50.

It is difficult to assess how successfully the author has achieved his aim of narrowing the gap between the theory of soil chemistry and the practice of soil analysis as described in laboratory manuals. It is certainly questionable whether this treatise provides "a means of comparing all known methods for a particular analysis and of selecting the most suitable, considering available facilities," because modern instrumental methods, often the most appropriate, are largely ignored. Such references as are made to flame-photometric and other spectrochemical methods indicate a lack of appreciation of such techniques. The reader is often left in some doubt regarding the reason for the preference of a particular method and is given no facts from which to draw his own conclusions. Nevertheless, this book provides a source of background information about

analytical procedures that is unobtainable elsewhere and its main consequence may be to encourage intelligent enquiry into the choice of appropriate methods.

Two introductory chapters consider the purpose of soil analysis, precision and accuracy, the presentation of results and the preparation of samples for analysis, but not the actual process of sampling, possibly the most important step of all. Only in the final chapter, dealing with water-logged soils, is field sampling mentioned, and here only in the context of the special problems that arise in anaerobic conditions.

Seven chapters cover the related subjects of soil reaction, acidity, exchangeable hydrogen, lime requirement, insoluble carbonates, soluble salts, cation and anion exchange, calcium and magnesium and potassium and sodium. Included with carbonates is a discussion of sulphur requirement, relating not to sulphur deficiency but to the amelioration of saline soils. This 130-page section probably fulfils most closely the objectives of the author and gives useful guidance, as well as raising many topics that the enquiring student will want to examine more intensively.

The four chapters dealing with nitrogen, carbon and organic matter, phosphorus and sulphur are equally instructive, but the discussions on iron, aluminium and manganese and on silicon, titanium and sesquioxides in the two chapters that complete the coverage of the major constituents of soil are more superficial and illustrate the somewhat irrational nature of the presentation. A more systematic treatment, with the elimination of irrelevant material and statements out of context, would undoubtedly improve the over-all acceptability of the text.

The longest chapter devotes 7 pages to total (elemental) analysis and 57 pages to thirty-three trace elements. The former discusses methods of bringing soil constituents completely into solution prior to analysis. While the author points out that such treatment is seldom relevant to trace-element investigations, many of the methods quoted for these elements are in fact for total content. The discussion on trace-element determinations is probably the least useful part of the book. After a brief mention of the minerals in which trace elements occur in mineral deposits, but which are seldom their form of occurrence in soils at trace level, one or two possible chemical techniques for each is mentioned. Without first-hand knowledge of these techniques it is difficult to assess how reliable they would prove at the levels at which most trace elements occur in extractable or total form in soils. The analyst untrained in such work would be well advised to test the methods on analysed soils or rocks of widely variable matrix composition, as even under the best laboratory conditions unexpected problems arise and these problems are inadequately considered in this discussion.

In the final chapters on oxidation - reduction potentials and poorly drained soils, the treatment is again more competent. Throughout the text the reviewer would have welcomed more consideration of differences between the major soil types of the temperate and tropical regions, in so far as they demand different analytical treatment, and quite different diagnostic assessment. While the author emphasises the need for field experimentation, more indication of the levels that the analyst could expect to find would have been welcome, as would a more wholehearted recommendation of the use of activity or amount - intensity relationships in assessing plant availability.

R. L. MITCHELL

STRUCTURAL ANALYSIS OF ORGANIC COMPOUNDS BY SPECTROSCOPIC METHODS. By WILHELM SIMON and THOMAS CLERC. Pp. viii + 195. London: Macdonald; New York: American Elsevier. 1971. Price £3·50.

This book is a translation of a specially revised version of the original German edition, published in 1967. It forms part of the University Chemistry Series edited by Professors Grundon, Roberts and Smith, and is intended for use both by undergraduate and graduate students. The text deals exclusively with the four main spectroscopic methods used to elucidate the structure of organic compounds. These are ultraviolet, infrared, nuclear magnetic resonance and mass spectroscopy—the four most important and useful techniques. The book consists almost wholly of compilations of spectroscopic data for all four methods of analysis, presented in tabular form for most of the common types of organic compound. Tables of data are also given for the individual techniques, which enable the user to interpret in terms of particular groupings or functionalities the over-all structure of the compound being examined.

A useful feature of the book is the Appendix, which contains spectra for fifty unknown compounds, the structures of which are to be worked out by the reader (answers are given later). This provides useful experience in interpreting spectroscopic data and undoubtedly will help to

create confidence in assigning particular structures to totally unknown substances. This is a most useful book, particularly for those not wholly concerned with spectroscopy, but who desire some working knowledge of how to interpret the various spectra. It forms a handy and inexpensive reference volume, which students and research workers will appreciate. WILLIAM I. STEPHEN

KINETICS OF ELECTRODE PROCESSES. By TIBOR ERDEY-GRÚZ. Pp. 482. London: Adam Hilger Ltd. 1972. Price £9.

Although primarily intended for physical chemists and electrochemists, this book will be useful to those analytical chemists who are interested in the rates and mechanisms of electrode processes. The first few chapters will also be useful reading for students attending M.Sc. courses in analytical chemistry that include this subject in the syllabus. A general treatment of the basic principles of the kinetics of electrode processes in which the rate-determining step is alternatively (*a*) charge transfer, (*b*) chemical reaction and (*c*) diffusion or mass transfer is followed by a chapter on particular electrodes, including extensive treatment of the oxygen and hydrogen electrodes and of the kinetics of electrolytic metal deposition and dissolution. Separate chapters then deal with electrode processes in molten salts and on semiconductor interfaces as well as with the kinetics of anodic film formation. The translation is good and the literature is covered up to mid-1970 with useful coverage of the Russian literature. The general treatment will be very useful for students, as there are not many texts in which this information is presented with sufficient care or detail, but the later sections will be of limited appeal mainly to those chemists who are working in the particular fields covered. J. M. OTTAWAY

QUANTITATIVE EVALUATION OF SPECTROGRAMS BY MEANS OF *l*-TRANSFORMATION. By TIBOR TÖRÖK and KÁROLY ZIMMER. Pp. viii + 40, 40–114 loose leaf. London, New York and Rheine: Heyden and Son Ltd. 1972. Price £3·50; $9; DM32.

From the earliest days of quantitative emission spectrochemical analysis, efforts have been made to find some simple function of the transmittance of the emulsion that will result in a straight-line emulsion calibration graph. This book gives a general review of most of the commonly used blackening transformations, such as those of Kaiser and Baker-Sampson, and discusses in more detail the *l*-transformation developed by the authors. The process of obtaining the *l*-transformation from the emulsion calibration data, and its application to quantitative analysis, are fully explained with the aid of numerical examples. The treatment of the various transformations is given in terms of the Gaussian subtraction logarithm of the blackening, which will probably be unfamiliar to many spectrographers, but this treatment has the advantage of unifying the various transformations and the more important expressions are also given in terms of the more commonly used parameter, the transmittance of the emulsion. One omission is a discussion of the effect of particular transformations on the accuracy of the calculated concentrations. Unless it can be shown that significant improvements in accuracy are obtained by using the *l*-transformation, it is unlikely that spectrographers will change from their existing methods of evaluation. Since, so far as I am aware, no review of these blackening transformations has previously been published, and the work on the *l*-transformation has appeared mainly in Hungarian journals, the book serves a most useful purpose. Although a few typographical and translation errors have crept in, the treatment is generally clear and concise and the numerical examples are a valuable feature. The book can be thoroughly recommended to all analytical spectrographers.

It seems a pity that the book could not have been produced in a stouter form. The main text is only 39 pages in length and the remaining 74 pages consist of loose cards that give the numerical tables that are required when using the *l*-transformation method. Considering the computing facilities that are generally available at the present time, these tables are of somewhat doubtful value. They are retained in an inadequate flap at the end of the book and would prove inconvenient to use and be easily mislaid. The binding is flimsy and would not survive hard use.

B. L. TAYLOR

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A Simplified Method of Extraction of Diosgenin from *Dioscorea* Tubers and its Determination by Gas - Liquid Chromatography

A simple method for determining diosgenin in *Dioscorea* tubers involving extraction and gas - liquid chromatography has been developed and used in our laboratory for the last 6 years. A small amount (600 mg) of the ground, dried tuber is hydrolysed by refluxing it for 4 hours with a 1 + 1 mixture of 3 N aqueous hydrochloric acid solution and xylene, after adding a suitable internal standard. After centrifuging the mixture, a portion of the xylene layer is injected on to the gas-chromatographic column, without any further manipulations. Hydrolysis conditions were examined to ensure that the release of diosgenin was complete and its degradation at a minimum. The proposed method enables pennogenin to be separated from diosgenin, but yamogenin is poorly resolved. The relative standard deviation for the determination of diosgenin was about 1·5 per cent. Good agreement with a gravimetric method was found.

A. ROZANSKI
Fine Chemical Division, The Upjohn Company, Kalamazoo, Michigan, U.S.A.

Analyst, 1972, **97**, 968–972.

Spectrophotometric Determination of Diosgenin in *Dioscorea composita* Following Thin-layer Chromatography

A rapid and accurate method is described for the determination of diosgenin in *Dioscorea composita* roots. After acid hydrolysis and solvent extraction of the root, pure diosgenin is isolated by thin-layer chromatography, treated with sulphuric acid - methanol, and the resultant chromophore is measured by spectrophotometry.

Similarly, gas - liquid chromatography is used to evaluate statistically the accuracy of the method.

GULLIVER LÓPEZ SÁNCHEZ, JORGE C. MEDINA ACEVEDO and ROBERTO RAMÍREZ SOTO
Syntex, S.A. División Farmacéutica, Dirección de Desarrollo Analítico y Control, P.O. Box 10–821, Mexico D.F., Mexico.

Analyst, 1972, **97**, 973–976.

Thin-layer Chromatographic Separation and Colorimetric Determination of Rhamnose, Quinovose, Fucose and Glucose

A thin-layer chromatographic method has been developed for the separation and subsequent determination of rhamnose, quinovose, fucose and glucose: 50 to 200 nmol of each of the four sugars can be adequately separated in a single ruled lane 20 mm wide on the plate; after elution the individual sugars are determined colorimetrically with the phenol - sulphuric acid reagent. The method requires the use of only simple apparatus and should have wide application. It has advantages over the gas - liquid and paper chromatographic methods and is less time consuming.

S. SINGH and B. E. STACEY
Department of Chemistry, Sir John Cass School of Science and Technology, City of London Polytechnic, Jewry Street, London, EC3N 2EY.

Analyst, 1972, **97**, 977-980.

Determination of 5-Hydroxymethyl-2-furaldehyde in Honey

Because existing methods for the determination of 5-hydroxymethyl-2-furaldehyde in honey were found to be unsatisfactory, a method that involves the use of column adsorption with activated charcoal for clean-up and determination by either a visual or spectrophotometric colorimetric method has been devised. It has been found to be adequate in respect of precision and accuracy.

A. K. DHAR and B. R. ROY
Central Food Laboratory, 3 Kyd Street, Calcutta 16, India.

Analyst, 1972, **97**, 981–985.

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Solvent Extraction of Trace Amounts of Cobalt from Pure Reagent Chemicals and its Spectrophotometric Determination with 2-Nitroso-5-dimethylaminophenol

Trace amounts of cobalt in pure reagent chemicals have been determined spectrophotometrically with 2-nitroso-5-dimethylaminophenol. Cobalt in amounts below 0·6 μg in the aqueous phase is extracted into 1,2-dichloroethane (5 ml) at pH 4 to 8. The excess of reagent and metal complexes other than the cobalt complex that are extracted into the organic phase are removed by extraction of the latter with 1 N potassium hydroxide solution followed by hydrochloric acid solution (1 + 2). The organic phase is then filtered through a dry filter-paper, and the absorbance of the organic phase measured in a cell of 50-mm path length at 456 nm. By using the above procedure, trace amounts of cobalt (10^{-8} to 10^{-4} per cent.) in sodium, potassium and iron(III) salts, etc., were determined by the standard addition method.

S. MOTOMIZU

Department of Chemistry, Faculty of Science, Okayama University, Tsushima, Okayama-shi, Japan.

Analyst, 1972, **97**, 986–992.

The Determination of Chloride by Atomic-absorption Spectrophotometry

A method for the determination of trace amounts of chloride ion in aqueous solution is described. The chloride is converted into phenylmercury(II) chloride, which is extracted into chloroform and subsequently determined in ethyl acetate solution by means of atomic-absorption spectrophotometry. Alternatively, but less conveniently, the phenylmercury(II) chloride can be extracted into isopropyl acetate and this solution, after concentration, is sprayed into the spectrophotometer. As little as 0·015 p.p.m. of chloride can be detected in a 250-ml sample of water, and 0·1 p.p.m. of chloride can be determined with a relative mean error of 4·6 per cent. Interferences are minimal but bromide, iodide, thiocyanate and cyanide must be absent from the solution. The present method compares favourably with a previously described gas - liquid chromatographic method, and is only slightly less sensitive.

R. BELCHER, A. NADJAFI, J. A. RODRIGUEZ-VAZQUEZ and W. I. STEPHEN

Department of Chemistry, University of Birmingham, P.O. Box 363, Birmingham, B15 2TT.

Analyst, 1972, **97**, 993–997.

Determination of Organic Hydroxyl Groups by Nuclear Magnetic Resonance Spectroscopy by Using Trimethylsilyl Derivatives

A nuclear magnetic resonance spectroscopic method is presented for the determination of hydroxyl values in mixtures of organic compounds. A nine-fold enhancement of sensitivity is obtained by the formation of trimethylsilyl derivatives, which makes the method especially useful for the determination of low hydroxyl values. The method can be applied to the rapid determination of the hydroxyl values of fatty acids, esters, polymerised fatty acids, polyesters, tertiary alcohols and certain phenols. *o*-Nitrophenols and highly hindered phenols fail to give correct results, as do hydroxy-compounds of low relative molecular mass, which give volatile trimethylsilyl derivatives. Epoxides and non-enolised ketones do not interfere in the determination. Certain shortcomings of methods for the determination of hydroxyl values based on acetylation and differential titration are discussed.

ANNELI HASE and TAPIO HASE

Helsinki University of Technology, Otaniemi, Finland.

Analyst, 1972, **97**, 998–1002.

The Determination of Tin in Biological Material by Using Neutron-activation Analysis

A method for the determination of tin in biological material, by using neutron-activation analysis and radiochemical separation of tin-121, is described. Decontamination from sodium and antimony was shown to be satisfactory by tracer techniques, and the method was applied to biological standard materials and other samples. The precision is ± 10 per cent. at the submicrogram level, and the sensitivity (which could be lowered by increasing the neutron flux) was found to be 10 to 20 ng.

H. J. M. BOWEN

Chemistry Department, Reading University, Whiteknights, Reading, Berkshire, RG6 2AD.

Analyst, 1972, **97**, 1003–1005.

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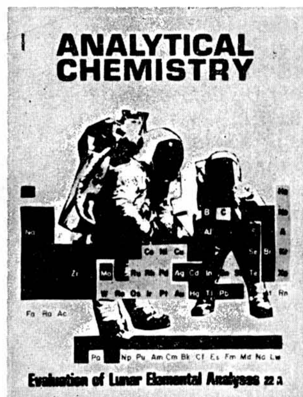

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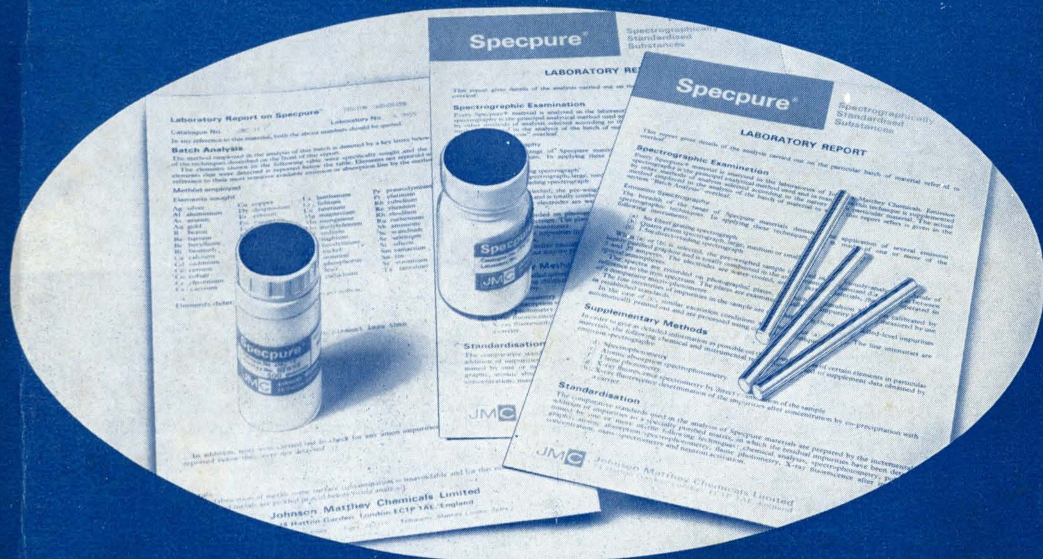

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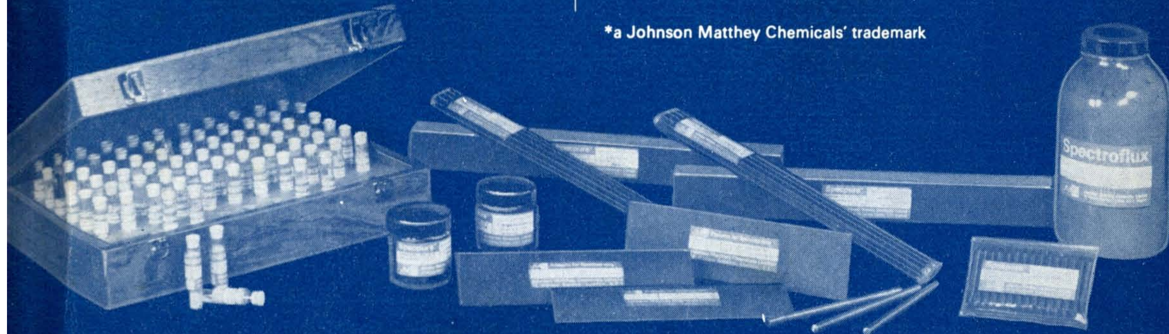

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Volume 97 No. 1161, Pages 929-1008 December, 1972

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Printed for the Society for Analytical Chemistry by W. Heffer & Sons Ltd., Cambridge, England
Communications to be addressed to the Editor, J. B. Attrill, 9/10 Savile Row, London, W1X 1AF
Enquiries about advertisements should be addressed to J. Arthur Cook, 9 Lloyd Square, London, WC1X 9BA

Entered as Second Class at New York, U.S.A., Post Office.