THE ANALYST

A Monthly Publication dealing with all branches of Analytical Chemistry: the Journal of the Society of Public Analysts and Other Analytical Chemists

Editor: F. L. OKELL, F.R.I.C.
7-8, IDOL LANE, LONDON, E.C.3
Telephone: MANsion House 6608

Published for the Society by
W. HEFFER & SONS. LTD. CAMBRIDGE, ENGLAND

Volume 78 Price 6s. 6d.
Subscription Rato, inclusive of Abscract. C, 80/- per annum, Post Free
No. 930, Pages 509-568 September, 1953

No. 13 September, 1953

THE SOCIETY OF PUBLIC ANALYSTS AND OTHER ANALYTICAL CHEMISTS

BULLETIN

FORTHCOMING MEETINGS

Ordinary Meeting of the Society, October 7th, 1953

AN Ordinary Meeting of the Society will be held at 7 p.m. on Wednesday, October 7th, 1953, in the Meeting Room of the Chemical Society, Burlington House, Piccadilly, London, W.1.

At this meeting there will be a discussion on "The Destruction of Organic Matter." The subject will be introduced by Dr. G. Roche Lynch, O.B.E., D.P.H., F.C.G.I., L.M.S.S.A., F.R.I.C. Two papers will follow and will be open to discussion—

"The Preparation of Biological Material for the Determination of Trace Metals. Part II. A method for the Destruction of Organic Matter in Biological Material," by G. Middleton, B.Sc., F.R.I.C., and R. E. Stuckey, B.Sc., Ph.D., F.R.I.C., Ph.C.

"The Determination of Lead in Foodstuffs," by H. C. Lockwood, Ph.D., F.R.I.C.

Note—The first part of the paper by G. Middleton and R. E. Stuckey, "The Preparation of Biological Material for the Determination of Trace Metals. Part I. A Critical Review of Existing Procedures," is printed in the current (September, 1953) issue of *The Analyst*, pp. 532-542.

Ordinary Meeting of the Society, October 23rd, 1953

AN Ordinary Meeting of the Society, organised by the Physical Methods Group, will be held at 5 p.m. on Wednesday, October 23rd, 1953, in Southampton University, **Southampton.**

The subject of the meeting will be "Paper Electrophoresis," and the following papers will be presented and discussed—

An introductory paper by F. H. Pollard, B.Sc., Ph.D. (University of Bristol).

"Paper-strip Electrophoresis of Serum Protein," by A. L. Latner.

"Use of Paper Electrophoresis in the Study of Nucleic Acids," by Dr. Roy Markham.

The meeting will be preceded by an afternoon visit to the Esso Refinery at Fawley.

Joint Meeting of the Biological Methods Group with the Crop Protection Panel of the Agriculture Group of the Society of Chemical Industry, the Association of Applied Biologists and the Pharmacological Society, October 2nd, 1953

A JOINT meeting of the Biological Methods Group with the above bodies will be held at 10.30 a.m. to 5 p.m. on Friday, October 2nd, 1953, in the Large Chemistry Lecture Theatre, Imperial College, London, S.W.7.

The subject of the meeting will be "Organo-phosphorus Insecticides."

Joint Meeting of the North of England Section and the Microchemistry Group with the Liverpool and North-Western Section of the Royal Institute of Chemistry, September 26th, 1953

A JOINT meeting of the North of England Section and the Microchemistry Group with the Liverpool and North-Western Section of the Royal Institute of Chemistry will be held at 2.30 p.m. on Saturday, September 26th, 1953, in the Mayor's Parlour, Town Hall, **Southport.**

The subject of the meeting will be "The Training and Education of Microchemists," and the following papers will be read and discussed—

"The Academic Approach," by Cecil L. Wilson, D.Sc., Ph.D., F.R.I.C.
"Technical Aspects," by Gerald Ingram, A.R.I.C.
"Industrial Requirements," by Rudolph Rothwell.

PAPERS ACCEPTED FOR PUBLICATION IN *THE ANALYST*

THE following papers have been accepted for publication in *The Analyst*, and are expected to appear in the near future. It is not possible to enter into correspondence about any of them.

"A Colorimetric Determination of Dihydrostreptomycin," by G. C. Ashton, M. C. Foster and M. Fatherley.

> A colorimetric method for the determination of dihydrostreptomycin is presented. It is based on a reaction between guanidino materials and diacetyl, alkali and α-naphthol. The colour formed is not subject to interference at high salt concentrations and can be used in the routine analysis of factory samples. Penicillin and its compounds do not interfere with the reaction, so the method can be applied to the determination of dihydrostreptomycin in mixtures with penicillin.
>
> The standard error on analyses made by the method is ±1·3 per cent., which compares favourably with that found in microbiological assay.

"A Systematic Approach to the Choice of Organic Reagents for Metal Ions," by R. J. P. Williams.

> The choice of an organic reagent for any particular metal ion is limited by the nature of the free energy change occurring on the formation of the complex of the reagent and the metal ion in aqueous solution. A broad general division of such reactions is made that is based upon whether the entropy change or the heat change of the reaction is the most important term in the free energy change. Small cations of large charge (usually ions of low electronegativity) are divided from the larger and more electronegative cations in this way. The selection of the type of reagent suitable for selective reaction with a particular metal ion can be based on these principles. Minor factors, such as the nature of the available orbitals, also affect the free energy of formation of complexes in special cases. Many examples of the successful use of organic reagents are examined and some suggestions are made for the design of further reagents.

"An Isopiestic Method for the Micro-determination of Molecular Weights," by Miss J. E. Morton, A. D. Campbell and T. S. Ma.

> A simple method is described for the determination of the molecular weight of non-volatile compounds; it is based on Sinclair's isopiestic method for the determination of vapour pressures. A solution of the compound in a volatile solvent is rocked at constant temperature in a small desiccator with a solution, in the same solvent, of a substance of known molecular weight. When the two solutions are isopiestic, the molecular weight of the compound is calculated from the molecular weight of the standard substance and the concentrations (by weight) of the two solutions. For 3 to 7-mg samples concordant results have been obtained with a range of solvents.

"Micro-determination of Iodides by Arresting the Catalytic Reduction of Ceric Ions," by B. Rogina and M. Dubravčić.

A method is described for the determination of small amounts, 0·01 to 1·00 μg, of iodine as iodide. The method is based on the catalytic effect of iodides on the reduction of a ceric salt by arsenious acid. The rate of reduction is readily determined by arresting the reaction at a given time by the addition of ferrous and thiocyanate solutions and by measuring the resulting red colour of ferric thiocyanate by means of a photometer. The method offers advantages over that described by Chaney, in which the rate of reduction is measured while the reaction is in progress.

Results are quoted to show the accuracy of the proposed method. A reproducibility of ±0·002 μg has been attained in the range from 0·01 to 0·10 μg of iodide in 8 ml of aqueous solution.

"The Determination of Potassium and Traces of Sodium in Some Potassium Salts," by C. Jackson.

For determining sodium in certain potassium salts of weak acids the sample is titrated with 0·2 *N* perchloric acid in glacial acetic acid, a small controlled excess being added. After the precipitated potassium perchlorate has been removed by filtration, the filtrate is evaporated to dryness and the sodium is determined as sodium zinc uranyl acetate. It is thus possible to estimate volumetrically the total equivalent alkali metal and gravimetrically both sodium and potassium on one sample.

The method can be applied to the determination of sodium in most potassium salts.

"The Determination of Small Amounts of *m*-Dinitrobenzene in Nitrobenzene," by F. G. Angell.

Small amounts of *m*-dinitrobenzene in nitrobenzene can be concentrated by chromatographic adsorption on alumina and elution with a mixture of benzene and light petroleum. The adsorbed *m*-dinitrobenzene is extracted with ethanol from the dried extruded alumina column and determined polarographically for concentrations greater than 0·02 per cent. or absorptiometrically with acetone and sodium hydroxide for concentrations less than 0·02 per cent.

"The Analysis of Rosin Size," by D. E. Davis and K. Linke.

Methods are suggested for the determination of all the constituents of rosin size. They include methods for free alkali and for free acid in the presence of alkali. Results are given that indicate that the proportions of free acid and alkali in rosin size may be large.

"An Apparatus for Simplifying Titration in a Controlled Atmosphere," by R. H. Prince.

The construction and use of equipment for carrying out titrations in controlled atmospheres is described in detail. It is especially suited to Fischer determinations of moisture, and such a determination of moisture in transformer oil is described; the apparatus is simpler than any other assembly for this determination. A comparison between titrations of moisture in *n*-butanol to the visual end-point in the apparatus described and to the dead-stop end-point in an apparatus of the type described by Bonner shows good agreement.

"The Determination of Copper in Plant Material," by W. A. Forster.

In the presence of the ammonium salt of ethylenediaminetetra-acetate, copper can be determined colorimetrically as the diethyldithiocarbamate complex without interference from aluminium, cobalt, iron, manganese, nickel, zinc, calcium, magnesium and phosphorus. The method is rapid, and analyses of 24 plant digest solutions by the new method and by a dithizone procedure, respectively, were in close agreement.

NOTICES

The Physical Society Colour Group

THE next meeting of the Physical Society Colour Group will be held at the Institute of Ophthalmology, Judd Street, London, W.1, at 3.30 p.m. on Wednesday, November 4th, 1953, when papers will be presented by Dr. W. A. H. Rushton, F.R.S., and Dr. R. A. Weale.

Conference on Applied Mass Spectrometry

A CONFERENCE on applied mass spectrometry, organised by the Mass Spectrometry Panel of the Institute of Petroleum Hydrocarbon Research Group, is to be held on Thursday and Friday, 29th and 30th October, 1953, at the Institution of Electrical Engineers, Savoy Place, London. Its main object is to bring together American, British and European workers in the field of applied mass spectrometry to discuss problems of current and future interest. The majority of papers are designed to interest those who use mass spectrometers in their daily work, but all of the papers on the first day are devoted to subjects in analytical chemistry for which mass spectrometry has proved particularly valuable. It is hoped that these papers will give rise to discussions between mass spectrometrists and those who carry out similar analyses by alternative methods.

In all there will be 18 papers from workers in Britain, America, Canada, France, Germany and Holland, and they will cover broadly—

(1) Quantitative analysis of gases, liquids and solids, including the continuous monitoring and control of product composition by mass spectrometry.

(2) Applications to the study of molecular structure, free radicals and electron - molecule collision processes.

(3) Recent developments in instrumentation and computing techniques.

To cover the cost of preprints and other conference expenses a registration fee of £2 2s. 0d. will be charged. Applications for further particulars and for registration forms should be made to W. J. Brown, Hon. Secretary, Mass Spectrometry Panel, c/o Metropolitan-Vickers Electrical Co., Ltd., Trafford Park, Manchester, 17, as soon as possible.

London Meetings of the Royal Sanitary Institute

THE Royal Sanitary Institute, 90, Buckingham Palace Road, London, S.W.1, will hold the following meetings in London—

Wednesday, November 11th, 1953, at 2.30 p.m., at the Institute. Discussion on "Air Pollution and the London Fog of December, 1952," by E. T. Wilkins, M.Sc., Ph.D., a Principal Scientific Officer to the Fuel Research Station, Department of Scientific and Industrial Research.

Wednesday, December 9th, 1953, at 2.30 p.m., at the Institute. Discussion on "Investigations into Detergency in Kitchens and Laundries," to be opened by S. G. Burgess, Ph.D., B.Sc., F.R.I.C., F.Inst.Pet., Deputy Chemist, D. Burns, B.Sc., A.R.I.C., Senior Assistant Chemist, Chemical Branch, Public Health Department, and C. W. Tidy, A.M.I.H.V.E., Senior Assistant, Chief Engineer's Department, all of the London C.C.

Enquiries about these meetings should be addressed to the Secretary of the Royal Sanitary Institute, 90, Buckingham Palace Road, London, S.W.1

PRINTED BY W. HEFFER & SONS LTD., CAMBRIDGE, ENGLAND

THE ANALYST

PROCEEDINGS OF THE SOCIETY OF PUBLIC ANALYSTS AND OTHER ANALYTICAL CHEMISTS

BIOLOGICAL METHODS GROUP

THE Summer Meeting of the Group was held on Friday, June 19th, 1953, and 28 members and guests attended. After lunching together, the party paid a most interesting visit to the Ministry of Agriculture and Fisheries' Veterinary Laboratory, New Haw, Weybridge, Surrey, and were shown various aspects of the work in progress. Departments visited included Biochemistry, Manufacture of Vaccines, Tuberculin Production, Poultry Diseases and Manufacture of S.19 *Brucella abortus* vaccine.

At the conclusion of the meeting Dr. H. O. J. Collier, Chairman of the Group, moved a vote of thanks to the Director, Dr. Stableforth, and his colleagues for their hospitality.

The Determination of Ergosterol in Yeast

Part I. The Ultra-violet Absorption of Purified Ergosterol

BY W. H. C. SHAW AND J. P. JEFFERIES

(*Presented at the meeting of the Society on Wednesday, May 20th, 1953*)

Most published records agree on the wavelengths of the three main peaks in the ultra-violet absorption curve of ergosterol, but there are discrepancies in the extinction values reported for the various maxima, apparently because of the difficulty of preparing pure ergosterol by direct recrystallisation of the commercial material and to a lesser extent because of small variations in the moisture content of the hydrated sterol. Simple recrystallisation does not always yield a pure product; the best means of purification is by recrystallising a suitable ester, with subsequent regeneration of the sterol.

The preparation and purification of ergosterol benzoate is described, and the physical properties and ultra-violet absorption (in various solvents) of a purified specimen of ergosterol are recorded.

METHODS for determining small amounts of ergosterol are mostly based either on colour reactions or on measurements of ultra-violet absorption. A number of colour reactions are known[1,2,3] and some can be applied quantitatively to pure solutions. Similar colours, however, are given by many other sterols. The colorimetric method with acetic anhydride and zinc chloride,[4,5] modified by Pesez and Herbain,[6] appears to be specific for ergosterol amongst the yeast sterols, but this method was found to give insufficiently reproducible results, even when applied to purified ergosterol.

The most satisfactory method of detecting and determining small amounts of ergosterol is based on ultra-violet absorption measurements. The absorption of ergosterol is of high intensity and characteristic of Δ-5:7 unsaturated sterols, amongst which 7-dehydrocholesterol[7] is the only other one of importance known to occur naturally. Well-defined maxima occur at 271·5, 282 and 293·5 mμ in absolute alcohol, with a marked inflection at about 263 mμ

and a smaller inflection in the region of 253 mμ (Fig. 1). Most literature reports agree on the position of the maxima, but discrepant results are found for the extinction value of the pure compound at the main maximum (Table I).

The methods of purifying the samples used in obtaining the figures in Table I require some comment. No details of methods are given in the first three publications mentioned.

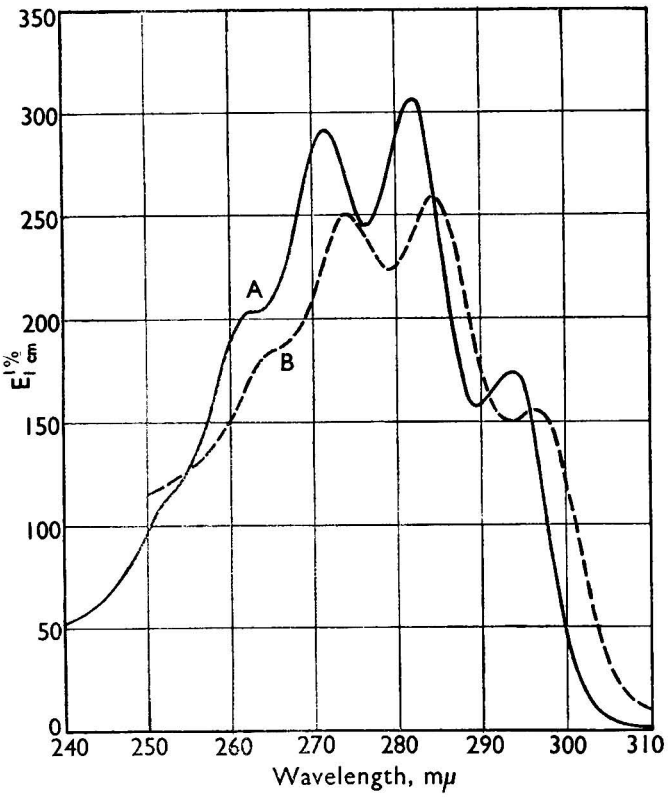

Fig. 1. Absorption curves. Curve A, ergosterol in absolute alcohol; curve B, ergosterol benzoate in chloroform. Concentrations approximately 0·002 per cent. w/v

Hogness *et al.*[11] recrystallised ergosterol from ethyl alcohol and benzene and then twice from *iso*octane. Subsequently, Huber *et al.*[7] showed that Hogness's values were too low and attributed this to decomposition during the several recrystallisations. They stated that the extinction values were highest on recrystallising the commercial material once only from a mixture of alcohol and benzene. Lamb, Mueller and Beach,[12] unable to confirm this, preferred recrystallisation from acetone containing 1 per cent. of water and obtained results about 4 per cent. higher. It thus appeared desirable to investigate again the extinction values in the solvents it was proposed to use subsequently for the determination of ergosterol.

TABLE I

RECORDED EXTINCTION VALUES OF ERGOSTEROL AT 281·5 TO 282 mμ (MAX.)

Authors	$E_{1\,cm}^{1\%}$	ε
Morton, Heilbron and Kamm[8]	—	10,200
Morton and Gillam (quoted by Bacharach, Smith and Stevenson[9])	333	13,800*
Morton and de Gouveia[10]	—	11,700
Hogness, Sidwell and Zscheile[11]	—	10,600
Huber, Ewing and Kriger[7]	—	11,500
Lamb, Mueller and Beach[12]	289	12,000*

* ε calculated, assuming the monohydrate composition.

The chief impurities likely to be encountered in commercial ergosterol are zymosterol and 5-dihydroergosterol. Callow[13] stated that the former, but not 5-dihydroergosterol, could be eliminated by recrystallisation from an alcohol - benzene mixture. By recrystallisation of the benzoate and subsequent regeneration of the sterol he obtained a purified product, but unfortunately did not record its ultra-violet absorption values. Our experience supports the view that recrystallisation of the sterol does not invariably yield a pure product and that purification is most satisfactory after recrystallisation of a suitable ester.

Ergosterol crystallises from hydrated solvents with one molecule of water of crystallisation ($C_{28}H_{44}O.H_2O$ requires 4·3 per cent.), but the moisture content as found by analysis normally differs a little from the theoretical figure, and it appears necessary to carry out this determination on any material to be used as standard. To avoid any confusion, our results, unless otherwise stated, are expressed throughout in terms of anhydrous ergosterol.

Experimental

Spectrophotometers—The greater part of this work was carried out with a Unicam spectrophotometer (model SP 500), the more important results being confirmed on a second Unicam or a Uvispek instrument. The wavelength scale was checked as a routine with the 486·1-mμ hydrogen line and occasionally with the mercury lines at 313·2 mμ. The maximum error in wavelength near the ergosterol maxima appeared to be about 0·2 mμ.

The optical density scale and cell thickness (1 cm) were checked by means of 0·006 per cent. w/v potassium dichromate in slightly acid solution, in the manner described by Cama, Collins and Morton,[14] with whose figures ours were in close agreement ($E^{1\%}_{1cm}$ equal to 124·6, 144·8, 48·8 and 106·8 at 235 mμ (min.), 257 mμ (max.), 313 mμ (min.) and 350 mμ (max.), respectively).

The two main maxima on the ergosterol absorption curve (Fig. 1) are sharp and the band-width used has some effect on the measured optical density at the maxima for any given solution. The maximum permissible band-width does not have to be found when a spectrophotometer designed to be operated at a constant band-width of the order of 0·5 mμ is used. Nevertheless, spectrophotometers requiring a constant energy level should be operated at the narrowest slit-width (and hence band-width) compatible with adequate electrical sensitivity of the instrument at the particular wavelength used. In practice, on a Unicam instrument, slit-widths up to 0·5 mm at 282 mμ and 0·6 mm at 271·5 mμ may be used without perceptibly lowering the observed optical densities. These slit-widths correspond, according to the manufacturer's formula, to nominal band-widths of 1·8 and 1·9 mμ, respectively. To satisfy these requirements the hydrogen lamp must be of high emission at the wavelengths used and any solvent used must be of good transmittancy.

Solvents—

Absolute alcohol—Usually the laboratory reagent grade of absolute alcohol is satisfactory without further treatment.

cyclo*Hexane*—The commercial grade of *cyclo*hexane usually contains traces of benzenoid impurities that cause a marked decrease in transmittancy below 282 mμ. As a rule these are insufficient to affect readings at the 282-mμ ergosterol maximum, but, when additional readings are required at the 271·5-mμ maximum, it is necessary to use *cyclo*hexane containing as little as possible of these impurities. Alternatively, *cyclo*hexane specially purified for spectroscopy can be used.

Chloroform—Analytical reagent grade chloroform usually requires no further treatment.

Ethylene dichloride—Commercial ethylene dichloride can be freed from traces of acid and other impurities by shaking it twice with a small volume of 50 per cent. w/w aqueous potassium hydroxide. The aqueous washings are discarded and the solvent is filtered through a dry paper, dried with phosphorus pentoxide, filtered again and distilled; the first and last tenths are discarded.

Preparation and purification of ergosterol—

Direct recrystallisation—The three methods generally used for the direct purification of ergosterol involve recrystallisation from a mixture of 1 part of 95 per cent. alcohol and 2 parts of benzene,[7] 95 per cent. alcohol[13] or acetone containing 1 per cent. of water.[12] To test the efficiency of these methods of purification, 50-g portions of commercial ergosterol

of good quality ($E^{1\%}_{1cm}$ (anhyd.) at 282 mμ = 294 in absolute alcohol) were recrystallised a number of times from each solvent. After each recrystallisation the ergosterol was dried *in vacuo* over calcium chloride and the moisture determined in a "pistol"-type apparatus with boiling toluene (b.p. 110° C) in the outer jacket. The ultra-violet absorption in absolute alcohol was determined after each recrystallisation; the results are recorded in Table II.

TABLE II

RECRYSTALLISATION OF ERGOSTEROL

Solvent	Number of recrystallisation	Amount lost at 105° C *in vacuo*, %	$E^{1\%}_{1cm}$ (calculated as anhydrous) 271·5 mμ (max.)	282 mμ (max.)	293·5 mμ (max.)
Alcohol (95 per cent.) - benzene mixture (1 + 2)	1	5·0	283·0	297·3	169·5
	2	4·9	283·3	298·4	169·7
	3	4·8	284·5	299·0	170·3
	4	4·9	285·1	300·5	170·4
	5	5·0	287·2	303·1	172·3
	6	4·9	285·8	301·0	171·1
Alcohol (95 per cent.)	1	4·5	279·5	294·3	167·5
	2	4·5	280·3	295·0	167·9
	3	4·2	279·2	294·6	167·6
	4	4·6	282·7	298·0	168·8
	5	4·8	282·8	297·2	169·0
Acetone containing 1 per cent. of water	1	4·8	280·1	295·4	167·7
	2	3·7	281·4	296·7	168·6
	3	4·9	283·0	298·9	170·3
	4	4·9	284·3	299·3	170·1
	5	4·9	282·5	297·9	169·4

They show that alcohol - benzene appears to be the most satisfactory solvent, although acetone with 1 per cent. of water is only slightly less efficient. Extinction values, however, are 1 to 2 per cent. lower than those subsequently obtained after purification by means of the benzoate.

Purification through ergosterol benzoate—A 50-g portion of the same commercial sample as that used for the recrystallisations just recorded was benzoylated in dry pyridine, substantially as described by Callow.[13] The resulting material (46 g) was recrystallised five times from ethyl acetate to give a product having physical constants (Table III) close to those quoted by Callow. The ultra-violet absorption curve for the purified benzoate in chloroform is recorded in Fig. 1.

TABLE III

RECRYSTALLISATION OF ERGOSTEROL BENZOATE FROM ETHYL ACETATE

Recrystallisation	Melting point, °C	$(\alpha)^{20}_D$	$(\alpha)^{20}_{546\cdot1\,m\mu}$	$E^{1\%}_{1cm}$ (max.) at 284 to 284·5 mμ in chloroform approx. 0·002% w/v
1	169·8 to 170·4	−66·5	−87·5	255·0
2	170·8 to 171·5	−67·7	−87·1	259·0
3	170·8 to 171·3	−67·6	−87·5	257·4
4	170·8 to 171·3	−67·7	−87·2	257·5
5	171·0 to 171·3	−68·0	−88·0	258·9*

* $E^{1\%}_{1cm}$ (max.) at 273·5 to 274 mμ = 250·5, $E^{1\%}_{1cm}$ (max.) at 296 mμ = 156.

The purified benzoate (10·4 g) was hydrolysed with 3 per cent. w/v alcoholic potassium hydroxide; the regenerated sterol was washed with alcohol and water and then recrystallised once from 95 per cent. alcohol (yield 7 g). All recrystallisations and the hydrolysis were carried out in an atmosphere of nitrogen to prevent oxidation.

The purified hydrated sterol was obtained as colourless crystals, m.p. 163·6 to 164·4° C, $(\alpha)^{20}_D$ −129·5° (−136·0° as anhydrous), $(\alpha)^{20}_{546\cdot1\,m\mu}$ −165·6° (−174·0° as anhydrous), loss *in vacuo* at 105° C, 4·8 per cent. ($C_{28}H_{44}O.H_2O$ requires 4·3 per cent.).

Portions of this material were stored in sealed nitrogen-filled ampoules kept in a refrigerator. The absorption curve in absolute alcohol is shown in Fig. 1 and the specific

and molecular extinction coefficients in the solvents mentioned above are recorded in Table IV.

Discussion of results

The discrepancies among the extinction values of ergosterol found in the literature appear to have arisen through failure to prepare pure samples by direct recrystallisation and, to a lesser extent, through small differences in moisture content of the material after recrystallisation. By recrystallisation of commercial samples, notably from a mixture of alcohol and benzene, almost pure ergosterol can be obtained, but the most reliable method involves purification through a suitable ester such as the benzoate. Huber *et al.*[7] prepared several nitrobenzoyl esters, but did not use them as means of purifying ergosterol. The extinction values in absolute alcohol for the hydrated sterol shown in Table IV are about 5 per cent. higher than those recorded by Huber *et al.*[7] and very slightly higher than those of Lamb, Mueller and Beach,[12] with whose results we are in substantial agreement. For the exceptionally high result obtained in 1933 by Morton and Gillam, and quoted by Bacharach, Smith and Stevenson,[9] we have no explanation to offer.

Table IV

Mean extinction values for purified ergosterol

Solvent	Number of determinations	Wavelengths of maxima (±0·5 mμ), mμ	$E^{1\%}_{1cm}$ of hydrated ergosterol*	$E^{1\%}_{1cm}$ of anhydrous ergosterol	Molecular extinction coefficient, ε
Absolute alcohol ..	9	271·5	276·3	290	11,500
		282·0	291·1 (±0·34)†	306	12,150
		293·5	165·9	174	6,900
Chloroform	2	274·5	240·7	253	10,000
		284·5	260·2	273	10,850
		296·0	158·3	166	6,600
Ethylene dichloride ..	4	273·5	258·9	272	10,800
		283·5	274·7	289	10,450
		295·5	159·0	167	6,650
*cyclo*Hexane	4	271·5	268·6	282	11,200
		282·0	284·7	299	11,900
		294·0	162·4	171	6,750

* Loss at 105° C *in vacuo*, 4·8 per cent. Results for hydrated ergosterol are given to four figures, as observed. The remaining extinction values have been rounded off to be consistent with the precision of the measurements.

† Standard error of the mean.

In view of these discrepancies we thought it advisable to have the main extinction values of our material checked by Professor Morton, who kindly undertook an independent examination of a portion of the purified hydrated sterol. He reported a maximum at 282 mμ, $E^{1\%}_{1cm}$ of 291, in absolute alcohol and a maximum at 282·5 mμ, $E^{1\%}_{1cm}$ of 286, in *cyclo*hexane (not corrected for moisture). He also observes that $E^{1\%}_{1cm}$ at 281·5 mμ must be taken as between 286 and 290 for the monohydrate of ergosterol, which would correspond with 299 to 303 for the anhydrous material, if the hydrate contains ergosterol and water in molar proportions.

He adds that, in the Liverpool laboratories, the maximum absorption is generally found to be at 281·5 mμ, compared with 281·8 mμ estimated on our particular instrument.

It will be seen that agreement between the values obtained at Liverpool and by us for purified ergosterol is excellent. Confidence in the results is increased when it is remembered that agreement was also good for potassium dichromate solutions measured by different observers with photo-electric instruments of different types.

Any analyst proposing to use ultra-violet absorption methods for determining ergosterol should check the wavelength scale of his particular instrument by means of a suitable light source emitting in the region of 282 mμ and check the optical density readings against dichromate in the manner described. Further calibration with specially purified ergosterol should not then be necessary.

In certain instances an analyst may desire to work against a "standard preparation" of the purest ergosterol available. Provided that the examination of a reference standard such as dichromate does not reveal any instrumental errors and the extinction values of

his material do not depart significantly from the values given in Table IV, he can safely use his own figures for analytical purposes.

REFERENCES

1. Lieberman, C., *Ber.*, 1885, **18**, 1803.
2. Rosenheim, O., *Biochem. J.*, 1929, **23**, 47.
3. Heilbron, I. M., and Spring, F. S., *Ibid.*, 1930, **24**, 133.
4. Bruckner, J., *Biochem. Z.*, 1934, **270**, 346.
5. ——, *Ibid.*, 1934, **274**, 465.
6. Pesez, M., and Herbain, M., *Bull. Soc. Chim. France*, 1949, **5**, 760.
7. Huber, W., Ewing, G. W., and Kriger, J., *J. Amer. Chem. Soc.*, 1945, **67**, 609.
8. Morton, R. A., Heilbron, I. M., and Kamm, E. D., *J. Chem. Soc.*, 1927, 2000.
9. Bacharach, A. L., Smith, E. L., and Stevenson, S. G., *Analyst*, 1933, **58**, 128.
10. Morton, R. A., and de Gouveia, A. J. A., *J. Chem. Soc.*, 1934, 911.
11. Hogness, T. R., Sidwell, A. E., and Zscheile, F. P., *J. Biol. Chem.*, 1937, **120**, 239.
12. Lamb, F. W., Mueller, A., and Beach, G. W., *Ind. Eng. Chem., Anal. Ed.*, 1946, **18**, 187.
13. Callow, R. K., *Biochem. J.*, 1931, **25**, 79.
14. Cama, H. R., Collins, F. D., and Morton, R. A., *Ibid.*, 1951, **50**, 48.

GLAXO LABORATORIES LTD.
GREENFORD, MIDDLESEX

December 22nd, 1952

The Determination of Ergosterol in Yeast

Part II. Determination by Saponification and Ultra-violet Absorption Spectroscopy

BY W. H. C. SHAW AND J. P. JEFFERIES

(*Presented at the meeting of the Society on Wednesday, May 20th*, 1953)

Part of the ergosterol found in yeast occurs in a combined form and treatment to liberate the free sterol is a necessary preliminary to its determination. Most published methods make use of alcoholic or aqueous alkali hydroxides for this purpose, and the variety of strengths prescribed suggested that further investigation was necessary.

The conditions for the destruction of the yeast cells and maximum recovery of ergosterol have been studied. It was found that boiling under reflux with alcoholic sodium or potassium hydroxide did not break down the yeast sufficiently. Aqueous sodium hydroxide at strengths from 10 to 50 per cent. w/w gave low and erratic results, additional ergosterol being recovered by subsequent alcoholic saponification of the residue.

Concentrations of potassium hydroxide above 20 per cent. w/w in aqueous solution proved satisfactory, the strength required depending on the length of boiling under reflux. For the method described, 40 per cent. w/w aqueous potassium hydroxide has been selected, as it allows considerable latitude in time of boiling and in concentration.

The ergosterol in the extracted unsaponifiable matter is separated by digitonin precipitation from non-sterol substances showing irrelevant absorption in the same region as ergosterol. This precipitation has been shown to be quantitative, the separated sterol digitonides yielding solutions spectroscopically indistinguishable at wavelengths above 260 mμ from those of purified ergosterol.

A METHOD for determining ergosterol in yeast based on ultra-violet absorption is unlikely to suffer interference from other sterols present in yeast; 5-dihydroergosterol and zymosterol do not possess the conjugated di-ene system of ergosterol[1,2] and show no absorption in the ergosterol range. The minor sterols, ascosterol, fecosterol and episterol,[1] are isomers of zymosterol and would be expected to behave similarly. Hence, provided the sterols of yeast can be separated quantitatively from matter possessing irrelevant absorption, the ultra-violet method should be specific for ergosterol.

Separation of the sterols from yeast is normally carried out after destruction of the yeast cells by boiling alkalis. However, Bills[3] proposed a method in which matter showing

irrelevant absorption is removed by a preliminary treatment of the yeast with 75 per cent. methanol. After filtration, the yeast is dried under reduced pressure at 70° C and the ergosterol is then extracted from the dry powder with successive small amounts of a boiling mixture of alcohol and benzene (2 + 1). The filtered extracts are evaporated to dryness and the residue is dissolved in absolute alcohol for measurement of absorption at the 282-mμ maximum.

This method was given an extended trial, but was not found satisfactory; it appeared that matter possessing irrelevant absorption at 282 mμ had not been entirely removed by the methanol treatment. In addition, extraction by alcohol and benzene was not efficient, even with an increased number of extractions for twice the prescribed period; additional ergosterol was still recovered from the extracted residues by applying the saponification procedure developed subsequently.

At least a part of the ergosterol of yeast appears to be present in a combined form not readily extracted by solvents, and complete recovery of ergosterol can only be attained after breaking down the complex. This is associated with destruction of the yeast cells and is normally carried out by heating with aqueous or alcoholic alkali hydroxides,[4,5,6,7] although formic acid has been used for this purpose.[8] The variety of conditions proposed for saponification suggested that further investigation was necessary.

Experimental

Digitonin—Commercial samples of digitonin normally show little absorption in the ultra-violet region. The absorption of an 0·5 per cent. w/v alcoholic solution in a 1-cm cell of the particular sample used was negligible over the range 200 to 400 mμ.

Yeast—Most of the work was carried out on samples of fresh bakers' yeast (*Saccharomyces cerevisiae*) of a strain relatively high in ergosterol. Each sample was mixed thoroughly upon receipt, transferred to a closed container and stored in a refrigerator. The ergosterol content of these samples was found to be constant for at least a fortnight, after which they were discarded.

Recovery of ergosterol as digitonide—Digitonin has long been used as a precipitating agent for sterols and the weight of the complex formed provides an approximate means of determining total free sterols.[9] However, variations in composition of the complex preclude its use for accurate gravimetric purposes. Ergosterol digitonide is insoluble in ethyl alcohol of strengths between 80 and 95 per cent. v/v, but in absolute alcohol it is sufficiently soluble for spectroscopic purposes. Recovery of about 1-mg amounts of ergosterol was checked in the following manner.

Two-millilitre portions of an alcoholic solution of ergosterol containing 0·456 mg per ml were treated in stoppered centrifuge tubes with 2·50 ml of 0·5 per cent. w/v digitonin solution in absolute or in 80 per cent. v/v alcohol and with sufficient alcohol or water or both to make up 5 ml and to adjust the alcoholic strength to within the desired range. The tubes were warmed for a few minutes in a water-bath at 70° C and set aside overnight. Any precipitates were separated by centrifugation and decanting the supernatant liquid. The residues were dissolved in warm absolute alcohol and the cooled solutions diluted to 50 ml for spectroscopic examination. The percentage recoveries, recorded in Table I, show that precipitation is quantitative from 90 per cent. v/v alcohol, although the precise concentration is not critical, and also that 1 mg of ergosterol as digitonide is almost completely soluble in 5 ml of absolute alcohol. In addition, they confirm earlier findings[4] that the extinction of ergosterol is unaffected by combination with digitonin. A full absorption curve was determined on a specimen of digitonide derived from yeast and the curve above 260 mμ was found to be indistinguishable from that of purified ergosterol.

Table I

Recovery of ergosterol after digitonin precipitation

Ethyl alcohol, % v/v	99 to 100	95·0	90·0	85·0	80·0
Ergosterol recovered, % ..	negligible	98·9	99·5	99·4	99·4

Effect of boiling alkalis on purified ergosterol—The effect of boiling aqueous or alcoholic sodium or potassium hydroxide solutions on purified ergosterol was ascertained in the following manner.

Approximately 20-mg amounts of ergosterol, *i.e.*, the amount normally derived from about 5 g of yeast cake, were treated with 10 ml of *N* alcoholic sodium or potassium hydroxide or with strong aqueous alkali diluted with the amount of water present in 5 g of sample. The mixtures were heated under reflux for 2 hours, cooled, diluted and extracted three times with ether (anaesthetic grade). The combined ether extracts were washed with water, and the ether was then removed by distillation. The residue was examined spectroscopically at a suitable dilution in absolute alcohol. Ergosterol was recovered quantitatively and in spectroscopically pure condition whether 20 or 50 per cent. w/w aqueous or 1·0 *N* alcoholic potassium or sodium hydroxide was used (Table II).

TABLE II

RECOVERY OF ERGOSTEROL AFTER ALKALI TREATMENT

Alkali treatment	Recovery after treatment with sodium hydroxide, %	Recovery after treatment with potassium hydroxide, %
N alcoholic	99·3	100·2
20 per cent. w/w aqueous	99·7	99·6
50 per cent. w/w aqueous	99·7	99·7

That similar conclusions apply in the presence of yeast solids was shown during later experiments, in which no loss of ergosterol was observed during prolonged periods of heating with alkalis of the strength required for quantitative liberation of the free sterol.

EFFECT OF ALKALI CONCENTRATION AND OTHER CONDITIONS ON THE SAPONIFICATION OF YEAST—

Alcoholic solutions—Most authors appear to favour alcoholic solutions for the saponification, and extended trials were given to both sodium and potassium hydroxide, usually in concentrations close to 1·0 *N*. These experiments showed that alcoholic sodium hydroxide solution was unsatisfactory, as the yeast solids were largely insoluble and bumping during the heating under reflux was severe. Alcoholic potassium hydroxide solution was better in both respects and considerable attention was paid to the conditions prescribed by Castille and Ruppol.[4] Results by their method, however, gave values only about 75 per cent. of those attained by the aqueous saponification method described below. For example, two samples of yeast cake gave 0·29, 0·32 per cent. and 0·40, 0·44 per cent. by the respective methods.

The possibility of increasing the strength of potassium hydroxide in alcoholic solution in order to give higher recovery is limited by solubility considerations.

Aqueous solutions—In this series of determinations 5-g portions of fresh bakers' yeast were heated under reflux with 10 ml of alkali for different lengths of time. After dilution with water the solutions were extracted with ether in the manner described above for pure ergosterol. The unsaponifiable matter was dissolved in absolute alcohol to 50 ml and the sterols in a 2·5 or 5·0-ml aliquot were precipitated with an equal volume of 0·5 per cent. w/v digitonin solution in 80 per cent. v/v alcohol. The separated digitonide was finally dissolved in absolute alcohol to give a concentration suitable for spectroscopic examination.

Initially, aqueous alkali at concentrations of below 20 per cent. w/v was used; in particular, 20 per cent. sodium hydroxide was studied in detail, as saponification periods of more than 1 hour yielded little additional ergosterol. However, no independent method was available to check the validity of the results. The procedure described above was therefore modified so that the ergosterol was extracted after saponification from the undiluted aqueous solution; the residual aqueous layer was then diluted with alcohol to give an alkali concentration of about 1·0 *N*. After further heating under reflux, the solution was again extracted with ether, and any extracted ergosterol was separated as digitonide for spectroscopic determination. The results obtained in this way on two samples of yeast are shown in Table III. They show that 20 per cent. aqueous sodium hydroxide (equivalent to about 15 per cent. w/v in the saponification mixture, owing to the water in the sample) does not liberate all the ergosterol in yeast and that an additional amount of ergosterol, usually 5 to 15 per cent. of the total, is obtained by subsequent alcoholic saponification.

Attempts were made to carry out the aqueous and alcoholic saponifications successively, that is, without intervening extraction of the ergosterol liberated in aqueous solution, but results were invariably low and erratic (*e.g.*, 0·23, 0·26 per cent. on sample A, Table III). These differences were probably due to adsorption of ergosterol by the yeast solids precipitated on the addition of alcohol; they could not be redissolved by reasonable dilution with water.

TABLE III

RECOVERY OF ERGOSTEROL FROM YEAST BY SAPONIFICATION

Yeast sample	Amount obtained by saponification with 20 per cent. w/v sodium hydroxide, %	Amount obtained by subsequent alcoholic saponification, %	Total, %
Sample A	0·316	0·031	0·347
	0·309	0·038	0·347
Sample B	0·289	0·032	0·321
	0·292	0·021	0·313

The effects of increasing the alkali strength and of using potassium hydroxide in place of sodium hydroxide were then studied, whilst the additional alcoholic saponification was applied as a check on complete recovery in those tests of which the results are marked with an asterisk in Table IV. In those tests destruction of yeast solids during the aqueous saponification was so severe that the previously noted precipitation was not observed on the addition of alcohol.

TABLE IV

EFFECT OF ALKALI AND CONCENTRATION ON RECOVERY OF ERGOSTEROL IN YEAST

Saponification conditions (5 g of yeast + 10 ml of alkali)	Reflux period			
	½ hour	1 hour	1½ hours	3 hours
Sodium hydroxide, 20 per cent. w/v	—	0·292 0·289	—	—
Sodium hydroxide, 50 per cent. w/w	0·218	0·244	—	0·328
Potassium hydroxide, 50 per cent. w/w ..	0·333	0·359	0·359* 0·359*	0·355
Potassium hydroxide to a concentration of 50 per cent. w/w in the saponification mixture	0·338* 0·325*	—	0·328 0·313	—

* Subsequent alcoholic saponification yielded no additional ergosterol.

The results in Table IV show that recoveries of ergosterol are low and erratic when sodium hydroxide is used and confirm an earlier observation[7] that results are more satisfactory with potassium hydroxide. In order to establish the most satisfactory concentration of potassium hydroxide and the time required for saponification, additional tests were carried out. The results, shown in Table V, are compared with those for similar concentrations of sodium hydroxide, and they show that potassium hydroxide at concentrations of between 20 and 50 per cent. w/w can be used, according to the time allowed for saponification. A saponification time of 2 hours and a concentration of 40 per cent. w/w were selected, as they allow considerable margin in both time and concentration.

Some destruction of ergosterol appears to occur when the strength of potassium hydroxide is raised to 50 per cent. w/w in the mixture, whereas with sodium hydroxide at all strengths results are low and variable.

METHOD

REAGENTS—

Potassium hydroxide, 40 per cent. w/w—Dissolve 100 g of analytical reagent grade potassium hydroxide, containing not less than 85 per cent. w/w of potassium hydroxide, in 113 ml of water.

Digitonin—Specially selected for low absorption in the range 270 to 310 mμ.

Digitonin solution, 0·5 per cent. w/v—Dissolve 0·5 g of digitonin in 80 per cent. w/v ethyl alcohol to produce 100 ml. Filter if necessary.

PROCEDURE—

Weigh 5 ± 0·01 g (Note 1) of fresh yeast into a 100-ml flask provided with a ground-glass connection. Add 10 ml of 40 per cent. w/w potassium hydroxide solution, avoiding contamination of the ground-glass connection. Attach an air condenser, lubricating the joint with a few drops of water, and boil gently for 2 hours (conveniently on a hot plate).

Cool, transfer the contents of the flask to a 250-ml separator with the aid of 2 to 3 ml of water and extract successively with three 50-ml portions of "anaesthetic grade" ether, using the first portion to rinse the condenser and flask. Mix the ether extracts in a second

TABLE V

EFFECT OF ALKALI CONCENTRATION AND SAPONIFICATION TIME ON RECOVERY OF ERGOSTEROL FROM YEAST

Results are given as percentage of the result obtained under the conditions recommended (40 per cent. w/w potassium hydroxide for 2 hours)

Alkali strength* (per cent. w/w)	Saponification time: Potassium hydroxide 1 hour	Potassium hydroxide 2 hours	Potassium hydroxide 3 hours	Sodium hydroxide 1 hour	Sodium hydroxide 2 hours	Sodium hydroxide 3 hours
10	21	23	23	21	22	25
20	51	96	98	69	88	94
30	87	100	100	76	81	82
40	97	100	100	57	72	62
50	97	98	99	85	87	91
50 in sap. mixture	96	88	88	—	—	—

* 5 g of yeast and 10 ml of alkali.

separator and wash with two 25-ml portions of water. Discard the aqueous washings and filter the ether through a small plug of absorbent cotton-wool into a distillation flask, rinsing the separator and filter with three 10-ml portions of ether. Distil the ether and dry the residue in a gentle stream of air.

Dissolve the residue in absolute alcohol by warming, cool and dilute with absolute alcohol to 50 ml in a graduated flask. If a correction procedure is to be applied, dilute accurately a portion of this solution and measure its ultra-violet absorption at the required wavelengths. Usually 2·50 ml diluted to 50 ml in absolute alcohol is a satisfactory dilution. We return to this matter in our next paper.[10]

Mix together in a stoppered centrifuge tube a suitable aliquot (2·50 ml, see Note 1) of the solution and when necessary dilute to 5·0 ml with absolute alcohol. Add an equal volume of digitonin solution, warm in a water-bath at about 70° C and set aside overnight.

Centrifuge to clear the supernatant layer and then decant as much as possible, avoiding loss of precipitate. Suspend the precipitate in about 5 ml of absolute alcohol and transfer the suspension to a 50-ml graduated flask. Wash the centrifuge tube with more absolute alcohol to a volume of about 40 ml. Warm to dissolve the precipitate, cool and dilute to volume with absolute alcohol. Measure the optical density (E) of the solution at 282 mμ (max.) in a 1-cm cell, using the plain solvent in the blank cell.

The percentage of anhydrous ergosterol in the sample is given by—

$$\frac{E \times 50 \times 100}{306 \times 2{\cdot}5 \times 2 \times \text{weight taken.}}$$

NOTE 1—The amounts of sample and alkali, and the dilutions prescribed, are satisfactory for yeast cake containing about 72 per cent. of water and 0·4 per cent. of anhydrous ergosterol. Samples containing a higher proportion of water should be dried to about 70 per cent. moisture; alternatively, a stronger potassium hydroxide solution can be used to maintain the correct strength during saponification. In testing dried yeast, each gram of sample requires the addition of 4 ml of water and 10 ml of 40 per cent. w/w potassium hydroxide.

NOTE 2—It is essential that the final solution be free from even the slightest trace of turbidity. If necessary, the solution should be filtered, loss by evaporation being avoided. As a check on clarity the optical density at 310 mμ should be less than 0·01 for a reading of 0·5 to 0·6 at the 282-mμ maximum.

REFERENCES

1. Fieser, L. F., and Fieser, M., "Natural Products Related to Phenanthrene," Reinhold Publishing Corp., New York, 1949.
2. Smedley-Maclean, I., *Biochem. J.*, 1928, **22**, 22.
3. Bills, C. E., Massengale, O. N., and Prickett, P. S., *J. Biol. Chem.*, 1930, **87**, 259.
4. Castille, A., and Ruppol, E., *Bull. Acad. Méd. Belg.*, 1933, **13**, 48.
5. Heiduschka, A., and Lindner, H., *Hoppe-Seyl. Z.*, 1929, **181**, 15.
6. Goering, K. J., U.S. Patent 2,395,115; *Chem. Abstr.*, 1946, **40**, 2930.
7. Giral, F., and Dupont, M. G., *Rev. Soc. Mex. Historia Natural*, 1944, **5**, 173.
8. Pesez, M., and Herbain, M., *Bull. Soc. Chim. France*, 1949, **5**, 760.
9. Pénau, H., and Hardy, Z., *J. Pharm. Chim.*, 1929, **9**, 145.
10. Shaw, W. M. C., and Jefferies, J. P., *Analyst*, 1953, **78**, 519.

GLAXO LABORATORIES LTD.
GREENFORD, MIDDLESEX

December 22nd, 1952

The Determination of Ergosterol in Yeast

Part III. Corrections for Irrelevant Absorption in Solutions of Ergosterol

BY W. H. C. SHAW AND J. P. JEFFERIES

(*Presented at the meeting of the Society on Wednesday, May 20th*, 1953)

The unsaponifiable matter of yeast normally contains substances that possess ultra-violet absorption in the same region as ergosterol, so that the extinction at the ergosterol maximum (282 mμ in absolute alcohol) cannot be used directly as a measure of the concentration of ergosterol present. Although the digitonin purification described in Part II may be used for separating ergosterol in a spectroscopically pure condition, considerable time can be saved by applying a correction to the gross absorption.

Two such procedures have been studied: (*i*) a correction of the Morton and Stubbs type, based on the 282-mμ maximum and two subsidiary wavelengths at which the purified compound possesses six-sevenths of the maximum absorption, and (*ii*) a new four-point correction based on the three ergosterol maxima (271·5, 282 and 293·5 mμ) with a fourth reading at 310 mμ, at which ergosterol shows little absorption.

Procedure (*i*) suffers from the disadvantage that the maxima in the absorption curve of ergosterol are sharp and observations at the subsidiary wavelengths must be taken on steep portions of the curve. In consequence small errors in measuring wavelengths have a large effect on the correction factor and any instrument used for this procedure must be calibrated with pure ergosterol. Procedure (*ii*) is free from this disadvantage, but requires a greater range of wavelengths and linearity of absorption must be assumed over it.

Results by both correction procedures are compared with those by the full digitonin method. The degree of agreement is generally good, but depends on the shape and amount of irrelevant absorption, which has been determined for a number of different strains of yeasts.

SOLUTIONS of ergosterol obtained after saponification of yeast are spectroscopically "impure" and therefore the absorption at the main maximum (282 mμ) cannot be used directly as a measure of ergosterol content. The digitonin purification previously described[1] is available for separating free ergosterol from matter possessing irrelevant absorption; although this purification is efficient, it is time-consuming. The possibility of avoiding the digitonin precipitation by applying some form of correction to the gross absorption, provided this could be done without undue loss of accuracy, was therefore investigated.

The well-known Morton and Stubbs[2] correction for irrelevant absorption is based on measurements of absorption at a maximum on the curve and at two subsidiary wavelengths, usually one on either side of the maximum. If the irrelevant absorption is assumed to be linear over the three selected wavelengths, it is possible by a geometrical construction to

correct for the irrelevant absorption at the maximum. If the maximum occurs at wavelength λ_1, and the two subsidiary wavelengths are λ_2 and λ_3, and the extinction values of the pure compound at these wavelengths are respectively $E\lambda_1$, $E\lambda_2$ and $E\lambda_3$, the following general formula can be applied for calculating the corrected absorption at the maximum—

$$E\lambda_1 \text{ (corrected)} = \frac{E_1k_1k_2(\lambda_3 - \lambda_2) - E_2k_1k_2(\lambda_3 - \lambda_1) - E_3k_1k_2(\lambda_1 - \lambda_2),}{k_1(\lambda_2 - \lambda_1) + k_1k_2(\lambda_3 - \lambda_2) + k_2(\lambda_1 - \lambda_3)}$$

where $k_1 = E\lambda_1/E\lambda_2$, $k_2 = E\lambda_1/E\lambda_3$ and E_1, E_2 and E_3 are, respectively, the observed extinctions at wavelengths λ_1, λ_2 and λ_3.

If the two subsidiary wavelengths are taken at points where the absorptions of the pure compound are equal, *i.e.*, where $E\lambda_1/E\lambda_2 = E\lambda_1/E\lambda_3$ and hence $k_1 = k_2 = K$, the following simpler expression can be used—

$$E\lambda_1 \text{ (corrected)} = K\left[\frac{E_1(\lambda_3 - \lambda_2) - E_2(\lambda_3 - \lambda_1) - E_3(\lambda_1 - \lambda_2)}{(\lambda_2 - \lambda_1) + K(\lambda_3 - \lambda_2) + (\lambda_1 - \lambda_3)}\right]$$

It is in the second form, or some derived form, that the correction procedure has been applied extensively to spectroscopic determinations of vitamin A. The subsidiary wavelengths chosen are those at which the absorption of the pure compound is some convenient fraction, usually six-sevenths, of the maximum. In the same form and by use of the same fraction, the correction can be applied to spectroscopically impure solutions of ergosterol. The main ergosterol maximum at 282 mμ is, however, much sharper than the maximum of vitamin A, so that great care is required in measuring the absorptions, particularly at the subsidiary wavelengths, which are on steep portions of the absorption curve. Fig. 1 shows on an extended scale the absorption curves of a purified specimen of ergosterol in the region of the 282-mμ maximum. On an instrument calibrated as described previously[3] the main maximum was found at 281·8 mμ and the subsidiary wavelengths for six-sevenths absorption at 278·3 and 284·3 mμ with absolute alcohol as solvent. In *cyclo*hexane the corresponding wavelengths were 282·2, 279·0 and 284·6 mμ.

It can be calculated from Fig. 1 that a wavelength error of +0·2 mμ in setting the instrument at both subsidiary wavelengths gives a correction about 8 per cent. and an error of +0·5 mμ a correction approximately 19 per cent. too high. The absorption at the maximum is much less affected by small errors in wavelength and, since it is sharply defined, any appreciable shift of the maximum is normally encountered only in grossly impure solutions.

The results in Table I show that the Morton and Stubbs type of correction can be applied in this way with a fair measure of success. It appears essential, however, that apart from the usual check, the wavelength scale of any spectrophotometer used in this manner must be especially calibrated for purified ergosterol in the region of the maximum. The precise wavelengths found for six-sevenths of maximum absorption can then be used for this correction. In using the four-point correction described below, this careful calibration is unnecessary.

The following formulae were used in calculating the results in Table I—

(*i*) in alcohol, $$F = 7 - 2{\cdot}68 \times \frac{\text{E at } 278{\cdot}3}{\text{E at } 281{\cdot}8} - 4{\cdot}32 \times \frac{\text{E at } 284{\cdot}3}{\text{E at } 281{\cdot}8},$$

(*ii*) in *cyclo*hexane, $$F = 7 - 2{\cdot}99 \times \frac{\text{E at } 279{\cdot}1}{\text{E at } 282{\cdot}2} - 4{\cdot}01 \times \frac{\text{E at } 284{\cdot}6}{\text{E at } 282{\cdot}2}.$$

F is the factor to be applied to the gross extinction at the 282-mμ maximum.

FOUR-POINT CORRECTION—

Consideration of the characteristic absorption curve of ergosterol suggested that a correction based on the three maxima should not require the careful wavelength calibration with pure ergosterol necessary for the usual Morton and Stubbs correction.

In addition, experience showed that the absorption of an alcoholic ergosterol solution at 310 mμ is a useful guide to purity, since the extinction of pure ergosterol at that wavelength is comparatively small ($E^{1\%}_{1cm} = 0{\cdot}8$). Thus a direct indication of the irrelevant absorption is obtainable at that wavelength.

From the four selected points on the ergosterol curve (at the maxima at 271·5, 282 and 293·5 mμ and at 310 mμ) three three-point corrections are obtained for E at 282 (corrected)

by substituting the appropriate values in the general Morton and Stubbs expression given above—

(*i*) 4·288 × E at 282 — 2·242 × E at 271·5 — 2·046 × E at 293·5,

(*ii*) 3·228 × E at 282 — 2·347 × E at 271·5 — 0·880 × E at 310,

(*iii*) 26·62 × E at 282 — 45·16 × E at 293·5 — 18·55 × E at 310.

A fourth correction can be obtained by eliminating the E at 282 reading between expressions (*i*) and (*ii*), thus—

(*iv*) 6·232 × E at 293·5 — 2·675 × E at 271·5 — 3·559 × E at 310.

Correction (*iii*), based on observations at 282, 293·5 and 310 mμ, is unsatisfactory, because it so happens that these three points are almost in a straight line on the absorption

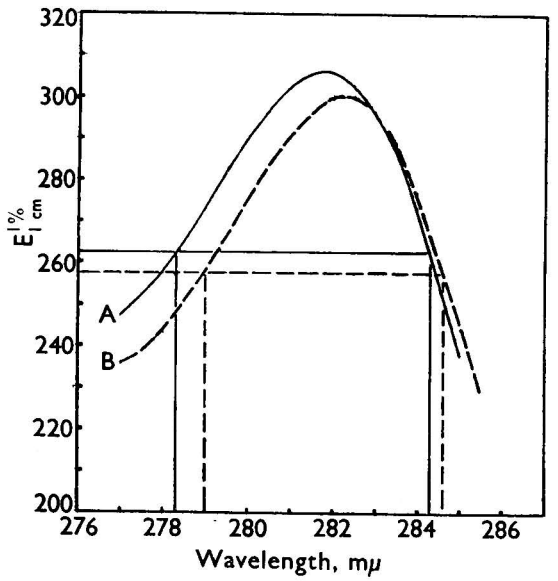

Fig. 1. Absorption curves for anhydrous ergosterol in the region of the 282-mμ maximum. Curve A: in absolute alcohol; curve B: in *cyclo*hexane. Concentration approximately 0·002 per cent. w/v. The wavelengths for $^6/_7$ths maximum absorption required for a Morton and Stubbs type of correction are indicated

curve for pure ergosterol. In consequence, the coefficient of each term of the correction is unusually high.

The remaining three corrections can be applied individually as a test for linear irrelevant absorption in the manner described by Gridgeman.[4] Correction (*iv*) is open to the criticism that it places undue reliance on the reading at the subsidiary maximum at 293·5 mμ. A more balanced equation can be obtained by taking the mean of (*i*), (*ii*) and (*iv*) in the form of the following four-point correction—

E at 282 (corrected) = 2·505 × E at 282 + 1·395 × E at 293·5
— 2·421 × E at 271·5 — 1·479 × E at 310.

E at 282, E at 293·5, E at 271·5 and E at 310 are the observed extinction values of the particular solution (in absolute alcohol) at the three respective maxima and at 310 mμ. If a 1-cm cell is used, the percentage (w/v) of anhydrous ergosterol in the test solution is given by E at 282 (corrected)/306.

The corresponding four-point correction (*cyclo*hexane as solvent) is—

E at 282 (corrected) = 2·513 × E at 282 + 1·205 × E at 294
— 2·327 × E at 271·5 — 1·389 × E at 310,

and the percentage of anhydrous ergosterol (w/v) in the solution under test is given by E at 282 (corrected)/299.

Table I shows a number of results obtained on various organisms by the full digitonin method previously described.[1] For comparison, those obtained by applying both correction procedures described above to solutions of the unsaponifiable matter before digitonin precipitation are included.

TABLE I

ERGOSTEROL CONTENT OF VARIOUS YEASTS

	Sample	Total solids, %	Ergosterol: By digitonin precipitation, %	Ergosterol: By four-point correction, %	Ergosterol: By Morton and Stubbs type correction, %	Calculated† to the dry sample, %
A.	Bakers' yeast (*Saccharomyces cerevisiae*)	28·0	0·441	0·435	0·434	1·56
			0·430	0·429	0·431	
			0·435	0·430	0·432	
			0·443	0·439	0·452	
B.	Bakers' yeast (*S. cerevisiae*) ..	28·0	0·420	0·420	0·407	1·49
			0·414	0·413	0·408	
C.	Bakers' yeast (*S. cerevisiae*) ..	26·5	0·363	0·359	—	1·36
			0·358	0·352	—	
				0·360*	0·343*	
				0·356*	0·333*	
				0·358*	0·324*	
D.	*Rhodotorula gracilis*	11·5	0·0681	0·0728	0·0645	0·59
			0·0680	0·0741	0·0687	
E.	*S. Carlsbergensis*	28·0	0·523	0·522	0·526	1·87
			0·525	0·525	0·516	
F.	*S. Carlsbergensis*	28·5	1·389	1·354	1·427	4·88
G.	Brewers' yeast (top fermentation)	21·8	0·0511	0·0589	0·0534	0·23
			0·0512	0·0585	0·0530	
H.	Brewers' yeast (sample G after washing)	19·1	0·0538	0·0568	0·0564	0·28
			0·0548	0·0575	0·0582	
J.	Brewers' yeast (bottom fermentation)	26·8	0·0350	0·0386	0·0402	0·13
			0·0361	0·0390	0·0406	
K.	Brewers' yeast (*S. cerevisiae*) ..	20·5	0·0636	0·0669	0·0660	0·31
			0·0639	0·0704	0·0677	
L.	*S. italicus*	31·8	0·898	0·887	0·888	2·82
M.	*S. chevalieri*	61·2	1·778	1·799	1·841	2·91
N.	Vacuum-dried yeast	—	1·86	1·86	—	1·86
			1·86	1·87	—	
O.	Roller-dried yeast (*S. cerevisiae*) ..	—	0·455	0·443	0·478	0·45
P.	Roller-dried yeast (*S. cerevisiae*) ..	—	0·365	0·344	0·364	0·36

* *cyclo*Hexane as solvent. † By digitonin precipitation.

IRRELEVANT ABSORPTION—

The success of the kind of geometrical correction procedure considered depends on the validity of assuming linear irrelevant absorption. In vitamin-A analysis it is not possible to test this assumption experimentally[5] and so the question remains a subject of controversy.

With the digitonin treatment described earlier,[1] it is possible to separate the ergosterol quantitatively from yeast in a "spectroscopically pure" solution; the irrelevant absorption in the mother liquors after digitonin precipitation can then be determined. Fig. 2 shows the irrelevant absorption determined in this way on certain of the samples listed in Table I.

When the proportion of ergosterol in the dry matter is high, as in samples F and M, the relative amount of irrelevant absorption is small and little correction is required.

Bakers' yeasts Q, R and S, containing less ergosterol, gave satisfactory corrected results because the irrelevant absorption, although higher, was nearly linear. Brewers' yeasts J, G and K, and the *Rhodotorula* strain, D, contained only small amounts of ergosterol, so that not only was the irrelevant absorption a much higher proportion of the gross extinction, but it also showed marked deviation from linearity. For such materials no geometrical adjustment will give more than an approximation to the correct value; for accurate results the full digitonin procedure must be used. The samples of brewers' yeast contained a high proportion of fermentable matter, which appeared to be the source of some of the irrelevant

absorption. This is illustrated by sample G, which gave about half the original irrelevant absorption (curve H) after being washed with water. This procedure should always be applied to yeast containing little ergosterol if it is desired to obtain an approximate result by the correction procedure for estimating ergosterol content.

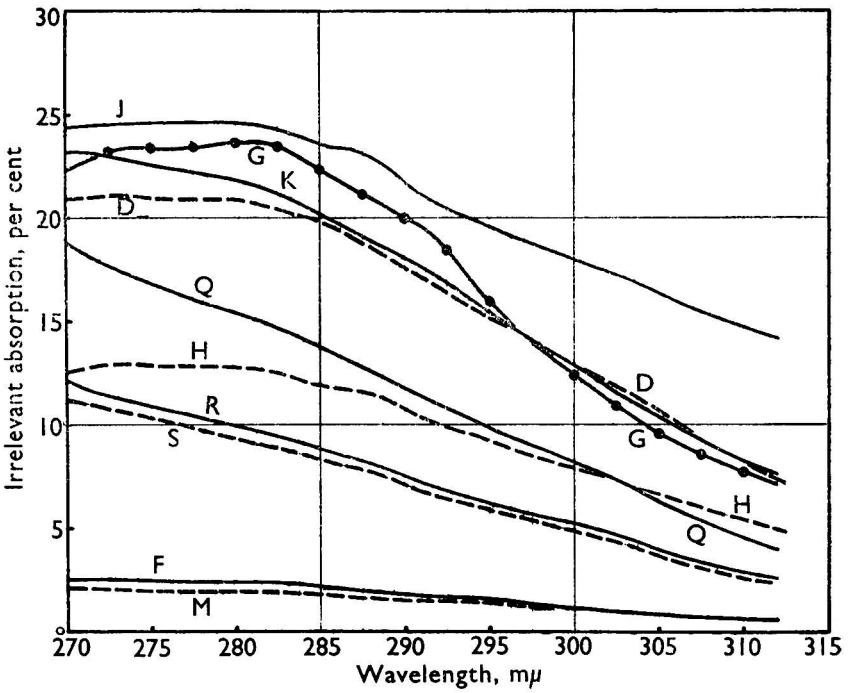

Fig. 2. Irrelevant absorption of yeasts expressed as a percentage of the ergosterol present. For key to samples, see Table I. Samples Q, R and S are representative bakers' yeasts (*Saccharomyces cerevisiae*) containing, respectively, 1·07, 1·42 and 1·50 per cent. of ergosterol in the dry matter

References

1. Shaw, W. H. C., and Jefferies, J. P., *Analyst*, 1953, **78**, 514.
2. Morton, R. A., and Stubbs, A. L., *Ibid.*, 1946, **71**, 348.
3. Shaw, W. H. C., and Jefferies, J. P., *Ibid.*, 1953, **78**, 509.
4. Gridgeman, N. T., *Ibid.*, 1951, **76**, 449.
5. Bagnall, H. H., and Stock, F. G., *J. Pharm. Pharmacol.*, 1952, **4**, 81.

NOTE—References 1 and 3 are to parts II and I of this series.

GLAXO LABORATORIES LTD.
GREENFORD, MIDDLESEX

December 22nd, 1952

The Determination of Ergosterol in Yeast

Part IV. A Short Method Based on Ultra-violet Absorption

By W. H. C. SHAW and J. P. JEFFERIES

(*Presented at the meeting of the Society on Wednesday, May 20th, 1953*)

An accurate method for determining ergosterol in yeast has been described previously; it involves saponification, extraction of unsaponifiable matter, digitonin precipitation and subsequent spectroscopic determination and has proved too lengthy for the examination of large numbers of samples.

For this purpose, a short semi-micro method is described; it involves the saponification of a portion of the yeast sample containing about 1 mg of ergosterol with a small volume of 40 per cent. w/w aqueous potassium hydroxide in a specially designed flask and extraction of the unsaponifiable matter with a single accurately measured volume of *cyclo*hexane. The filtered extract is suitable for spectroscopic examination. The four-point correction procedure, also previously described, is applied to allow for irrelevant absorption; the ergosterol content of the sample is calculated from the corrected extinction at the 282-mμ maximum.

The method requires a shorter manipulation time than the full digitonin procedure, but, because a geometrical correction and a single extraction only are used, gives results slightly lower and a standard deviation of a single determination a little greater than those got with the full method.

In a previous paper,[1] a method was described of determining ergosterol in yeast. Although it is accurate and therefore of special use for research purposes, the time necessary for a single determination by it is considerable because of the manipulations involved in saponification, three extractions with ether, distillation of the ether, precipitation with digitonin and spectroscopic examination of the digitonide in solution. If any irrelevant absorption present is assumed to be linear, the digitonin precipitation may be omitted[2] with some saving of time, but not sufficient for an examination of many small samples of yeast, for which a short simple method was required, even though it might entail some loss of accuracy.

Ultra-violet absorption provides an accurate means of identifying and determining small amounts of ergosterol, so that, provided the ergosterol can be liberated and quantitatively recovered, correspondingly small samples of yeast can be assayed. With this in mind, we have studied the possibility of saponifying about 0·2 g of fresh yeast in a small volume of aqueous potassium hydroxide and then extracting once with an immiscible solvent.

Experimental

Effect of strength and volume of potassium hydroxide—

Initially, saponifications were carried out on 0·2 g of yeast cake with 0·4 ml of 40 per cent. w/w aqueous potassium hydroxide solution, because it is desirable with a single solvent extraction to keep the volume of the aqueous phase as small as possible. Under these conditions ergosterol could generally be satisfactorily recovered by one extraction with 50 ml of solvent, but certain yeasts containing little ergosterol gave gelatinous residues from which the ergosterol could not be extracted quantitatively. Moreover, even with a specially-designed apparatus, it was difficult to avoid undue concentration of alkali and consequent destruction of some ergosterol.[1]

About 1·5 ml appeared to be the smallest volume in which the saponification could reasonably be carried out; a convenient apparatus for this and the subsequent extraction is shown in Fig. 1.

The conditions necessary for the quantitative liberation of ergosterol from yeast have been investigated during the work already reported.[1] However, it became apparent that with only one extraction the concentration of the potassium hydroxide solution had to be controlled over a narrower range than formerly (Table I).

The low results obtained when the concentration during extraction was much over 40 per cent. w/w appeared to be due to some adverse effect on the distribution of ergosterol between solvent and aqueous phase. Also, concentrations appreciably below 32 per cent. w/w led to low recovery, possibly on account of incomplete saponification.

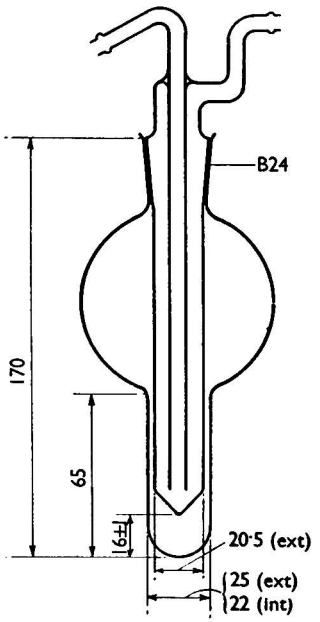

Fig. 1. Pyrex-glass flask for saponification and extraction. Dimensions are in millimetres

SOLVENTS—

Under the saponification conditions described various solvents were tried for the subsequent extraction.

Ether—As diethyl ether is normally used in the determination of unsaponifiable matter, a few trials were carried out with it. Although separations were rapid, the extracts were

TABLE I

EFFECT OF POTASSIUM HYDROXIDE CONCENTRATION ON RECOVERY OF ERGOSTEROL

Strength of potassium hydroxide reagent,* % w/w	Concentration of potassium hydroxide during extraction, % w/w	Maximum recovery, %
30	27	82
35	32	100
40	36·5	100
40	41†	99
40	46†	97
40	51†	86

* 1·5 ml with 0·2 g of yeast cake, saponified 2 hours.
† Extra solid potassium hydroxide added after saponification.

invariably turbid and loss by evaporation occurred during the necessary filtration. In addition, the ether appeared to dissolve some water and the potassium hydroxide extracted the preservative (probably hydroquinone) from the anaesthetic grade of ether used. These effects seriously altered the ultra-violet transmission of the solvent and specially prepared ether was necessary. Apart from this, quantitative manipulation of the ether at room temperatures above 25° C became almost impossible.

iso*Propyl ether, b.p.* 68° *C*—Spectroscopically pure *iso*propyl ether was not available commercially and purification proved troublesome. In addition, this solvent suffered from some of the defects of diethyl ether.

Ethylene chloride (1:2-*dichloroethane*), *b.p.* 84° *C*—Giral and Dupont[3] recommended substituting this solvent for ether in the extraction of ergosterol. Although it gave satisfactory extraction, the material available had to be purified before it could be used for spectroscopy. After extraction with this solvent separation was slow, for the density of ethylene chloride is much nearer that of 40 per cent. w/w potassium hydroxide than is that of any other solvent considered. Further, the extracts had to be dried and filtered for spectroscopic examination.

cyclo*Hexane*, *b.p.* 81° *C*—This proved to be the most satisfactory solvent. It yields extracts that can easily be clarified by filtration; alternatively, after being allowed to stand for sufficient time, the clear solvent can be decanted directly for examination. It is unaffected by shaking with 40 per cent. w/w potassium hydroxide solution and suitable batches of commercial material can be selected for use without further treatment.

CORRECTION FOR IRRELEVANT ABSORPTION—

The four-point and other geometrical corrections[2] depend for their validity on the assumption that the irrelevant absorption is linear over the selected wavelengths. For many samples of washed yeast containing much ergosterol, this assumption has been shown[2] to be valid, and results obtained by the correction procedure are reliable. When, however, it is desired to determine ergosterol in yeast from a strain not previously examined, it seems essential to apply the full digitonin method previously described[1] and to check by direct observation on the digitonin mother liquors that the irrelevant absorption is indeed linear over the wavelengths used.

TABLE II

COMPARISON OF ERGOSTEROL DETERMINATIONS BY THE DIGITONIN AND SHORT METHODS

Sample	Total solids, %	Anhydrous ergosterol		
		By digitonin method, %	By short method	
			No. of determinations	Mean and its standard error, %
Bakers' yeast (*S. cerevisiae*)	29·0	0·345	4	0·348 (±0·0036)
Bakers' yeast (*S. cerevisiae*)	26·5	0·397	15	0·394 (±0·0016)
Vacuum-dried yeast (*S. cerevisiae*) ..	—	1·75	4	1·68 (±0·0104)
S. carlsbergensis	28·5	1·55	4	1·49 (±0·0112)
S. carlsbergensis	30·8	1·71	4	1·66 (±0·0087)

Correction procedures are most seriously in error when a break in the irrelevant absorption occurs near the main ergosterol maximum at 282 mμ. In using the four-point correction[2] with observations at the maxima (at 271·5, 282 and 294 mμ) and at 310 mμ, it is sometimes possible to detect irrelevant absorption of this type by direct inspection of the observed optical densities. For purified ergosterol in *cyclo*hexane the ratio of E at 271·5 to E at 282 is 0·94. When the ratio is less than this, or when it approaches this value with a high reading at 310 mμ, non-linear irrelevant absorption must be suspected and the full digitonin method will have to be applied if accurate results are wanted. The reading at 310 mμ is a direct indication of the amount of irrelevant absorption at that wavelength, where the absorption due to ergosterol is small.

RESULTS—

In Table II figures for representative samples of yeast of good quality, obtained by the method described below, are shown in comparison with those obtained by the full digitonin method previously described.[1] Results for materials containing much ergosterol are reproducible and generally in good agreement with those given by the full method. Recovery is usually within the range 96 to 100 per cent. For samples containing little ergosterol, or when the irrelevant absorption shows any marked deviation from linearity, greater divergence than this must be expected.

It is clear that the values by the short method are, for the richer yeasts, significantly, but only slightly (under 10 per cent.), lower than those found by the digitonin method (mean of duplicate determinations); for the two poorer yeasts there is no real difference. The standard error of a single observation calculated for all thirty-one analyses is ±0·0130.

METHOD

APPARATUS—

Reflux flask (*see Fig.* 1)—The flask is made from a suitable length of 1-inch diameter tubing and a 150-ml flask. The condenser should be of such diameter that the space between it and the side of the base of the flask is as narrow as possible, so that moisture does not condense in the upper portion of the flask when it is in use. The distance between the bottom of the condenser and the base of the flask should be within the limits specified, although the thickness of the slip of paper placed in the ground-glass connection during the heating allows a small measure of control.

Hot-plate—This is covered with a layer of sand about 0·5 cm thick.

*"Wrist-action" shaking machine**—If necessary the length of two of the opposed arms of the shaker should be increased, *e.g.*, from 10 to 20 cm, so that the vertical movement of the flasks during shaking is about 1·5 cm.

REAGENT—

Potassium hydroxide solution, 40 per cent. w/w—Dissolve 100 g of analytical reagent grade potassium hydroxide, containing not less than 85 per cent. w/w of potassium hydroxide, in 113 ml of water.

SOLVENT—

cyclo*Hexane*—The optical density of a 1-cm cell filled with the solvent measured against air should be not more than 0·2 at and above 271·5 mμ.

PROCEDURE—

Weigh accurately into the base of the reflux flask sufficient of the sample to contain about 1 mg of ergosterol. Add 1·50 ml of potassium hydroxide solution, avoiding contamination of the upper portion of the flask with either sample or alkali. Insert the condenser and place a slip of paper in the neck to provide a vent. Attach the condenser to a water supply and place the bottom of the flask in the sand on the hot-plate. Heat gently under reflux for 2 hours, swirling occasionally. Remove the flask from the hot-plate, disconnect the water supply and remove the slip of paper. Allow to cool, add 50 ml of *cyclo*hexane from a pipette, discard the water in the condenser, and fix the condenser in the flask by means of a short length of adhesive tape. Extract in an inverted position on the shaking machine for not less than 10 minutes. Allow the phases to separate for at least 15 minutes, and then filter the solvent through a pleated Whatman No. 42 filter-paper. Avoid loss by evaporation during filtration and reject the first few millilitres of filtrate.

Determine the optical density of the filtrate in a 1-cm cell, with plain solvent in the blank cell. Take observations at the maxima at 271·5, 282 and 294 mμ and at 310 mμ, obtaining the four readings, E at 271·5, E at 282, E at 294 and E at 310, respectively.

Calculate the corrected extinction at 282 mμ from the expression—

$$\text{E at 282 (corrected)} = 2{\cdot}513 \times \text{E at 282} + 1{\cdot}205 \times \text{E at 294} - 2{\cdot}327 \times \text{E at 271·5} - 1{\cdot}389 \times \text{E at 310.}$$

The percentage of anhydrous ergosterol in the sample under test is then given by—

$$\frac{\text{E at 282 (corrected)} \times 50}{299 \times \text{weight taken}}.$$

The authors wish to express their thanks to Mr. J. L. Holmes for assistance with the practical work and to Mr. H. J. Bunker (Barclay Perkins & Co. Ltd.), Dr. J. I. Webb (A. Guinness Sons & Co. Ltd.) and Dr. J. S. Lowe for kindly supplying samples of yeast.

REFERENCES

1. Shaw, W. H. C., and Jefferies, J. P., *Analyst*, 1953, **78**, 514.
2. ——, ——, *Ibid.*, 1953, **78**, 519.
3. Giral, F., and Dupont, M. G., *Rev. Soc. Mex. Historia Natural*, 1944, **5**, 173.

NOTE—References 1 and 2 are to parts II and III of this series.

GLAXO LABORATORIES LTD.
GREENFORD, MIDDLESEX

December 22nd, 1952

* A "Microid" shaker, modified as described, has been found satisfactory.

DISCUSSION ON THE FOREGOING FOUR PAPERS

DR. R. E. STUCKEY asked what was the best method of determining the purity of commercial ergosterol samples and whether there was a great difference between the result determined on the 282-mμ peak alone and that obtained by the authors' four-point correction method.

MR. SHAW said that the validity of using the extinction at the main maximum for calculating the percentage of ergosterol in commercial material depended on the impurities present. If these were only the minor sterols of yeast, which had no absorption in the ergosterol range, the observed extinction required no correction and would give the true percentage. If any non-sterol matter possessing absorption at 282 mμ was present, the four-point correction could be applied and should give a reliable result provided the irrelevant absorption was linear. Even if it was not linear, the four-point correction would disclose the presence of irrelevant absorption, and it was then preferable to precipitate a portion of the sample with digitonin. The percentage of ergosterol could be calculated from the extinction of the digitonide at 282 mμ.

In practice, optical rotation of commercial ergosterol was normally determined as well as ultra-violet absorption, but in their experience it was generally impossible to correlate the two results, probably owing to the presence of minor sterols, which contributed to the optical rotation but not to the ultra-violet absorption.

The Determination of Microgram Amounts of Calcium

BY G. E. HARRISON AND W. H. A. RAYMOND

(*Presented at the meeting of the Society on Wednesday, May 20th*, 1953)

A micro-method for the determination of calcium is described; it is based on the precipitation of calcium molybdate and the subsequent formation of molybdenum thiocyanate, which is determined absorptiometrically. As little as 4 μg of calcium can be estimated to within $\pm$10 per cent.

The method is especially useful for determining calcium in serum from young children or from small laboratory animals.

A comparison of the results with those found by other methods is included.

WE have recently had to determine the calcium in small samples of neutral fluid containing about 20 μg of calcium to within $\pm$5 per cent. Calcium determinations by gravimetric,[1] volumetric,[2,3] colorimetric[4,5,6] and nephelometric[7] methods have been described, but in all of them the accuracy falls considerably for amounts of calcium less than 40 μg. Titration of the supernatant fluid with the sodium salt of ethylenediaminetetra-acetic acid, with murexide as indicator,[8,9] gave consistent results down to 50 to 100 μg of calcium, but was incapable of giving the accuracy we required. The following method, based on the absorptiometric estimation of a molybdenum thiocyanate,[10,11] has been found to give reproducible results within $\pm$2 per cent. (0·5 μg of calcium per sample). A special advantage of the method is that the presence of phosphate, ferric or ferrous iron, magnesium, copper or aluminium does not affect the calcium determination. The method can also be used for the estimation of strontium and barium together with lanthanum, yttrium and the rare earths, although, of course, in a mixture, it is impossible to distinguish between them.

METHOD

REAGENTS—

Sodium molybdate - pyridine reagent—Make a 5 per cent. solution in 50 per cent. alcohol from sodium molybdate of analytical reagent grade. Filter off any precipitate that forms on standing; this frees the molybdate solution from calcium. Mix 75 ml of the calcium-free solution with 25 ml of pyridine.

Potassium thiocyanate, 5 per cent solution.

Stannous chloride reagent—Make a 0·8 per cent. solution in 4 *N* hydrochloric acid by dissolving 0·5 g of tin in 4 *N* hydrochloric acid and making up to 100 ml with the acid. This solution should be freshly prepared for use.

iso*Amyl alcohol.*

Standard calcium solution—A solution containing about 2 mg of calcium per 100 ml.

PROCEDURE—

Put a known aliquot of the solution for assay containing up to 30 μg of calcium in a 15-ml centrifuge tube and add 1 ml or more of the sodium molybdate reagent. Place the mixed solutions in a bath of water at 70° C for about 30 minutes. Allow the sample to cool and then centrifuge it at 2500 r.p.m. at 15 cm radius for 10 minutes. Discard the supernatant liquor and drain the tube on filter-paper. Wash the precipitate three times with 50 per cent. alcohol, draining the tube well after each washing.

Run 2·5 ml of the 5 per cent. potassium thiocyanate solution into a 50-ml separating funnel from a burette and add 5 ml of *iso*amyl alcohol from a second burette. Dissolve the calcium molybdate precipitate in the centrifuge tube in 4 ml of hot stannous chloride reagent and pour the solution into the funnel. Wash the tube with 4 ml of 4 *N* hydrochloric acid and then with distilled water until the total volume of liquid in the funnel is about 20 ml. Stopper the funnel and gently invert it two or three times, and then set it aside to allow the alcoholic extract to rise to the top. Run off the aqueous layer and pour the coloured extract into a 10-ml calibrated flask. Return the aqueous solution to the funnel and again extract the molybdenum thiocyanate with a further 3 ml of *iso*amyl alcohol, repeating the inversion and separation. Add the second extract to the first and make the total volume up to 10 ml with *iso*amyl alcohol. Invert the flask a few times to mix the solution.

CALIBRATION STANDARDS—

Prepare a calibration graph by carrying out the procedure on a series of aliquots of the standard calcium solution; volumes of 0·2, 0·4, 0·6, 0·8 and 1·0 ml are convenient. As it is the molybdate ion that in fact is being estimated, confirmation of the completeness of the precipitations of calcium in this calibration can be made by using equivalent amounts of sodium molybdate. This was done in our calibration procedure and the results are shown in Table I. It can be seen that there is close agreement between the absorptiometric readings, and we concluded that the precipitation and recovery of calcium as molybdate was complete.

TABLE I

ABSORPTIOMETER READINGS FOR VARIOUS AMOUNTS OF MILLIMOLAR CALCIUM SOLUTION

Volume taken, ml	Calcium, μg	Change in wedge reading	Mean reading
0·1	4	19·2*	
		21·0	19·5
		19·3	
		19·0	
		19·1	
0·2	8	35·3	
		36·2	35·7
		35·6*	
0·3	12	53·7	
		57·2	54·0
		51·1	
0·4	16	65·1	
		65·6	67·2
		72·1	
		66·0	
0·5	20	89·1*	
		88·6*	
		88·8	89·1
		90·1	
0·6	24	99·4	
		106·5	103·3
		104·0	
		103·1	
0·7	28	122·2	122·1
0·8	32	146·9	
		142·5	
		145·2*	143·5
		142·1	
		140·9	

* Equivalent volumes of sodium molybdate reagent taken for these readings.

ABSORPTIOMETRY—

The absorptiometer used was a conventional double-beam instrument in which a 6-volt 30-watt small tungsten-filament incandescent lamp was used as a single light source. The barrier-type selenium photo-cells connected in opposition were used with a portable reflecting galvanometer to register the difference in light intensity between the two beams. A 10-mm absorption cell, a colour filter (Ilford Blue No. 602) and an annular neutral wedge of density range 0 to 1 were inserted in each beam. One of the absorption cells was filled with distilled water and remained a standard of reference. The other cell was filled successively with *iso*amyl alcohol and the alcoholic extract of molybdenum thiocyanate. With one wedge pre-set at a suitable value, the other was so adjusted as to bring the galvanometer spot back to the scale zero. In this way the absorption of each solution was measured in terms of an angular displacement of one of the neutral wedges. This procedure had the advantage that the wedge readings were independent of the linearity of response of the photometric cells and of variations in the light source.

RESULTS

INORGANIC SOLUTIONS—

The results of applying the procedure to aliquots of a standard solution containing 4·0 mg of calcium per 100 ml are shown in Table I.

The angular change in wedge reading is a linear function of calcium concentration in the specimens, so that Beer's law is obeyed over the range of the observations. From the results we conclude that 4 μg of calcium can be estimated to within $\pm$10 per cent. Moreover, the colours are nearly stable over several hours. This stability is shown by the following wedge rotation readings for an alcoholic extract of molybdenum thiocyanate recorded at different times after separation of the alcoholic extract: after 0·3 hour, 87·6; after 1·5 hours, 88·4; after 20 hours, 86·8.

A comparison of the molybdate, ethylenediaminetetra-acetic acid (EDTA) and oxalate methods of determining calcium is shown in Table II, in which the volumes taken for each type of asasy are also shown. It will be seen that although the molybdate and EDTA estimations agree well, the standard oxalate procedure gives low values when the sample contains 0·2 mg of calcium or less.

TABLE II

COMPARISON OF THE PROPOSED MOLYBDATE METHOD WITH OXALATE AND EDTA METHODS WHEN USED FOR DETERMINING CALCIUM IN STANDARD SOLUTIONS OF CALCIUM CHLORIDE

Standard solution of calcium, millimoles per litre	Calcium found, millimoles per litre, by: oxalate method, 10 ml of solution taken	EDTA method, 5 ml of solution taken	molybdate method, 1 ml of solution taken
0·5	0·42	0·53	0·50
0·3	—	0·32	0·30
0·2	0·16	0·22	0·21

ORGANIC SOLUTIONS—

A specially useful application of this method is to the determination of serum calcium in young children and in small laboratory animals, for which the volume of the blood sample is strictly limited. The presence of protein in serum makes it necessary to oxidise these specimens by adding a few drops of nitric acid and ammonium nitrate and taking to dryness on a sand-bath at about 200° C. Thereafter the method of analysis is similar to that for inorganic solutions. We have investigated this application by taking as little as 0·05 ml of horse serum, for which the molybdate method gave a result of 17·2 mg of calcium per 100 ml. The same value was found on applying the molybdate method to 0·2 ml of the serum. The mean result of several determinations on the same serum by the ethylenediaminetetra-acetic acid method, for which 0·5-ml samples were taken, was 17·0 mg per 100 ml.

Conclusions

The estimation of calcium based on precipitation of calcium oxalate from an alkaline solution fails if the sample contains 0·2 mg of calcium or less, owing to the incomplete recovery of calcium at these levels. The ethylenediaminetetra-acetic acid method is both simple and rapid, especially for the biologically important determination of calcium in serum. Again, however, samples containing at least 50 μg are required, and, if the phosphorus content is as high as 10 per cent., the estimation fails owing to inhibition of the end-point in the titration. On the other hand, the proposed molybdate method gives average recoveries of 97 per cent. when the sample contains 4 μg of calcium, and is unaffected by the presence of phosphorus.

References

1. Vogel, A. I., "Textbook of Quantitative Inorganic Analysis," Second Edition, Longmans, Green & Co. Ltd., London, 1951, p. 587.
2. Kolthoff, I. M., and Sandell, E. B., "Textbook of Quantitative Inorganic Analysis," The Macmillan Co., New York, 1947, p. 605.
3. Salomon, J., Gabrio, B. W., and Smith, C. F., *Arch. Biochem.*, 1946, **11**, 433.
4. Kultner, T., and Cohen, H. R., *J. Biol. Chem.*, 1927, **75**, 517.
5. Roe, J. H., and Kahn, B. S., *Ibid.*, 1929, **81**, 1.
6. Tsao, M. U., *Ibid.*, 1952, **199**, 251.
7. Murayama, M., *J. Lab. Clin. Med.*, 1948, **33**, 906.
8. Holtz, A. H., and Seekles, L., *Nature*, 1952, **169**, 870.
9. Debney, E. W., *Ibid.*, 1952, **169**, 1104.
10. Braun, A. D., *Z. anal. Chem.*, 1867, **6**, 86.
11. Hurd, L. G., and Allen, A. O., *Ind. Eng. Chem., Anal. Ed.*, 1936, **7**, 396.

Medical Research Council
Radiobiological Research Unit
Atomic Energy Research Establishment
Harwell, Didcot, Berks.

January 7th, 1953

Discussion

Mr. N. L. Allport said that this was an interesting approach to the problem of determining calcium colorimetrically. Like other available methods it was an indirect one in that the substance determined was the molybdate ion attached to the calcium. In presenting the paper, Dr. Harrison had said nothing about the specificity of the procedure; obviously lead, if present, would interfere, although, of course, one would not normally find lead in blood serum; if there had been medication with bismuth, the possible effects of this metal would need to be considered. A minute trace of zinc might well be present in many samples, as this metal was very widely distributed in animal tissues, and Mr. Allport asked if the authors had considered whether there was any disturbance from this factor and whether they had ensured that phosphates did not interfere.

Dr. Harrison, in reply, said that the specificity of the procedure was referred to in the first paragraph of the paper, but this had been omitted from the presentation. As Mr. Allport observed, lead was only of biological interest in exceptional circumstances, and no examination had been made of the interference from this ion or from that of bismuth. They had, however, found that ferrous, ferric, aluminium and phosphate ions were without effect even when present in large amounts, and also that zinc or magnesium present in amounts equivalent to that of the calcium in the sample did not affect the estimation.

Dr. J. H. Hamence asked what technique had been used for the determination of calcium by the oxalate method, and whether ethanol or acetone had been used in the initial precipitation and in the subsequent washing. Both these solvents had the effect of depressing the solubility of calcium oxalate in water and therefore of giving a better recovery when only microgram quantities of calcium were present.

Dr. Harrison replied that they had not used ethanol or acetone in the oxalate precipitation or in washing the precipitate. The oxalate had been precipitated according to the method of Clark and Collip (*J. Biol. Chem.*, 1925, **63**, 461), in which an excess of ammonium oxalate was added to an acidified solution of the calcium to be estimated and the acidity reduced to near the neutral point by dropwise addition of ammonium hydroxide, universal indicator being used. The precipitate was then heated and subsequently cooled for several hours. The precipitate was washed with a 2 per cent. ammonium hydroxide solution.

The Preparation of Biological Material for the Determination of Trace Metals

Part I. A Critical Review of Existing Procedures

By G. MIDDLETON and R. E. STUCKEY

A critical review is given of methods that have been used for the destruction of organic matter of biological origin preparatory to the determination of trace metals, and of the objections and criticisms that have been made of these methods.

The ever-growing interest in trace metals, whether as elements essential to normal biological development or as a potential source of danger to health, has resulted in the publication of numerous papers dealing with methods for the determination of such traces mixed or combined with large quantities of organic matter. For this purpose it is generally essential to isolate the mineral constituents after degradation or complete oxidation of the organic matter, and in determinations of this kind the emphasis has naturally been placed more on the determination itself and the results attained than on the preliminary treatment of the material.

Reports of such investigations are to be found in journals dealing with biology, medicine, public health, toxicology and food analysis, and are generally indexed under the names of the metals or elements in question and the material examined. As only a small proportion have anything new to say on the subject of the destruction of organic matter, the mere search for references to methods of destruction of organic matter in biological material is in itself tedious, and it does not appear that any attempt has been made to review the subject as a whole. In this paper an attempt is made to supply this deficiency, and there will be described in Part II (in the press) a method for the destruction of organic matter in biological tissues and products for which several advantages are claimed.

The destruction of organic matter

The reactions involved in the dry or wet ashing of organic matter, whether of animal or vegetable origin, are obscure and have not been studied in detail. They will vary, not only according to the reagents and methods used, but with the nature of the substances concerned, and it is impossible to treat the question as a whole without taking this into account.

The compounds that make up such material mostly belong to one of four main groups—

Carbohydrates—Carbohydrates, main constituents of many vegetable products, give on heating a residue of carbon that burns away readily in air at a fairly low temperature. They are oxidised without difficulty by the usual methods of wet combustion.

Fats—Fats are present, often in considerable amount, in both animal and vegetable tissues. On heating they are to a large extent lost by volatilisation, and the Association of Official Agricultural Chemists[1] recommends "smoking off" fat at about 350° C as a preliminary to ashing at a higher temperature. Fats are difficult to destroy by wet combustion, partly because they sublime on to the cooler upper parts of the vessel, and it is often advantageous to carry out the earlier stages of heating in an open vessel to facilitate loss of fat by volatilisation.

Proteins—Proteins may be present in large proportions in both animal and vegetable products. Certain animal proteins, apparently because of their high content of lysine, tyrosine and tryptophan, are among the most difficult substances to decompose by wet methods, while the difficulty of dry ashing may be due, at least in part, to their content of phosphates and other salts. Proteins decompose at a temperature that is generally below 350° C, and leave a black residue resembling carbon. It is, however, necessary to use a considerably higher temperature before the decomposition is complete.

Other compounds—Among other compounds that may be present in, or form the main part of, biological tissues and products are phosphatides, urea, bile salts, nucleic acid, porphyrins, resins, waxes and sterols.

Of the first three, and most important, groups mentioned above, carbohydrates are present chiefly in, and often make up the greater part of, vegetable tissues; proteins form the major constituent of animal tissues, meat products, nuts, and some seeds and fruits, and are generally associated with fats.

It is not surprising to find that methods for destroying organic matter that work satisfactorily when applied to substances of one type become troublesome when used for materials having quite a different composition: for example, the destruction of organic matter of vegetable origin and consisting largely of carbohydrates (*i.e.*, nearly all vegetable tissues except seeds) by methods of dry ashing or wet oxidation generally offers no special problems or particular difficulty; on the other hand, animal tissues (organs, meat products, blood, shell-fish and the like) often give trouble, especially where it is necessary to take a large quantity of tissue in order to obtain sufficient of the trace element for determination.

Scope of the review

The large number of proposed methods or variations of methods for dry or wet ashing of organic matter is due partly to the need for adaptation to the special character (volatility or liability to form insoluble products) of particular trace elements, and partly to a realisation of the unsatisfactory nature of existing procedures when applied to particular substances. Animal tissues especially (*e.g.*, liver or shrimps) offer practical difficulties in the application of existing methods. In the past insufficient attention has been given, in the application of different methods, to the nature of the material being destroyed; here this factor is given greater emphasis.

The present paper does not attempt to give a complete account of the preliminary methods used for the destruction of organic matter, but is restricted to certain applications. Synthetic organic compounds are ignored, and volatile elements such as boron and mercury require procedures of such a special character that they are better treated separately. Methods used for arsenic have been included, but for this element the principles are usually similar to those used for less volatile elements. Spectrographic methods, owing to the small quantity of material required for a determination, are also considered as a special subject and have not been included.

An account of methods that have been used for the destruction of organic matter of biological origin with the object of determining trace elements (with the limitations described in the preceding paragraph) is therefore given, together with objections that have been raised against them, and some account of our own experience in particular instances. These methods are grouped under a number of headings according to the general method or reagents used.

Dry methods

Methods—

Dry ashing is a method frequently used for the destruction of organic matter, and it is generally considered advisable that it should be carried out at as low a temperature as possible in order to reduce the possibility of loss of trace metals by volatilisation and by the formation of difficultly soluble compounds, as for example by reaction with silica. Whilst a number of workers have ashed materials directly at an indefinite temperature—presumably a more or less bright red heat—others are more specific. A temperature of about 500° to 550° C (equal to a dull red heat), which represents the lowest temperature at which combustion can generally be completed in a reasonable time, is most commonly recommended, especially when the trace metal in question is volatile.

For the determination of lead in animal tissues and similar material, Fairhall[2] recommends ashing at a temperature well below red heat, otherwise there is a loss by volatilisation; Schmidt[3] first moistens the sample with sulphuric acid and then ashes at a dull red heat; Kehoe, Edgar, Thamann and Sanders[4] use a temperature not over 600° C for ashing deposits from urine, as they find Fairhall's method unsatisfactory. Seiser, Necke and Müller[5] also disagree with Fairhall and consider that blood can only be ashed without loss of lead if sulphuric acid is first added—they recommend a temperature of 500° to 530° C, and state that lead sulphate is not volatile below 550° C. Tannahill[6] ashes the sample at a dull red heat when determining lead in tissues. Roche Lynch, Slater and Osler[7] consider that when ashing bone, a temperature above 550° C will result in loss of lead, and that it is possible that prolonged heating at even a lower temperature may result in some loss. Weyrauch and Müller[8] used a temperature of 500° to 550° C for the same purpose.

For zinc, dry ashing is used by Eggleton,[9] the temperature not exceeding 450° C, and by Rauschning,[10] at 500° C; Heller and Burke[11] use a "black heat," and Sylvester and Hughes,[12] followed by Allport and Moon,[13] find dry ashing at 500° to 550° C satisfactory, with no appreciable volatilisation below 900° C.

Richards[14] ashes at a "low red" heat for the determination of manganese. Forbes and Swift,[15] determining iron in meat products, ignite the sample "to a white ash" at an unspecified temperature; while Davies[16] determines copper and iron in milk and cheese by ashing the sample over a smoky flame in a wide tube as an improvised muffle furnace. In the procedure used by Ansbacher, Remington and Culp[17] for copper in eggs, oysters and so on, the temperature of ashing does not exceed 400° C, although repeated moistening with nitric acid and re-ashing may be necessary to complete the procedure. The procedure of McFarlane[18] for copper in blood is practically identical. Bertrand and Macheboeuf[19] ash the sample in a thin layer at a low temperature (unspecified) for cobalt and nickel in animal organs. Warburg[20] ashes serum and similar material in a special silica flask at a dark red heat for determining copper, iron and manganese. Sylvester and Lampitt[21] ash milk with a small quantity of sulphuric acid in a silica dish at 500° to 550° C for the determination of copper.

With aluminium there is some danger of the metal becoming insoluble or of forming silicates if too high a temperature is used for ashing. Eveleth and Myers[22] use a temperature not over 500° C for tissues, milk and urine, usually for a period of 24 hours, but it is sometimes necessary to digest the ash with sulphuric and nitric acids to remove the last remnants of carbon.

For tin, which also is liable to become insoluble, dry ashing methods are not usually used, but Misk[23] ashed tissues with the addition of magnesium nitrate, and Bamford[24] similarly used a mixture of magnesium oxide and nitrate for determining antimony and arsenic, following the method of Strzyowski.[25] In order to determine silica in tissues, King[26] fused the material with sodium carbonate in a platinum crucible—here, however, the quantity of material taken for the analysis was quite small. For determining cobalt in liver, Kidson, Askew and Dixon[27] found all methods of ashing with additions, whether of calcium acetate, calcium hydroxide, magnesium acetate or magnesium nitrate, to be unsatisfactory.

Often no attempt is made to burn all the carbon in a single operation. Tannahill[6] emphasises that tissues form a residue that must be repeatedly extracted before the entire char can be consumed, and that usually re-ashing is essential owing to fused inorganic salts preventing complete oxidation. Fairhall[2] and McHargue[28] extract the char with hydrochloric acid; Davies[16] extracts the ash of butter with water and re-ashes the residue; other workers complete the removal of carbon by treating the residue with nitric and sulphuric acids in a wet combustion procedure (Seiser, Necke and Müller[5]; Eveleth and Myers[22]; Richards[14]). Alternatively, re-ignition after moistening with nitric acid is sometimes used (Davies[16]; Tompsett and Anderson[29]; Glassmann and Barsutzkaja[30]). Essery[31] prefers to reduce the amount of sugars in wort by fermentation before ashing, followed by extraction of the residue with hydrochloric acid and re-ashing.

A method intermediate between dry and wet ashing is that of Wirthle[32]—digestion with sulphuric acid until a porous mass is formed, followed by the addition of soda and sodium nitrate and ashing.

Finally, mention should be made of the procedure of combustion in a closed vessel (bomb) in oxygen, used for the determination of arsenic by Bertrand,[33] by Remington, Coulson and Kolnitz,[34] and by Carey, Blodgett and Satterles.[35]

Among official methods for the determination of trace metals in biological products, those for gelatin in the British Pharmacopoeia, 1953,[36] and the United States Pharmacopoeia, 14th edition,[37] all recommend ashing at an unspecified temperature. The Dutch Pharmacopoeia, 1926,[38] recommends a sulphated ash method. The British Standard method[39] for gelatin is very indefinite—the material is incinerated in a platinum capsule "until a white ash is obtained" at a temperature that is specified as not high enough to cause loss of metals by volatilisation and not so low as to produce errors due to colouring of the resulting solutions with "carbon"; the alternative of wet ashing (with nitric and sulphuric acids) is permitted. For the determination of lead, the Association of Official Agricultural Chemists recommends ashing food products[40] at a temperature of 500° C with the addition of a proportion of aluminium and calcium nitrates; further portions of these salts or of nitric acid may be added if necessary. Wet combustion with nitric and sulphuric acids is recommended for a number of other elements.[41]

CRITICISMS AND OBJECTIONS—

Various objections have been raised to dry ashing. These are based on the possibility of losing volatile elements, the absorption or combination of trace metals with other ash constituents or with the material of the vessel used, the possibility of contamination from dust, and the difficulty in carrying out the procedure.

Many workers reject the method of dry ashing completely. For the determination of cadmium in tissues, Cholak and Hubbard[42] found that dry ashing was unsatisfactory whenever deflagration occurred and when samples containing more than 10 μg of cadmium were low in ash content. The addition of sodium phosphate resulted in a certain amount of improvement, but if too much was used the samples did not burn out completely and losses occurred when the ash was treated with nitric acid and re-ignited. Tompsett[43] obtained poor recoveries of copper and iron from diets when ashed in silica vessels, but more satisfactory ones from urine and faeces, and concluded that the addition of sodium phosphate was to be recommended in respect of diets. Cheftel and Pigeaud[44] rejected dry ashing for the determination of lead in sardines as "we know by our previous experience that it leads to losses."

With iron, the presence of chlorides gives rise to the danger of loss by volatilisation, as ferric chloride is appreciably volatile at 450° C (Monier-Williams[45]), whilst with iron in complex combination the recoveries are variable and low (Woiwood[46]); Davidson[47] found it necessary to remove silica with hydrofluoric acid before extracting iron from an ash.

Sulphuric acid may be used to eliminate chlorides before ashing, and such a procedure was used for the determination of iron in biological material by Woiwood,[46] but Weyrauch,[48] who also used a sulphated ash at 500° to 550° C, considered the method to be unsuitable for organs owing to the difficulty of finding suitable vessels—phosphates in the ash may attack glass, quartz takes up oxides, and platinum might alloy with trace metals. Reimann and Minot[49] also found the question of suitable vessels somewhat troublesome, since even when platinum is suitable, it is at times necessary to have a number of determinations in progress simultaneously. For determining manganese they used quartz beakers, specially selected for freedom from manganese, and after being ashed at 600° to 700° C, the partly burnt residue was treated with a mixture of sodium and potassium nitrates, sulphuric acid and hydrochloric acid, and then fused with the acid sulphates. Comrie,[50] who treated milk by a sulphated ash procedure, noted a loss of copper resulting from contact with silica.

In the process of dry ashing a good supply of air is necessary, and it is difficult to ensure the absence of dust containing, possibly, trace metals. Ansbacher, Remington and Culp[17] consider that the "dangers of loss and contamination in ashing in open dishes are not to be underrated." Woiwood[46] also considers it necessary to take precautions against aerial contamination.

COMMENTS AND CONCLUSIONS—

Although dry ashing—the most obvious method of destroying organic matter—would appear at first sight to be simple and reliable, in practice the simplicity is less apparent, because a first essential is the provision of special equipment in the form of a controlled-temperature muffle furnace with fume disposal.

The initial stage of carbonisation can be a troublesome process with animal tissues when large quantities are involved, as the outer layers harden and prevent the escape of steam and gases from the interior of the mass, and a voluminous froth results. It is better to dry the material first in an oven, but this is often very slow and does not completely eliminate the trouble. The next stage involves heating the dried mass in air at a temperature that is limited especially by the possibility of loss of trace elements by volatilisation and of formation of insoluble compounds such as silicates.

The progress of the combustion varies according to the nature of the original material—possibly in part because of variations in the content of salts and of phosphorus—and with some animal tissues it is extremely difficult to burn all the carbon. With vegetable products, *e.g.*, cocoa powder, the reverse is sometimes true—it is difficult to control the operation in such a way as to avoid incandescence.

With a char derived from animal tissues, ashing at a temperature not exceeding a first visible red (*i.e.*, about 480° C) is extremely slow, even in an electrically heated muffle furnace, and from the majority of writers quoted above it would appear that 500° to 550° C is required.

Even at this temperature the process is slow or, with some materials, extremely slow, so that it is necessary to facilitate the combustion by removal of ash constituents, moistening with nitric acid, or by some other procedure.

From this it can be seen that the application of dry ashing, especially to animal tissues, is often neither simple, rapid nor convenient, and that it does not necessarily lead to the recovery of the whole of the trace metals originally present.

Methods making use of chlorine oxy-acids (except perchloric acid)

Methods—

Probably the earliest record of a method for the determination of a trace element in animal tissues is that of Fresenius and v. Babo[51] for arsenic. They treated the organs with hydrochloric acid and potassium chlorate, filtering off and rejecting the fatty matter. Gusserow[52] adopted a similar procedure, Friedmann[53] modified the method by giving the sample a preliminary treatment for 24 hours with "antiformin" (a caustic alkaline hypochlorite solution) before adding hydrochloric acid and chlorate. The method of Kisskalt and Friedmann[54] is similar, and they showed also that the rejected fatty fraction generally contained only traces of lead. Schütz and Bernhardt,[55] Kuhn,[56] and Eckardt[57] all used similar procedures, while Bernhardt[58] used also euchlorine for decomposing bones. Harrold, Meek, Whitman and McCord,[59] determining indium in excreta and foods, boiled the material with nitric acid, then added concentrated hydrochloric acid and boiled to reduce the volume, and repeated this procedure twice more with more hydrochloric acid. Chloric acid was used for oxidation by Sonnenschein and Jeserich.[60]

The method of Fresenius and v. Babo is recommended by Bamford[61] as the best method for the detection of mercury in viscera and for the treatment of bones, although it is admitted that it has certain objections, namely, that it is long and tedious and that it involves the use of a number of reagents, some of which are difficult to obtain in a state of absolute purity.

Criticisms and objections—

These methods are troublesome and unsatisfactory, and with a number of elements the use of large quantities of chlorine compounds is undesirable on account of the danger of loss of trace elements by volatilisation. For example, Glassmann and Barsutzkaja[30] found that tin was lost by volatilisation as chloride by this procedure. Further, the method at most breaks down organic compounds; it does not generally destroy them completely, and when insoluble matter remains after the treatment, *e.g.*, with cellulosic materials, it has been shown that a large proportion of tin remains in this residue and can be recovered by fusion with alkali and nitrate (Manicka and Lauth[62]; Deussen[63]). A further disadvantage is that large quantities of salts are present in the final solution.

Direct oxidation with perchloric acid

Methods—

For the determination of tin in canned foods, Goss[64] boiled the material down with perchloric acid, or with a mixture of nitric and perchloric acids. Bolliger[65] also oxidised tissues by boiling with 60 per cent. perchloric acid and adding nitric acid drop by drop, followed by 30 per cent. hydrogen peroxide; the perchloric acid was removed by evaporation and any residue of ammonium perchlorate was destroyed by heat. This method was followed by Allcroft and Green[66] for the determination of arsenic in tissues, and they suggest that "the use of sulphuric acid in place of nitric acid . . . is equally serviceable . . . when the quantity of organic matter is sufficiently small for the analyst to view a possible conflagration . . . with equanimity." Hiscox[67] found the perchloric acid method to give the best recovery of cobalt from plant materials. Gerntz[68] also used nitric and perchloric acids, but without fuming off the excess of perchloric acid.

Criticism and objections—

In spite of possible advantages of this method, few workers have felt disposed to adopt it, no doubt because of a fear of explosions. Those named above apparently succeeded in avoiding such mishaps, and probably the method is safe if the conditions laid down are carefully adhered to, but a momentary negligence might easily lead to a disaster. Under

suitable conditions explosions resulting from perchloric acid and organic matter can be very violent.

Having previously experienced such an incident when using perchloric acid with sulphuric acid for oxidising organic matter, we have not felt inclined to follow up the method described above, especially when somewhat large amounts of organic matter are to be destroyed.

Wet combustion with sulphuric acid

Methods of wet combustion with sulphuric acid can be considered as based on the original Kjeldahl[69] procedure for the determination of nitrogen, in which potassium permanganate was used as oxidising agent. For the determination of trace metals, a non-metallic oxidant is naturally preferred, and generally nitric acid, perchloric acid, or a mixture of both, is used.

Methods—

The original procedure of Neumann for the determination of iron in proteins, urine and similar materials made use of a mixture of sulphuric acid and ammonium nitrate, but the procedure was soon simplified by replacing the latter by nitric acid.[70] Necke, Schmidt and Klostermann[71] used a mixture of sulphuric and fuming nitric acids for the destruction of organs and excreta, with the object of determining lead. Similar procedures were adopted by Schönheimer and Foshima,[72] Cox,[73] Lampitt and Sylvester,[74] Roche Lynch, Slater and Osler,[7] Hamence,[75] Klein and Wichmann,[76] Kidson, Askew and Dixon,[27] Wührer,[77] Coleman and Gilbert,[78] Adam and Horner,[79] Lunde, Aschehoug and Kringstad[80] and by Jones and Dawson.[81] Cribb and Still[82] merely digested canned peas with nitric and sulphuric acids and filtered off undissolved matter, completing the wet combustion on the filtrate.

Minot,[83] determining arsenic in tissues, digested them with nitric acid overnight, boiled the digest for 2 hours, then added a few millilitres of sulphuric acid and completed the operation as a normal wet combustion. Lakemann[84] used a mixture of sulphuric and fuming nitric acids for the determination of arsenic, but found it necessary to complete the decomposition by fusion with potassium and sodium nitrates. Gross[85] found that heterocyclic compounds remaining after the wet combustion of tobacco leaves with sulphuric and nitric acids interfered with the determination of arsenic, and that it was necessary to evaporate and ignite the digested solution.

A modified procedure was adopted by Lampitt and Rooke[86] for the determination of lead in canned sardines: the usual sulphuric - nitric acid treatment was continued until the liquid was straw-coloured, potassium sulphate was added and the heating was continued until the mixture was colourless; this took at least 4 hours.

Other workers have endeavoured to complete the sulphuric - nitric acid combustion by the addition of perchloric acid (Bertrand[87]; Kehoe, Thamann and Cholak[88]; Hubbard[89]; Morris and Calvery[90]; Cassil[91]; Aull and Kinard[92]; Cholak and Hubbard[42]; Meunier[93]; Feldstein and Klendshoj[94]). Kennedy[95] and Myers, Mull and Morrison[96] used sulphuric and perchloric acids only. McNaught[97] found that, after the digestion of animal tissues with nitric and sulphuric acids, it was necessary to fume off the sulphuric acid and to heat the residue for 5 minutes at 500° C in order to destroy residual organic matter.

Nitro-sulphuric acid combustion is specified in the British Standard method[39] for gelatin for the determination of arsenic, and as an alternative method for other metals.

Still others have dispensed with a special oxidising agent and relied on the oxidising action of sulphuric acid, the boiling point being raised by the addition of potassium sulphate. Such methods were used by de Giacomi[98] for the determination of tin in minced meats, the use of nitric acid being considered inadvisable owing to the danger of forming insoluble tin compounds, by Willard[99] for copper in oysters, by Schryver[100] for tin in canned meats, and by Chou and Adolph[101] for copper in urine and faeces. Catalysts as used for the determination of nitrogen have also been used, but are not favoured, as the action is slow and the most effective catalysts are metals or are liable to contain metallic impurities. Lawson and Scott[102] used sulphuric acid with potassium sulphate and copper sulphate for determining arsenic in body tissues. Hilger and Labande[103] used sulphuric acid with mercuric oxide when determining tin. In the method of Klein[104] for the determination of selenium, mercuric oxide is used with nitric and sulphuric acids, as it is claimed to "fix" the selenium and prevent its loss by volatilisation, but the method is not capable of destroying fats, which must be removed. This method has been adopted by the Association of Official Agricultural Chemists.[105]

Nitrosyl-sulphuric acid was used by Francis, Harvey and Buchan[106] to decompose the urea in urine as a preliminary to nitric acid treatment for the determination of lead. A reference to this reagent by Monier-Williams[107] suggests that this reagent has been used for the destruction of organic matter in general, but this does not appear to be so.

CRITICISMS AND OBJECTIONS—

Although carbon reduces sulphuric acid slowly at the boiling point, it is not practicable to oxidise animal matter with sulphuric acid alone, and even if a considerable quantity of sodium or potassium sulphate is added to raise the temperature, a number of heterocyclic compounds remain in the mixture undecomposed (Middleton and Stuckey[108]; Gross[85]), and the time required for the operation is extremely long when large quantities of organic matter are involved. The addition of catalysts such as mercury, selenium or copper is ineffective for dealing with large quantities of organic matter. In practice, therefore, sulphuric acid is nearly always used in conjunction with an oxidising agent, usually nitric acid. Although this procedure is generally convenient and satisfactory for vegetable products, it can be extremely troublesome and may even fail entirely when applied to animal tissues.

Details of the procedure as adopted vary: nitric acid may be allowed to drop slowly and continuously into the boiling sulphuric acid digest, or the digest may be allowed to cool before the addition of nitric acid. In the first variant of the procedure the nitric acid is not used to best effect, as most of it immediately passes into vapour.

Various comments have been made on the difficulty of carrying the digestion to a satisfactory conclusion (Kehoe, Thamann and Cholak[88]; Necke, Schmidt and Klostermann[71]; Gortner and Lewis[109]; Klein[104]; Woiwood[46]; Kidson, Askew and Dixon[27]; Remington, Coulson and v. Kolnitz[34]; Cary, Blodgett and Saterlee[35]). Discussing the determination of copper in foodstuffs, Monier-Williams says that "if the sample contained much fat the acid residue should be heated with ammonium oxalate,"[110] and "a slight yellow tinge . . . which is difficult to remove . . . appear to be caused by the action of nitric acid on fats with the production of substances which are extremely resistant. . . . Complete oxidation can usually be effected with perchloric acid. The yellow colour can sometimes be removed by heating with . . . ammonium oxalate."[111] In practice, perchloric acid treatment is ineffective in difficult cases, while the purpose of the addition of ammonium oxalate is to remove nitrogen acids from the digest (Minot[83]), but neither perchloric acid nor ammonium oxalate can be relied on to give a colourless digest.

In the determination of lead, precipitation of the lead as sulphate is one method that has been used for avoiding the difficulty caused by residual colour in the digest.[112]

In our own experience, in the most difficult cases no combination of sulphuric acid with nitric acid or perchloric acid or both is effective in producing a colourless digest, and nothing is gained by the addition of hydrogen peroxide, ammonium oxalate or other similar oxidants.

Peters[113] considered the Neumann process of wet combustion inferior to dry ashing, which saves time, ensures the destruction of oxidising agents and gives a neutral ash. Gross[85] and McNaught[114] complain of the inconvenience resulting from the necessity of evaporating large quantities of sulphuric acid, and Wirthle[32] also finds the large quantity of acid used in the so-called Kjeldahl procedure to be an unsatisfactory feature. With large quantities of different acids the blank may be appreciably higher than the amount of trace metal to be determined, and it is then necessary to distil the acids specially before use (Meunier[93]; Roche Lynch, Slater and Osler[7]; Francis, Harvey and Buchan[106]; Lunde, Ashehoug and Kringstad[80]). This is a simple procedure with nitric acid, but inconvenient and undesirable with sulphuric acid or perchloric acid.

USE OF NITRIC ACID ALONE

The earliest use of nitric acid alone for the oxidation or destruction of organic matter is in the well-known method of Carius[115] for the determination of sulphur, phosphorus, arsenic, and so on, in organic compounds. Temperatures of up to 320° C were used, with nitric acid of various concentrations up to that of the fuming acid, in a sealed tube. Although it is not clear whether Carius in fact applied his method to tissues, he suggests that it would be suitable for proteins, and also that it might be used for the determination of metals in organic combination.

Much later, Fontes and Thivolle[116] developed a procedure for the nitric acid combustion of tissues in which the use of sulphuric acid was avoided. The material (up to 50 g) was treated in a Pyrex-glass Kjeldahl flask with its own weight of nitric acid and 1 or 2 ml of 10-volume hydrogen peroxide—the latter to oxidise nitrous vapours and to reduce frothing. After boiling the mixture for a short time to disperse the tissue, 1 g of magnesium oxide was added. The flask was then heated in a bath of fused metal at 300° C. When the excess of acid had been evaporated, the remaining porous mass charred and ignited. The residue was re-evaporated with a few millilitres of nitric acid, leaving a dry white ash readily soluble in acids.

A procedure for the destruction of organs with pure nitric acid was recommended by Orfila, quoted by Danckwortt and Ude.[117] They digest organs in nitric acid for some hours (with fatty organs the action is so vigorous that the flask must be cooled). Fat is removed and the liquid is evaporated to a dark syrupy mass, which is ashed in small portions over a small flame. The lead is extracted from the still carbonaceous residue with nitric acid, as re-ashing after moistening with nitric acid is stated to give rise to loss of lead by combination with silica.

In a development of this method Danckwortt and Jurgens[118] digest bones for 14 days with nitric acid, and then heat in an acid-resistant cast-iron vessel. After the material has reached a syrupy stage there is a sudden "self-ignition" and a white ash is left. This operation was apparently carried out on successive small portions of the liquid. The authors claim to obtain a white ash without using a high temperature.

The method of Cholak[119] for bismuth in organs is somewhat similar, but the "self-ignition" is omitted. The samples are digested with nitric acid and the solution is evaporated and ashed in a muffle furnace at 450° to 500° C, the ash being moistened with nitric acid and re-heated. Because of difficulties due to the presence of phosphates, samples such as liver and brain were wet-ashed with sulphuric, nitric and perchloric acids.

Kludt[120] digested urine with nitric acid, evaporated the solution and heated the residue carefully. The mass showed localised incandescent spots (sparks), which should not spread through the mass. Faeces, treated similarly, gave a white ash without becoming incandescent. Vendevelde[121] used a similar procedure for gingerbread, and McCance and Widdowson[122] for faeces, urine and foodstuffs.

Milton, Hoskins and Jackman[123] used ammonium nitrate with nitric acid when determining alkalies and alkaline earths (and sulphur) in diets and food products. About 1 g of dry material, dissolved in nitric acid, was treated in a 250-ml Kjeldahl flask with 10 ml of a 50 per cent. solution of ammonium nitrate in 25 per cent. nitric acid and heated. The procedure was repeated with more reagent as necessary.

CRITICISM AND OBJECTIONS—

The method of Carius[115] is, of course, only suitable for small quantities of material, and even then is objectionable on account of the high pressures produced in the sealed tube. The methods both of Fontes and Thivolle[116] and of Danckwortt *et al.*[117,118] are effective, but involve a stage in which the blackened residue from the evaporation of the nitric acid solution suddenly ignites and burns in the nitric acid vapour (assisted in the former method by the presence of magnesium nitrate). The temperature attained locally for a short period is obviously quite high, and it is to be expected that such a vigorous reaction might lead to loss of trace metals. It is, however, evident that this incandescence does not occur under all conditions, at least with some materials.

Although these methods have the advantage of being simple and rapid, and give a soluble ash free from added reagents, they have not met with general acceptance.

COMPARATIVE TESTS

There are but few reports of comparative investigations of the suitability of various methods for a particular purpose. Allcroft and Green[66] compared different methods of destruction of tissues for the determination of arsenic, and obtained the following recoveries: Kjeldahl digestion with potassium sulphate and copper, 20 to 63 per cent.; nitric and sulphuric acids, 57 to 81 per cent.; potassium chlorate and hydrochloric acid, 0 to 49 per cent.; perchloric and nitric acids, 63 to 97 per cent.; ashing with magnesium nitrate, 47 to 90 per cent.

Hiscox[67] compared six methods for the destruction of plant materials, with the object of determining cobalt: (1) by using nitric acid and ashing by method of Kidson and Askew;

(2) using nitric and sulphuric acids; (3) by dry ashing; (4) by sulphuric acid digestion; (5) using perchloric and nitric acids; (6) by nitric, perchloric and sulphuric acid digestion, then taking to dryness. They found the best recoveries of cobalt by method (5) or by dry ashing.

Jackson[124] tried four methods of destruction for the determination of iron in biological material: (1) by dry ashing with the addition of sodium carbonate; (2) by sulphated ashing; (3) by dry ashing after covering with calcium carbonate; (4) by wet ashing with nitric, sulphuric and perchloric acids. The test solution contained glucose, urea, sodium chloride and calcium phosphate. Method (1) was found to be unsatisfactory; method (2) gave a recovery of 40 to 54 per cent.; method (3) of 80 to 87 per cent.; and method (4) of 100 per cent. They concluded that wet ashing was demonstrated to be the only acceptable method of those tested.

Manicka and Lauth[62] compared the recovery of tin by various methods with the following recoveries: potassium chlorate and hydrochloric acid, 75 per cent.; potassium chlorite and hydrochloric acid with hydrogen peroxide, 89 per cent.; potassium chlorate and hydrochloric acid, followed by sodium hydroxide and potassium nitrate, 95 per cent.; Wirthle method, 101 per cent.; Orfila method, 96 per cent.; sulphuric acid - nitric acid, 98 per cent.; sulphuric acid and hydrogen peroxide, 101 per cent.; sulphuric acid, hydrogen peroxide and nitric acid, 101 per cent.

Conclusions drawn from the results quoted above can only be valid in their own limited sphere of application. The success of the subsequent treatment of the product after destruction of the organic matter may depend on this preliminary treatment, and on the nature of the solution obtained. It is also necessary to take into account the chemical nature of the original material—whether of a protein, carbohydrate or fatty nature.

References

1. "Official Methods of Analysis of the Association of Official Agricultural Chemists," Seventh Edition, Washington, D.C., 1950, Chap. 24, § 57.
2. Fairhall, L. T., *J. Ind. Hyg.*, 1922/3, **4**, 9.
3. Schmidt, P., *Dtsch. Med. Wschr.*, 1928, **54**, 520.
4. Kehoe, R. A., Edgar, G., Thamann, F., and Sanders, L., *J. Amer. Med. Ass.*, 1926, **87**, 2081.
5. Seiser, A., Necke, A., and Müller, H., *Z. angew. Chem.*, 1929, **42**, 96.
6. Tannahill, R. W., *Med. J. Aust.*, 1929, **16**, (1), 194.
7. Roche Lynch, G., Slater, R. H., and Osler, T. G., *Analyst*, 1934, **59**, 787.
8. Weyrauch, F., and Müller, H., *Z. Hyg. InfektKr.*, 1933, **115**, 216.
9. Eggleton, W. G. E., *Biochem. J.*, 1939, **33**, 403; 1940, **34**, 991.
10. Rauschning, S., *Z. Untersuch. Lebensmitt.*, 1942, **84**, 30.
11. Heller, V. G., and Burke, A. D., *J. Biol. Chem.*, 1927, **75**, 85.
12. Sylvester, N. D., and Hughes, E. B., *Analyst*, 1936, **61**, 734.
13. Allport, N. L., and Moon, C. D. B., *Ibid.*, 1939, **64**, 395.
14. Richards, M. B., *Ibid.*, 1930, **55**, 554.
15. Forbes, E. B., and Swift, R. W., *J. Biol. Chem.*, 1926, **67**, 516.
16. Davies, W. L., *J. Dairy Res.*, 1932, **3**, 86.
17. Ansbacher, S., Remington, R. E., and Culp, F. B., *Ind. Eng. Chem., Anal. Ed.*, 1931, **3**, 314.
18. McFarlane, W. D., *Biochem. J.*, 1932, **26**, 1022.
19. Bertrand, G., and Macheboeuf, M., *Bull. Soc. Chim. France*, 1925, **37**, 934.
20. Warburg, A., *Biochem. Z.*, 1927, **187**, 255.
21. Sylvester, N. D., and Lampitt, L. H., *Analyst*, 1935, **60**, 376.
22. Eveleth, D. F., and Myers, V. C., *J. Biol. Chem.*, 1936, **113**, 449.
23. Misk, E., *Compt. Rend. Acad. Sci., Paris*, 1923, **176**, 138.
24. Bamford, F., *Analyst*, 1934, **59**, 101.
25. Strzyowski, C., *Pharm. Post*, 1906, **39**, 977.
26. King, E. J., *Biochem. J.*, 1939, **33**, 944.
27. Kidson, E. B., Askew, H. O., and Dixon, J. K., *N.Z. J. Sci. Tech.*, 1936/7, **18**, 601.
28. McHargue, J. S., *J. Agric. Res.*, 1925, **30**, 193.
29. Tompsett, S. L., and Anderson, A. B., *Biochem. J.*, 1935, **29**, 1851.
30. Glassmann, R., and Barsutzkaja, S., *Z. Untersuch. Lebensmitt.*, 1928, **56**, 208.
31. Essery, R. E., *J. Inst. Brew.*, 1945, **51**, 185.
32. Wirthle, F., *Chem. Ztg.*, 1900, **24**, 263.
33. Bertrand, G., *Compt. Rend. Acad. Sci., Paris*, 1903, **137**, 266.
34. Remington, R. E., Coulson, E. J., and v. Kolnitz, H., *Ind. Eng. Chem., Anal. Ed.*, 1934, **6**, 280.
35. Carey, F. P., Blodgett, G., and Satterlee, H. S., *Ibid.*, 1934, **6**, 327.
36. British Pharmacopoeia, 1953, p. 242.
37. United States Pharmacopoeia, Fourteenth Revision, 1950, p. 257.
38. Nederlandsche Pharmacopoeia, Fifth Edition, 1926, p. 220.
39. British Standard Specification No. 757:1944.

40. "Official Methods of Analysis of the Association of Official Agricultural Chemists," Seventh Edition, Washington, D.C., 1950, Chap. 24, § 57.
41. *Ibid.*, Chap. 24, § 3.
42. Cholak, J., and Hubbard, D. M., *Ind. Eng. Chem., Anal. Ed.*, 1944, **16**, 333.
43. Tompsett, S. L., *Biochem. J.*, 1934, **28**, 2088.
44. Cheftel, H., and Pigeaud, M. L., *Ann. Falsif.*, 1936, **29**, 76.
45. Monier-Williams, G. W., "Trace Elements in Food," Chapman & Hall Ltd., London, 1949, p. 257.
46. Woiwood, A. J., *Biochem. J.*, 1947, **41**, 39.
47. Davidson, J., *J. Ass. Off. Agric. Chem.*, 1931, **14**, 551.
48. Weyrauch, F., *Z. Hyg. InfektKr.*, 1930, **111**, 162.
49. Reimann, K., and Minot, A. S., *J. Biol. Chem.*, 1920, **42**, 329.
50. Comrie, A. A. D., *Analyst*, 1935, **60**, 532.
51. Fresenius, R., and v. Babo, L., *Ann. Chim. Pharm.*, 1844, **49**, 287.
52. Gusserow, *Virchow's Archiv.*, 1861, **21**, 443.
53. Friedmann, A., *Hoppe-Seyl. Z.*, 1914, **92**, 46.
54. Kisskalt, K., and Friedmann, A., *Z. Hyg. InfektKr.*, 1914, **78**, 500.
55. Schütz, F., and Bernhardt, H., *Ibid.*, 1925, **104**, 441.
56. Kuhn, B., *Z. anal. Chem.*, 1907, **46**, 62.
57. Eckardt, A., *Z. Untersuch. Nahr. -u. Genussm.*, 1909, **18**, 193.
58. Bernhardt, H., *Z. anal. Chem.*, 1925/6, **67**, 97.
59. Harrold, G. C., Meek, S. F., Whitman, N., and McCord, C. P., *J. Ind. Hyg.*, 1943, **25**, 233.
60. *Quoted in* Kobert, R., "*Kompendium der praktischen Toxicologie*," Verlag von Ferdinand Enke, Stuttgart, 1912.
61. Bamford, F., "Poisons: Their Isolation and Identification," Second Edition, J. & A. Churchill Ltd., London, 1947.
62. Manicke, P., and Lauth, H., *Pharm. Zentralh.*, 1927, **68**, 161.
63. Deussen, E., *Arch. Pharm.*, 1926, **264**, 360.
64. Goss, R. C., *J. Ind. Eng. Chem.*, 1917, **9**, 144.
65. Bolliger, A., *Aust. J. Exp. Biol. Med. Sci.*, 1932, **10**, 57.
66. Allcroft, R., and Green, H. H., *Biochem. J.*, 1935, **29**, 824.
67. Hiscox, D. J., *Sci. Agric.*, 1947, **27**, 136.
68. Gerntz, H. W., *Ind. Eng. Chem., Anal. Ed.*, 1935, **7**, 167.
69. Kjeldahl, J., *Z. anal. Chem.*, 1883, **22**, 366.
70. Neumann, A., *Hoppe-Seyl. Z.*, 1902/3, **37**, 115; 1904/5, **43**, 32.
71. Necke, A., Schmidt, P., and Klostermann, M., *Dtsch. med. Wschr.*, 1926, **52**, 1855.
72. Schönheimer, R., and Oshima, F., *Hoppe-Seyl. Z.*, 1929, **180**, 249.
73. Cox, H. E., *Analyst*, 1925, **50**, 3.
74. Lampitt, L. H., and Sylvester, N. D., *Ibid.*, 1932, **57**, 418.
75. Hamence, J. H., *Ibid.*, 1934, **59**, 274.
76. Klein, A. K., and Wichmann, H. J., *J. Ass. Off. Agric. Chem.*, 1945, **28**, 257.
77. Wührer, J., *Z. Untersuch. Lebensmitt.*, 1938, **76**, 338.
78. Coleman, D. R. K., and Gilbert, F. C., *Analyst*, 1939, **64**, 726.
79. Adam, W. B., and Horner, G., *J. Soc. Chem. Ind.*, 1937, **56**, 329T.
80. Lunde, G., Aschehoug, V., and Kringstad, H., *Ibid.*, 1937, **56**, 334T.
81. Jones, C. R., and Dawson, E. C., *Analyst*, 1945, **70**, 256.
82. Cribb, C. H., and Still, A. L., *Ibid.*, 1925, **50**, 286.
83. Minot, A. S., *J. Cancer Res.*, 1926, **10**, 293.
84. Lakemann, G., *Biochem. J.*, 1911, **35**, 478.
85. Gross, C. R., *Ind. Eng. Chem., Anal. Ed.*, 1933, **5**, 58.
86. Lampitt, L. H., and Rooke, H. S., *Analyst*, 1933, **58**, 733.
87. Bertrand, D., *Compt. Rend. Acad. Sci., Paris*, 1942, **215**, 590.
88. Kehoe, R. A., Thamann, F., and Cholak, J., *J. Ind. Hyg.*, 1933, **15**, 257.
89. Hubbard, D. M., *Ind. Eng. Chem., Anal. Ed.*, 1941, **13**, 915.
90. Morris, H. J., and Calvery, H. O., *Ind. Eng. Chem., Anal. Ed.*, 1937, **9**, 447.
91. Cassil, C. C., *J. Ass. Off. Agric. Chem.*, 1940, **23**, 297.
92. Aull, J. C., and Kinard, F. W., *J. Biol. Chem.*, 1940, **135**, 119.
93. Meunier, P., *Compt. Rend. Acad. Sci.*, Paris, 1936, **203**, 891.
94. Feldstein, M., and Klendshoj, N. C., *Analyst*, 1953, **78**, 43.
95. Kennedy, R. P., *J. Biol. Chem.*, 1927, **74**, 385.
96. Myers, V. C., Mull, J. W., and Morrison, D. B., *Ibid.*, 1928, **78**, 595.
97. McNaught, J. J., *Analyst*, 1939, **64**, 23.
98. de Giacomi, R., *Ibid.*, 1940, **65**, 216.
99. Willard, J. T., *J. Amer. Chem. Soc.*, 1908, **30**, 902.
100. Schryver, S. B., *J. Hyg.*, 1909, **9**, 253.
101. Chou, T. P., and Adolph, W. H., *Biochem. J.*, 1935, **29**, 470.
102. Lawson, W. E., and Scott, W. O., *J. Biol. Chem.*, 1925, **64**, 23.
103. Hilger, A., and Labande, L., *Z. Untersuch. Nahr. -u. Genussm.*, 1899, **2**, 795.
104. Klein, A. K., *J. Ass. Off. Agric. Chem.*, 1941, **24**, 363.
105. "Official Methods of Analysis of the Association of Official Agricultural Chemists," Seventh Edition, Washington, D.C., 1950, Chap. 24, § 84.
106. Francis, A. G., Harvey, G. O., and Buchan, J. L., *Analyst*, 1929, **54**, 725.
107. Monier-Williams, G. W., *op. cit.*, p. 84.
108. Middleton, G., and Stuckey, R. E., *J. Pharm. Pharmacol.*, 1951, **3**, 829.
109. Gortner, R. A., and Lewis, H. B., *Ind. Eng. Chem., Anal. Ed.*, 1939, **11**, 198.

110. Monier-Williams, G. W., *op. cit.*, p. 51.
111. ——, *op. cit.*, p. 85.
112. ——, *op. cit.*, p. 90.
113. Peters, V. R. A., *J. Physiol.*, 1912, **44**, 131.
114. McNaught, K. J., *N.Z. J. Sci. Tech.*, 1938, **20**, 14A.
115. Carius, L., *Annalen*, 1860, **116**, 1; 1865, **136**, 129; *Ber.*, 1870, **3**, 697.
116. Fontes, G., and Thivolle, L., *Bull. Soc. Chim. Biol.*, 1923, **5**, 782.
117. Danckwortt, P. W., and Ude, W., *Arch. Pharm.*, 1926, **264**, 712.
118. Danckwortt, P. W., and Jürgens, E., *Ibid.*, 1928, **266**, 492.
119. Cholak, J., *Ind. Eng. Chem., Anal. Ed.*, 1937, **9**, 26.
120. Kludt, B., *Hoppe-Seyl. Z.*, 1928, **174**, 28.
121. Vandervelde, A. J. J., *Z. Untersuch. Nahr. -u. Genussm.*, 1904, **7**, 676.
122. McCance, R. H., and Widdowson, E. M., *J. Physiol.*, 1942, **101**, 44.
123. Milton, R., Hoskins, J. L., and Jackman, W. H. F., *Analyst*, 1944, **69**, 299.
124. Jackson, S. H., *Ind. Eng. Chem., Anal. Ed.*, 1938, **10**, 302.

THE BRITISH DRUG HOUSES LIMITED
GRAHAM STREET, LONDON, N.1

April 27th, 1953

A Comparison of the Applicability to Plant Extracts of Three Methods of Determining Deoxyribonucleic Acid

BY MARGARET HOLDEN

The diphenylamine method (Dische, 1930) and a modification of the tryptophan - perchloric acid method of Cohen (1944) have been found to be satisfactory for determining deoxyribonucleic acid in extracts from plant tissue. The cysteine - sulphuric acid method (Stumpf, 1947) has given erratic results. A number of substances, some of which are likely to be present in plant extracts, have been tested under the conditions of the three methods.

METHODS for the quantitative determination of deoxyribonucleic acid (DNA) have been used almost exclusively on animal tissues, although recently the diphenylamine method of Dische[1] has been used by Ogur and Rosen[2] for DNA estimations on extracts from plant root tips and by McClendon[3] on fractions from tobacco leaves.

The diphenylamine method of Dische,[1] the cysteine - sulphuric acid method of Dische[4] and Stumpf[5] and the tryptophan - perchloric acid method of Cohen[6] have been investigated with a view to finding the most satisfactory one for determining DNA in extracts from plant tissues, with particular reference to the possible interference of pectic substances. Fructose and fructose derivatives, ascorbic acid and glyceraldehyde were found by Cohen[6] to interfere in both the diphenylamine and tryptophan - perchloric acid methods. Fructose and its derivatives also interfered slightly in the cysteine - sulphuric acid method.[5]

MATERIAL AND METHODS

Leaves from glass-house grown tobacco plants (*Nicotiana tabacum*, variety White Burley) were used for most of the work. The leaves were minced in a domestic meat mincer and the sap squeezed out by hand through strong cotton cloth. The fibre, *i.e.*, the residue in the cloth, which contains most of the DNA, was washed several times by suspending it in distilled water and squeezing. The washed fibre was stored at 4° C with chloroform added as a disinfectant.

PREPARATION OF EXTRACTS CONTAINING DEOXYRIBONUCLEIC ACID—

Fibre was first extracted with 0·2 *N* perchloric acid and then with a (3 + 1) mixture of ethanol and ether to remove lipids. Ribonucleic acid (RNA) was removed by soaking the fibre in *N* perchloric acid at room temperature overnight (Holden[7]). The DNA was extracted by incubating the fibre in *N* perchloric acid at 37° C overnight. Extracts made in this way were usually almost colourless, but sometimes yellow or light brown.

Total carbohydrate was determined in the extracts by the orcin method of Pirie.[8]

Reducing sugar was determined by the Hanes[9] modification of the Hagedorn and Jensen method. An equal volume of *N* sodium hydroxide solution was added to the sample to neutralise the *N* perchloric acid.

Uronic acid was determined by Tracey's method.[10] The *N* perchloric acid extracts were neutralised with potassium hydroxide solution and after leaving them in a refrigerator overnight the precipitated potassium perchlorate was removed by centrifugation before drying the samples in bulb tubes.

Nitrogen was determined by a micro-Kjeldahl method. Much of the perchloric acid was removed as potassium perchlorate, as large amounts of perchloric acid cause loss of nitrogen during incineration (Weeks and Friminger[11]).

Absorption spectra in the visible region were measured with a Unicam diffraction-grating spectrophotometer.

Diphenylamine method

The standard conditions used for the test were as follows. Three millilitres of solution containing DNA, or 3 ml of water for the blank, and 6 ml of Dische reagent (1 per cent. diphenylamine in a glacial acetic acid solution containing 2·75 ml of concentrated sulphuric

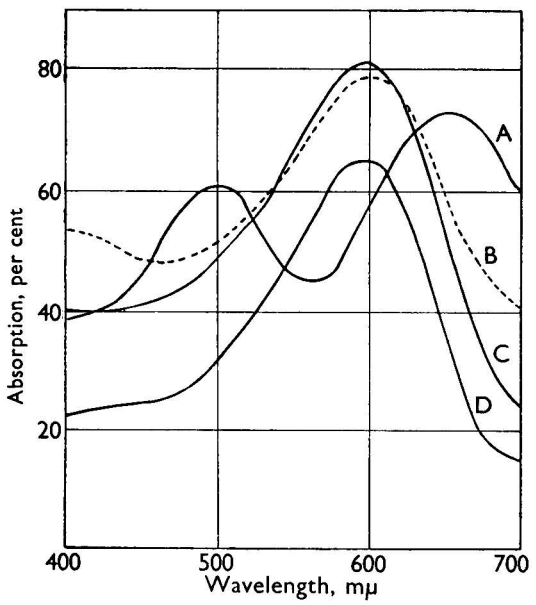

Fig. 1. Comparison of absorption curves of colours given by deoxyribose, DNA, a fibre extract and galacturonic acid with the diphenylamine reagent.
Curve A, 10 mg of galacturonic acid; curve B, fibre extract; curve C, 2 mg of DNA; curve D, 200 μg of deoxyribose

acid per 100 ml) were heated in glass-stoppered tubes in a bath of boiling water for 10 minutes. After cooling them in ice-water the solutions were transferred to matched tubes for measurement in an Evans Electroselenium photo-electric absorptiometer. The intensity of the blue colour given by deoxyribose and DNA was measured with the aid of Ilford No. 607 (maximum transmission 600 mμ) or No. 626 (maximum transmission 570 mμ) filters, the reagent blank being used for setting the zero reading of the instrument.

The diphenylamine solution was made up from the AnalaR grade of reagent (supplied by Hopkin and Williams Ltd.), which gave a colourless solution, without recrystallisation, when dissolved in AnalaR grade of glacial acetic acid. Some samples of acetic acid contained a contaminant that gave coloured blanks and an "off-colour" blue with DNA when the liquor from a bottle containing partly crystallised acid was used. Interference could be avoided by using only the frozen portion.

2-Deoxy-L-ribose (from Prof. M. Stacey) was used for deoxyribose standards. A dialysed solution of the sodium salt of thymus nucleic acid (supplied by British Drug Houses Ltd.)

was used for DNA standards. There was a linear relationship between the intensity of colour and the amount of deoxyribose with standards containing from 15 to 130 μg and with DNA standards containing from 50 to 600 μg (3·5 to 42 μg of phosphorus). Solutions of wheat germ DNA (from Prof. M. Stacey) gave the same intensity of colour as thymus DNA solutions of the same phosphorus content. It is well known that purine-bound, but not pyrimidine-bound, deoxyribose gives a blue colour with the diphenylamine reagent. Hypoxanthine deoxyribonucleoside (from Prof. A. R. Todd) gave the theoretical amount of deoxyribose under the standard conditions of the test, whilst thymidine gave only a faint blue-green colour.

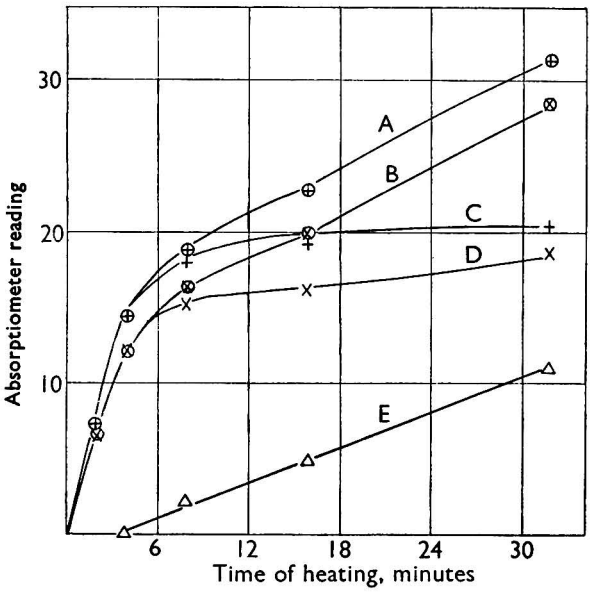

Fig. 2. Effect of time of heating with the diphenylamine reagent on the intensity of colour given by DNA, galacturonic acid, a fibre extract, DNA + galacturonic acid and fibre extract + galacturonic acid.
Curve A, 500 μg of DNA + 1 mg of galacturonic acid; curve B, 1 ml of fibre extract + 1 mg of galacturonic acid; curve C, 500 μg of DNA; curve D, 1 ml of fibre extract; curve E, 1 mg of galacturonic acid

The absorption curves in the visible region for the colours given by deoxyribose, DNA and a fibre extract, when heated with the diphenylamine reagent, are shown in Fig. 1. This shows that the absorption maxima are at 600 mμ, but that the fibre extract absorbs more strongly in the 400 to 450-mμ region. Ogur and Rosen[2] also found the maximum absorption to be at 600 mμ for DNA and plant tissue extracts. Deriaz, Stacey, Teece and Wiggins[12] stated that 580 mμ was the wavelength of maximum absorption for 2-deoxypentoses when heated with diphenylamine. The difference may be due to the different type of instrument used.

OTHER SUBSTANCES GIVING COLOURS WITH THE DIPHENYLAMINE REAGENT—

Galacturonic acid—Deriaz *et al.*[12] tested the method for interference with a large number of substances, but did not include galacturonic acid. As pectin or its breakdown products are likely to be present in extracts from most plant tissues, pectin and galacturonic acid were tested under the standard conditions of the method. Pectin gave a gelatinous precipitate and a faint green colour when heated with diphenylamine. Galacturonic acid gave a blue colour different from that with DNA. The absorption curve shown in Fig. 1 indicates that there are maxima at 500 and 650 mμ.

One to two millilitres of a fibre extract made with N perchloric acid was required for a DNA determination. The diphenylamine reagent was approximately 0·5 N with respect to sulphuric acid, so that the perchloric acid added significantly to the amount of strong acid present. The colour given by galacturonic acid was found to be decreased by decreasing

the concentration of strong acid. The diphenylamine was therefore dissolved in glacial acetic acid containing no added sulphuric acid. Without sulphuric acid diphenylamine solutions become coloured more quickly than did the usual reagent; a fresh solution had to be used for each batch. If a sample of less than 2 ml of a *N* perchloric acid extract was to be used for the test, *N* perchloric acid was added to ensure the presence of a total of 2 ml of it, and 2 ml of acid was added to the standards.

The development of the colours given by DNA, galacturonic acid and DNA plus galacturonic acid after different periods of heating with the diphenylamine reagent was investigated. The results with a No. 626 filter are shown in Fig. 2. The colour given by DNA developed quickly and reached a stable value, whereas the colour given by galacturonic acid did not develop at once, but developed slowly and was still increasing when the experiment was stopped after heating for 32 minutes. In other experiments with greater periods of heating

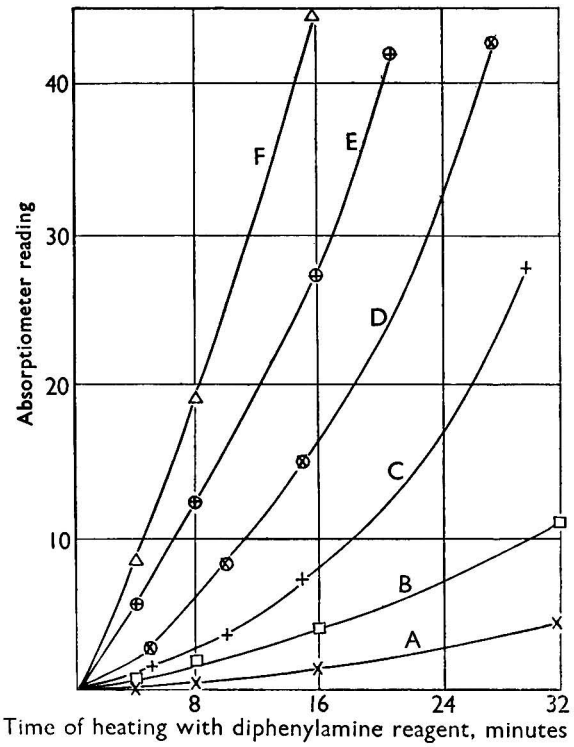

Fig. 3. Effect of degradation of pectin on intensity of colour with the diphenylamine reagent.

Curve A, pectin solution, 4·6 mg per ml in *N* perchloric acid: 0·58 mg of reducing sugar; curve B, pectin after heating for 15 minutes: 0·93 mg of reducing sugar; curve C, after heating for 30 minutes: 1·46 mg of reducing sugar; curve D, after heating for 1 hour: 2·33 mg of reducing sugar; curve E, after heating for 2·3 hours: 3·40 mg of reducing sugar; curve F, after heating for 4 hours: 4·00 mg of reducing sugar

the colour continued to develop, but the colour in the blanks also increased. On heating for only 4 minutes galacturonic acid caused no interference. Deriaz *et al.*[12] heated for only 3·25 minutes, but as the colour given by DNA has not reached its maximum in this time it is advisable to continue heating until a stable value is reached. Filter No. 607 has maximum transmission at the peak of the DNA curve, *i.e.*, at 600 mμ, but filter No. 626 with maximum transmission at 570 mμ gave lower values for galacturonic acid and was therefore used to diminish possible interference by galacturonic acid in the determination of DNA on fibre extracts.

Fig. 2 also shows the results of heating 1-ml samples of a fibre extract with the diphenylamine reagent for different times. With up to 16 minutes of heating the curve for the fibre extract ran parallel to the DNA curve, but for longer periods it gave more colour. Addition

of galacturonic acid caused marked divergence of the curves. The extract used in this experiment had a carbohydrate content of 2·6 mg per ml of which 0·8 mg per ml was reducing sugar and 1·4 mg per ml was uronic acid. Fibre extracts with higher carbohydrate contents showed greater divergence from the DNA curve, but this only occurred after heating for 8 minutes. A few extracts when heated with the diphenylamine reagent gave a gelatinous precipitate of pectic material that had to be removed before the absorptiometer reading was taken. The precipitate did not adsorb colour from the solution.

The increase in colour with the diphenylamine reagent when pectin is degraded is shown in Fig. 3. Citrus pectin (by British Drug Houses Ltd.) was extracted with hot 70 per cent. ethanol to remove reducing substances and its uronic acid content was found to be 75 per cent. of the dry matter. The pectin was dissolved by heating to boiling in *N* perchloric acid to give a solution containing 4·6 mg per ml. One millilitre of this solution was used for the initial graph of time of heating with diphenylamine. Its reducing sugar content expressed as galacturonic acid was determined and this was determined again after heating with perchloric acid for periods ranging from 15 minutes to 4 hours. The heating with acid was done in glass-stoppered tubes in a bath of boiling water. During the heating with acid, a white precipitate appeared in the solution and this was included in samples taken for heating with diphenylamine. The precipitate disappeared during the heating with diphenylamine in the samples that had previously been heated with perchloric acid for 1 hour or more. With samples that had been heated for less than 1 hour in perchloric acid a precipitate was still present after heating with diphenylamine; this was removed before the optical density was measured.

Yeast ribonucleic acid—This gave a faint green colour when heated with diphenylamine, but 5 mg caused an increase of less than 5 per cent. in the absorptiometer reading given by 200 μg of DNA.

Carbohydrates—Five milligrams of arabinose gave a faint green with diphenylamine, and 5 mg of starch and glucose gave no detectable colour. Pirie[8] found that agar, caragheen polysaccharides and aldehydo-sugars gave green colours with the diphenylamine reagent Two-milligram lots of agar, hepta-acetyl mannose, hepta-acetyl glucose, hepta-acetyl galactose, hexa-acetyl arabinose and hexa-acetyl rhamnose (all prepared by N. W. Pirie) were tested. Agar gave a bright green colour; the acetylated sugars gave only a faint green. Half of a milligram of 3:6-anhydro-α-methyl-D-galactoside (from Dr. D. J. Bell) gave a strong green colour and an absorption curve identical with that of 3 mg of agar. Digitoxose (by Hofmann la Roche), which is a 2:6-dideoxyhexose, or methyl deoxypentose gave a yellow-brown colour of low intensity.

Nitrogenous substances—Davidson[13] stated that most colorimetric reactions for determining sugars were prone to interference from proteins, and this has been confirmed for the diphenylamine method. Overend[14] found that amino-acids and purine and pyrimidine bases increased the intensity of the colour given by DNA in this method, but this interference of amino-acids was not confirmed.* Fibre extracts made with *N* perchloric acid at 37° C (40 ml of acid were added to fibre containing about 1 g of dry matter) have a nitrogen content of less than 0·1 mg per ml so that little interference is likely to be caused.

CYSTEINE - SULPHURIC ACID METHOD

The standard conditions of the method were as follows. One millilitre of sample containing DNA, or 1 ml of water for the blank, 0·1 ml of a solution of 5 per cent. w/v cysteine hydrochloride (by Roche Products Ltd.) and 10 ml of 70 per cent. w/w sulphuric acid were mixed and set aside at room temperature for 30 minutes for the pink colour to develop fully. The colour intensity was read with an Ilford No. 623 filter (maximum transmission at 495 mμ). There was a linear relationship between the amount of DNA and colour intensity for amounts of DNA between 150 and 800 μg. For less than 150 μg the relationship deviated from linearity, as found by Stumpf.[5]

2-Deoxy-L-ribose gave a more orange colour than DNA and the absorption curve (Fig. 4) showed strong absorption in the 400 to 430-mμ region. With deoxyribose as standard, hypoxanthine deoxyriboside under the standard conditions of the method gave the theoretical amount of deoxyribose, whilst thymidine gave about 70 per cent. of the theoretical value.

* Dr. W. G. Overend has informed me that he has since found that amino-acids did not increase the absorption due to DNA when other samples of diphenylamine were used.

Manson and Lampen[15] tested both deoxyribonucleosides and deoxyribonucleotides by this method. They found that the deoxyribosides of guanine and hypoxanthine and thymidine gave the same amount of colour per micromole of 2-deoxy-D-ribose, but that with cytosine deoxyriboside the colour developed more slowly and was considerably less. They also found that adenine and guanine deoxyribonucleotides gave approximately the same colour intensity as an equimolar amount of deoxyribose, the thymine derivative gave 74 per cent. of the theoretical value and cytosine deoxyribonucleotide gave no colour.

Most fibre extracts gave a colour different from that with DNA. For some extracts the difference was not great, but with others the colour was orange instead of purple-pink. This was partly because some extracts gave a pale orange-brown colour on addition of 70 per cent. sulphuric acid with no cysteine present. Extracts from cabbage leaf fibre gave a bright yellow at once on addition of sulphuric acid. The rate of colour development in a fibre extract was similar to that of DNA. Fig. 4 shows the absorption curves of the colours given

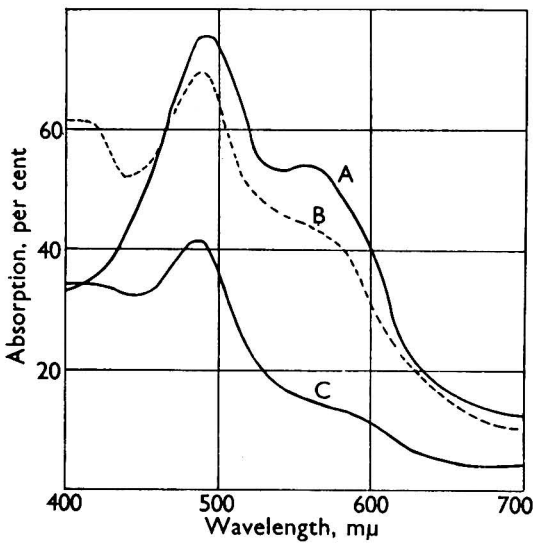

Fig. 4. Comparison of absorption curves of colours given by deoxyribose, DNA and a fibre extract with cysteine hydrochloride and sulphuric acid.

Curve A, 1 mg of DNA; curve B, 240 μg of deoxyribose; curve C, fibre extract

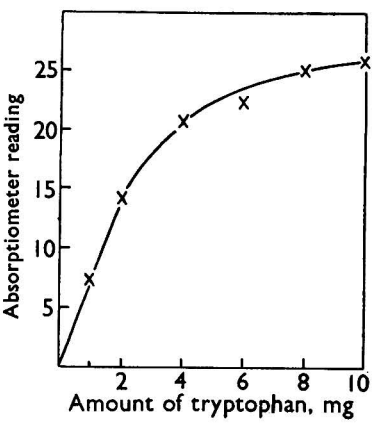

Fig. 5. Dependence of colour intensity given by DNA on the amount of tryptophan used in the Cohen method.

500-μg lots of DNA, 1 ml of 60 per cent. perchloric acid, 1 per cent. tryptophan solution and water to give 3 ml. Heated 20 minutes in bath of boiling water, cooled and then diluted with 6 ml of 20 per cent. perchloric acid

by a fibre extract, deoxyribose and DNA with cysteine and sulphuric acid. Stumpf's observation that the absorption maximum is at 490 mμ was confirmed, but there was also a shoulder at 560 mμ, which was not shown on his curve. This shoulder was also found in the deoxyribose curve. After several hours the colour of the solution became more purple and the shoulder at 560 mμ became more pronounced. The fibre extract had a maximum at 490 mμ, but there was also considerable absorption in the 400 to 450-mμ region.

INTERFERING SUBSTANCES—

Five milligrams of yeast RNA, starch, glucose, arabinose, pectin and galacturonic acid gave no colour with cysteine and sulphuric acid. Digitoxose, 0·5 mg, gave a strong brown colour. Agar gave no colour on standing for 30 minutes and a yellow-green after 18 hours. 3:6-Anhydro-α-methyl-D-galactoside gave a yellow after 30 minutes and a green after 18 hours.

The hepta-acetyl derivatives of glucose, mannose and galactose and the hexa-acetyl derivatives of rhamnose and arabinose gave no colour in 30 minutes. On standing for 18 hours the hexa-acetyl rhamnose was salmon-pink, but the other sugars were faintly straw coloured.

TRYPTOPHAN - PERCHLORIC ACID METHOD

The method as described by Cohen[6] was as follows. To 1 ml of solution containing DNA were added 0·2 ml of 1 per cent. tryptophan in 0·01 *N* sodium hydroxide solution and 1·2 ml of 60 per cent. perchloric acid, the mixture was heated at 100° C for 10 minutes and then rapidly cooled. To eliminate colours produced by substances other than DNA the pink colour caused by DNA was extracted with *iso*amyl alcohol, and the solution was used for absorptiometric determination. Some modifications were found necessary. A 1 per cent. w/v solution of DL-tryptophan could not be made at room temperature with 0·01 *N* sodium hydroxide, so a 0·1 *N* solution was used. With a final concentration of 30 per cent. of perchloric acid in the reaction mixture, DNA gave an orange colour of low intensity and the reagent blank was coloured. A final concentration of 20 per cent. gave the most satisfactory results, as the blank was colourless and the colour given by DNA was more intense and a clear pink. The intensity of colour was also affected by the amount of tryptophan and the time of heating. Fig. 5 shows the effect on the intensity of colour developed by 500 μg of DNA with 1 to 10-mg amounts of tryptophan. Fig. 6 shows the effect of time of heating in a bath of boiling water on the development of colour with DNA, deoxyribose, hypoxanthine

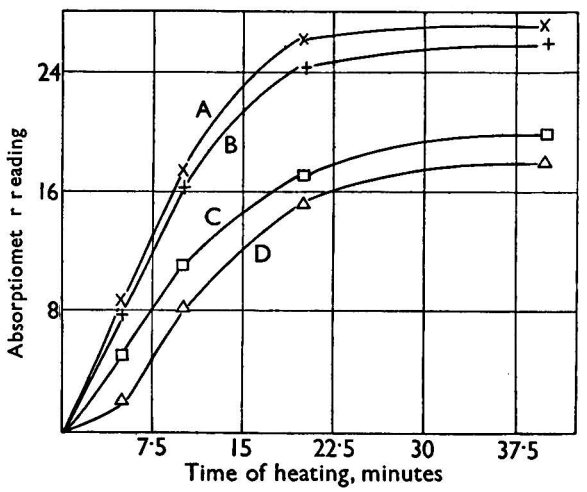

Fig. 6. Effect of time of heating with tryptophan and perchloric acid on the development of colour by DNA, deoxyribose, thymidine and hypoxanthine deoxyriboside.
Curve A, deoxyribose; curve B, hypoxanthine deoxyriboside; curve C, DNA; curve D, thymidine. 72 μg of deoxyribose and amounts of other substances calculated to contain 72 μg of deoxyribose

deoxyriboside and thymidine. Cohen[6] found that when DNA was heated with 30 per cent. perchloric acid for 40 minutes at 100° C all of the deoxyribose present reacted. With the conditions used in this investigation, hypoxanthine deoxyriboside gave over 90 per cent. of the theoretical amount of deoxyribose and thymidine only about 55 per cent. An increase in the concentration of perchloric acid to 30 per cent. did not give an increase of the optical density, but caused a brown coloration. There was little further increase in optical density when heating with tryptophan and 20 per cent. perchloric acid was continued for longer than 20 minutes.

The standard conditions of the method used in this investigation were as follows. One millilitre of solution (or 1 ml of water for the blank), 1 ml of 1 per cent. w/v tryptophan solution in 0·1 *N* sodium hydroxide solution and 1 ml of 60 per cent. perchloric acid were mixed and heated in glass-stoppered tubes in a bath of boiling water for 20 minutes. After they had been cooled in ice-water the samples were diluted with 6 ml of 20 per cent. perchloric acid and the optical density read with an Ilford No. 623 filter in the absorptiometer. The extraction with *iso*amyl alcohol was omitted. There was a linear relationship between amount of DNA and optical density with 0·05 to 0·5 mg of DNA.

The colour given by fibre extracts was often indistinguishable from that of a DNA standard, but was sometimes a brownish-pink. A comparison of the absorption curves of the colours given by a fibre extract, DNA and deoxyribose in this method is shown in Fig. 7. The absorption maximum for DNA and deoxyribose was at 500 mμ as found by Cohen.[6] The fibre extract also had an absorption maximum at 500 mμ, but as in the other methods there was strong absorption in the 400 to 450-mμ region. The rate of colour development in a fibre extract was similar to that of DNA on heating for as much as 20 minutes, but when

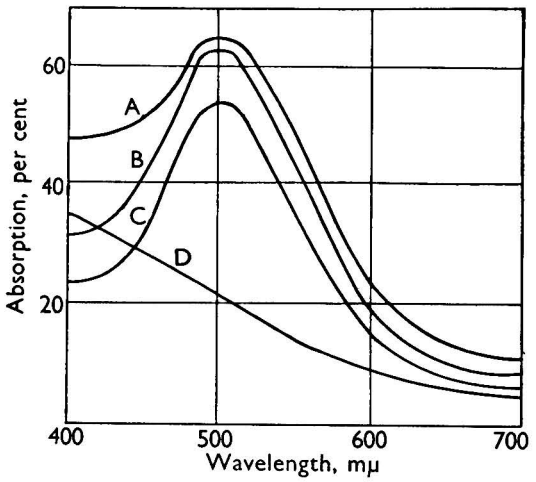

Fig. 7. Comparison of absorption curves of colours given by deoxyribose, DNA, galacturonic acid and a fibre extract with tryptophan and perchloric acid.
Curve A, fibre extract; curve B, 1·12 mg of DNA; curve C, 150 μg of deoxyribose; curve D, galacturonic acid

heating was continued for longer the fibre extract developed a greater colour intensity than the corresponding DNA standard and the difference in hues increased.

INTERFERING SUBSTANCES—

The following absorptiometer readings were obtained when 5 mg of various substances were tested under the standard conditions of the method; they are compared with the reading for 200 μg of DNA.

Absorptiometer reading—DNA, 14·0; yeast RNA, 3·9; pectin, 3·2; starch, 5·5; glucose, 4·2; arabinose, 3·7; galacturonic acid, 9·8.

As in the diphenylamine method, galacturonic acid interferes more than the other substances tested. The absorption curve for galacturonic acid is shown in Fig. 7. Digitoxose gave a pink colour similar to that given by DNA, but with an absorption maximum at 510 mμ instead of 500 mμ. Two milligrams of agar and 0·56 mg of 3:6-anhydro-α-methyl-D-galactoside gave dark brown colours with identical absorption curves. Hexa-acetyl rhamnose gave a light brown colour, but the hexa-acetyl derivative of arabinose and the hepta-acetyl derivatives of glucose, galactose and mannose gave only faint colours.

COMPARISON OF THE RESULTS BY THE THREE METHODS

The DNA contents of some fibre extracts determined by the three methods are compared in Table I. This shows that there is reasonable agreement between the results obtained with the diphenylamine and tryptophan methods, but that with the cysteine method the results are invariably lower and not a constant proportion of those found by the other methods. The difference between the measured phosphorus content of the solution and the value calculated for DNA phosphorus from the absorptiometric DNA determinations depends on how thoroughly the RNA has been removed from the tissue before extraction of DNA. In some experiments, when the preliminary removal of RNA was not thorough, the agreement between the diphenylamine and tryptophan methods was not as good as that shown in

Table I. The tryptophan method gave higher results, which suggested that the extraction of RNA is accompanied by removal of other interfering substances.

In a perchloric acid extract of leaf fibre the DNA will be present mainly as nucleotides. In the diphenylamine method the purine-bound deoxyribose only is estimated, and in the other two methods the deoxyribose attached to the purines reacts completely and the pyrimidine-bound only partly. If the ratio between purine and pyrimidine nucleotides is constant for all extracts and also the same in the DNA used for standards no difficulty arises. However, differences in the ratio may occur, and this should be borne in mind particularly in estimations of DNA in extracts prepared in different ways.

The interference of galacturonic acid in the diphenylamine and tryptophan methods might be considerable in extracts from plant tissues if treatments, such as heating at a high temperature in concentrated acid, which cause pectin breakdown are used. But it is possible with caution to use both methods with most extracts and get fair agreement between them. The disadvantages of the cysteine - sulphuric acid method are that addition of 70 per cent. sulphuric acid alone produces colours in many extracts and the results are erratic and bear little relation to those obtained by the other methods.

TABLE I

COMPARISON OF RESULTS ON PERCHLORIC ACID EXTRACTS OF TOBACCO LEAF FIBRE BY THREE METHODS OF DNA DETERMINATION

Phosphorus measured, μg per ml	Phosphorus in DNA		
	By diphenylamine method, μg per ml	By tryptophan method, μg per ml	By cysteine method, μg per ml
17	15	18	9
21	15	16	11
24	22	25	20
32	30	34	21
36	34	33	31
44	39	40	27
49	44	43	25
60	56	57	40
71	66	64	45
82	75	80	58

REFERENCES

1. Dische, Z., *Mikrochemie*, 1930, **8**, 4.
2. Ogur, M., and Rosen, G., *Arch. Biochem.*, 1950, **25**, 262.
3. McClendon, J. H., *Amer. J. Bot.*, 1952, **39**, 275.
4. Dische, Z., *Proc. Soc. Exp. Biol. Med.*, 1944, **55**, 217.
5. Stumpf, P. K., *J. Biol. Chem.*, 1947, **169**, 367.
6. Cohen, S. S., *Ibid.*, 1944, **156**, 191.
7. Holden, M., *Biochem. J.*, 1952, **51**, 433.
8. Pirie, N. W., *Brit. J. Exp. Path.*, 1936, **17**, 269.
9. Hanes, C. S., *Biochem. J.*, 1929, **23**, 99.
10. Tracey, M. V., *Ibid.*, 1948, **43**, 185.
11. Weeks, L. F., and Friminger, H. I., *Ind. Eng. Chem., Anal. Ed.*, 1942, **14**, 760.
12. Deriaz, R. E., Stacey, M., Teece, E. G., and Wiggins, L. F., *J. Chem. Soc.*, 1949, 1222.
13. Davidson, J. N., "Biochemistry of the Nucleic Acids," Methuen & Co. Ltd., London, 1950.
14. Overend, W. G., *J. Chem. Soc.*, 1951, 1484.
15. Manson, L. A., and Lampen, J. O., *J. Biol. Chem.*, 1951, **191**, 87.

ROTHAMSTED EXPERIMENTAL STATION
HARPENDEN, HERTS.

January 12th, 1953

The Micro-determination of Alkoxyl Values in Cellulose Esters

By W. C. EASTERBROOK and J. B. HAMILTON

Modifications of the Vieböck and Brecker method for the micro-determination of alkoxyl groups are described. These include (1) a spiral scrubbing trap containing a buffered cadmium sulphate - sodium thiosulphate solution, which has been found satisfactory for removing all likely impurities, (2) an upward sloping arm leading from the trap to prevent spray being carried over into the absorption solution and (3) removal of the ground-glass joint from below to above the condenser in order to prevent leakage and to facilitate manipulation of the joint. Bumping, caused by superheating of the reaction mixture, is obviated by special digestion flasks constructed with thin walls and bases. Results by the modified method as applied to α-methyl-D-glucoside, α-methyl-D-mannoside and phenacetin are given.

Results were erratic when the method was applied to some samples of cellulose esters in the form of film. Replicate determinations on film from the same batch after grinding to powder in a mill were in good accord. Deterioration of ethyl cellulose during drying can be avoided by heating it *in vacuo* at 80° C for 1 hour.

The method was devised for cellulose esters. General applicability is not claimed. Modification may be necessary for substances other than those mentioned here.

In Vieböck and Brecker's method[1,2] for the micro-determination of alkoxyl groups the sample substance is digested with hydriodic acid and the volatile alkyl iodide so formed is swept over by a stream of inert gas into a buffered solution containing bromine, the excess of which is subsequently removed with formic acid. Sulphuric acid and potassium iodide are then added and the liberated iodine titrated with sodium thiosulphate with starch solution as the indicator. Some samples of ethyl cellulose gave abnormal results. It is generally agreed that errors in the method arise principally from one or more of the following causes: (*i*) the use of impure reagents, (*ii*) superheating of the reaction mixture during distillation, which causes irregular passage of carbon dioxide through the receiver, (*iii*) leakage at the ground-glass joint between the flask and the condenser owing to attack on the glass by hydriodic acid, and (*iv*) inhomogeneity of the samples.

These points were investigated as follows.

The use of impure reagents—The critical reagent in this method is the hydriodic acid and our work was therefore devoted mainly to finding possible impurities in it. On working with AnalaR hydriodic acid and purified AnalaR hydriodic acid we observed that high blank values were caused by impurities and by hydrogen iodide vapour being carried over in the gas stream to the absorption solution. Similar findings have been reported in the literature.[3,4] The impurities normally present in the reagent are hydrogen sulphide and free iodine. Hydrogen sulphide in minute amounts did not affect the determination, but its presence in quantity was liable to decolorise the bromine in the absorption solution and to cause erroneous results.

Because of possible contamination with impurities, only the purest hydriodic acid was used for all subsequent work. This was stabilised immediately before use by the addition of 0·1 ml of hypophosphorous acid to retard the oxidation of the hydriodic acid to iodine and water. An excess of hypophosphorous acid must be avoided, for it may lead to the formation of phosphine and to consequent reduction of the bromine absorption solution.

The effect of small amounts of hypophosphorous acid on hydriodic acid was found by a number of alkoxyl determinations on α-methyl-D-mannoside. The results are shown in Table I.

In these experiments the modified spray trap contained 1 ml of 5 per cent. cadmium sulphate solution, 1 ml of 5 per cent. sodium thiosulphate solution, buffered by 2 ml of 20 per cent. sodium acetate solution, see Table II.

The results of successive experiments on the same charge of hydriodic acid without the addition of hypophosphorous acid show reasonably good reproducibility, but the results were abnormally low after the third experiment (see Table I). Furthermore, as the number of determinations increased, the end-point of the titration became increasingly difficult to see as the blue colour with starch indicator kept recurring. At this stage, the digestion mixture showed marked deterioration owing to the amount of free iodine that had accumulated in it. This deterioration was confirmed when the same procedure was applied to a sample of ethyl cellulose. Phenol was added to the digestion mixture to serve as a dispersing medium for cellulose esters[5,6] and no difficulty was experienced in subsequent experiments with material of viscosities between 7 to 1200 centipoise units.

Additional tests to ascertain how many determinations could be carried out on a fresh charge of digestion mixture with hypophosphorous acid present showed at least 16 determinations to be possible. After the seventh determination the digestion mixture became highly

TABLE I

ALKOXYL DETERMINATION ON α-METHYL-D-MANNOSIDE, (1) WITHOUT HYPOPHOSPHOROUS ACID, AND (2) WITH HYPOPHOSPHOROUS ACID IN THE DIGESTION MIXTURE

Composition of digestion mixture	$-OCH_3$ Found, %			$-OCH_3$ Theory, %
(1) *Without hypophosphorous acid*—				
5 ml of M.A.R. hydriodic acid and 2·5 ml of phenol	15·92	15·95	15·84	15·98
	15·64	15·55		
(2) *With hypophosphorous acid* (0·1 *ml*)—				
5 ml of M.A.R. hydriodic acid and 2·5 ml of phenol	15·91	15·92	15·87	
	15·94	16·00	16·10	
	16·01			,,
Further addition of 0·1 ml of hypophosphorous acid	15·94	15·92	15·95	
	15·90	15·94	15·90	
	15·85	16·00	15·98	

coloured by the iodine that had gradually accumulated during the decomposition of the sample, but at this stage it was decolorised by the further addition of 0·1 ml of hypophosphorous acid. The results showed reasonably good reproducibility.

Superheating of the digestion mixture—Numerous authors[5,7,8,9,10] have reported bumping during the course of the digestion with hydriodic acid and in spite of the many improvements recommended this trouble still persists. During this investigation bumping was particularly noticeable in one piece of apparatus, which gave high blanks. In spite of the remedial measures tried, which included the use of glass beads, glass wool and alundum granules, no improvement was effected. It was observed, however, after an accidental fracture, that the digestion flask had an unusually thick base, this flask having been blown and sealed off in such a way as to leave the lowest point of the base thicker than the wall. When the flask was re-blown, care was taken to ensure that it had a thin wall and base. This modification overcame the bumping and the subsequent blank values were normal.

Leakage at the ground-glass joint between the digestion flask and the condenser—Leakage at the ground-glass joint between the digestion flask and the condenser was troublesome and difficulty was experienced in disconnecting the joint. For these reasons the ground joint was transferred to the upper side of the condenser, where the effects of acid vapour and extreme temperatures were negligible.[11] This modification of the apparatus entailed the use of a longer handled weighing spoon (see Fig. 1), but otherwise the procedure for carrying out the determination was unchanged.

Because of difficulties with ineffective scrubbing of the gas stream, the scrubbing trap was modified as follows—

1. A spiral scrubber was introduced into the trap to give longer contact of the gas stream with the scrubbing solution; this effectively purified the alkyl iodide vapours.

2. The side-arm was made to slope upwards as a precaution against spray being carried over into the absorption solution. Numerous tests were carried out with the modified

scrubbing trap and in no instance was there evidence of liquid having been carried over to the absorption receiver. With this modified trap and scrubbing solutions of various compositions, a number of blank determinations were carried out. Some typical results are shown in Table II.

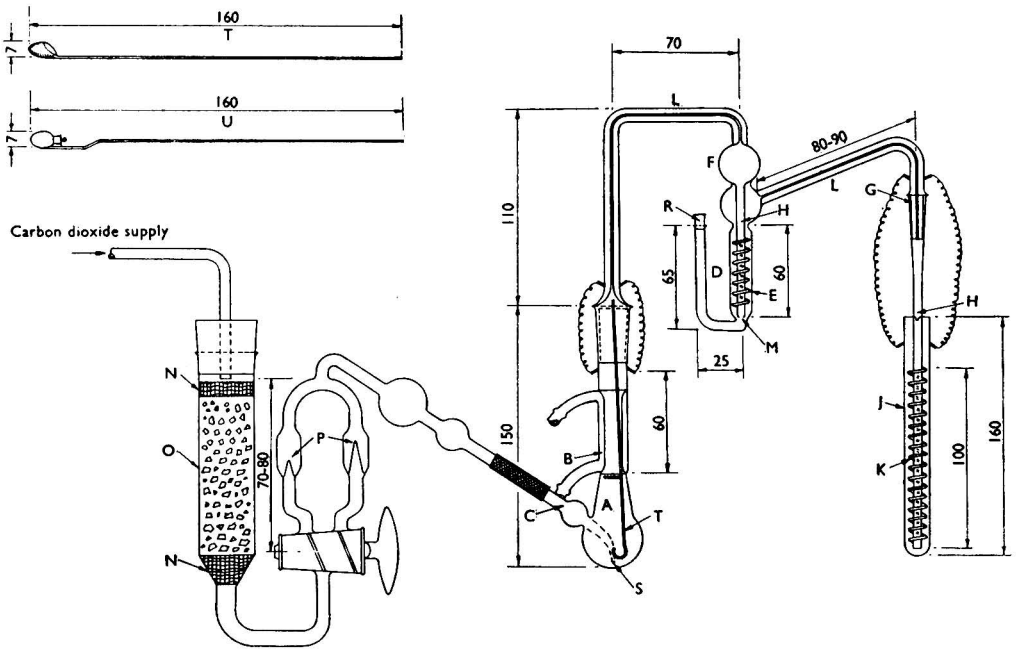

Fig. 1. Modified apparatus for micro-determination of alkoxyl groups. (All dimensions in millimetres)

A	Digestion flask	Ext. dia. 35	Capacity 25 ml
B	Water condenser fitted with B14 joint complying with B.S. 572	„ „ 17 Int. „ 10	Length 60 mm
C	Safety bulb	Ext. „ 15	
D	Scrubbing trap	„ „ 12	
	Side-arm	„ „ 5	
E	Glass spiral. Seven turns in 40 mm of 3 mm rod wound on ¼-inch mandrel and held in position by pip on glass tube.		
F	Safety bulbs	„ „ 25	
G	Glass joint B7 complying with B.S.572.		
H	Delivery tube	„ „ 5 to 5·5	
J	Receiver	„ „ 15	
K	Spiral. Seventeen turns of 3 mm glass rod wound on ¼-inch mandrel and held in position by pip on glass tube	Int. „ 6	Length 100 mm
L	Capillary tube	„ „ 2	
M	Jet of delivery tube	„ „ 1	
N	Cotton wool plugs.		
O	Calcium chloride.		
P	Jets: flow speeds 10 to 12 ml and 2 ml approximately per minute.		
R	Rubber stopper.		
S	Clearance between side-arm and base of flask, 1 mm.		
T	Weighing spoon.		
U	Weighing tube.		

These results show that the most efficient scrubbing solution is the buffered salt solution containing cadmium sulphate, sodium thiosulphate and sodium acetate,[12] which remove hydrogen sulphide, iodine and hydriodic acid, respectively. This solution was used in all subsequent tests. Water has been recommended for scrubbing by various

investigators,[2,4,13] but it failed to remove completely the impurities responsible for high blanks, even with the more efficient spiral scrubber.

TABLE II

COMPARISON OF VARIOUS SCRUBBING SOLUTIONS ON BLANK DETERMINATIONS

Composition of digestion mixture	Scrubbing solution in modified trap	Blank value, ml of 0·01 *N* $Na_2S_2O_3$
5 ml of M.A.R. hydriodic acid, 0·1 ml of hypophosphorous acid and 2·5 ml of phenol	3 ml of distilled water	0·59
,,	1·5 ml of 5 per cent. cadmium sulphate 1·5 ml of 5 per cent. sodium thiosulphate	0·32
,,	Suspension of red phosphorus in 3 ml of 5 per cent. cadmium sulphate	0·40
,,	1 ml of 5 per cent. cadmium sulphate 1 ml of 5 per cent. sodium thiosulphate 2 ml of 20 per cent. sodium acetate	0·19 0·20

METHOD

APPARATUS—

The apparatus for the determination is shown in Fig. 1 and is used in conjunction with a Drikold generator with a variable pressure lute so as to give carbon dioxide at a constant pressure through a jet adjusted to pass 10 to 12 ml of gas per minute. The micro-Zeisel apparatus for the determination of alkoxyl groups has been adopted with some slight modifications as a British Standard (B.S. 1428, Part C1).

REAGENTS—

Hydriodic acid (*M.A.R.*), *sp.gr.* 1·7—Preferably stored in 5-ml ampoules.

Hypophosphorous acid, 30 *per cent.*—Pure free from sulphate.

Preparation of bromine solution—Dissolve 17·6 g of potassium hydroxide in 227 ml of glacial acetic acid and add 2 ml of bromine free from iodine.

All other reagents should be of recognised analytical grade.

PROCEDURE—

Clean the apparatus with chromic - sulphuric acid mixture, wash it with distilled water and dry in a water-oven. Transfer the contents of a 5-ml ampoule of M.A.R. hydriodic acid to the digestion flask and then add 0·1 ml of hypophosphorous acid and 2·5 g of phenol. Connect the flask to the condenser making certain that the ground-glass joint fits securely. Make the scrubbing solution by mixing 1 ml of 5 per cent. cadmium sulphate, 1 ml of 5 per cent. sodium thiosulphate and 2 ml of 20 per cent. sodium acetate solution and place the mixture in the scrubbing trap. Add 5 ml of bromine solution to the receiver and connect it to the apparatus by means of springs or rubber bands. Carry out a blank determination by boiling the digestion mixture for 30 minutes. At the end of this period remove the receiver and pour the contents into a 250-ml glass-stoppered flask containing 5 ml of 25 per cent. sodium acetate solution and wash out the receiver with water. Add formic acid to the solution drop by drop, insert the stopper and shake the flask until there is no trace of bromine, then add 2 ml of 10 per cent. potassium iodide solution and 5 ml of 10 per cent. sulphuric acid solution. Titrate the liberated iodine with freshly standardised 0·01 *N* sodium thiosulphate using starch solution as indicator.

Determine the alkoxyl groups in organic compounds by weighing 5 to 10 mg of the prepared and dried sample in a special glass weighing spoon or weighing bottle (see Fig. 1), and after transferring both spoon and sample to the digestion flask, follow the same procedure as described above for the blank.

1 ml of 0·01 *N* $Na_2S_2O_3$ = 0·0517 mg of $-OCH_3$
0·0751 mg of $-OC_2H_5$

To test the accuracy of the method a number of alkoxyl determinations were made on α-methyl-D-glucoside (re-crystallised five times from anhydrous methanol and adopted as a standard for this work) and pure phenacetin. The results are shown in Table III.

TABLE III

ALKOXYL CONTENT OF α-METHYL-D-GLUCOSIDE (REFERENCE STANDARD) AND PURE PHENACETIN FOUND UNDER ROUTINE CONDITIONS

Sample	Alkoxyl content Found, %						Theory, %
α-Methyl-D-glucoside ..	15·95	16·00	16·08	15·98	15·92	as $-OCH_3$	15·98 as $-OCH_3$
	16·05	16·05	15·97	15·86	16·09		
Phenacetin	25·09	25·12	25·10	25·20	as $-OC_2H_5$		25·15 as $-OC_2H_5$
	25·20	25·21					

APPLICATION OF THE METHOD TO METHYL AND ETHYL CELLULOSE—

Methyl and ethyl celluloses were normally prepared for test in the form of films. (*a*) Methyl cellulose or water-soluble films were prepared as follows: weigh 1 g of the material and wet it thoroughly with 9 ml of hot water (90° to 100° C). Allow to cool while swelling of the sample takes place and when the mixture is cold add 10 ml of cold water, gradually, with stirring. Spread this solution in a glass Petri dish using approximately 0·4 g of solution per sq. cm and dry the dish to constant weight at 90° to 100° C. Strip from the glass the film of cellulose derivative so formed and use a weighed quantity for analysis. (*b*) Ethyl cellulose films are usually cast from a mixed solvent and are prepared as follows: weigh 1 g of the material and dissolve it in 19 g of toluene - butanol mixture (80 + 20, v/v) or other suitable solvent to form an approximately 5 per cent. solution. Spread this solution on a glass Petri dish, using approximately 0·4 g of solution per sq. cm and allow the solvent to evaporate gradually by exposure to air at room temperature. In this way a film of the cellulose derivative is formed. When the solvent has evaporated, strip the film from the glass, boil it in water for 10 to 15 minutes and dry to constant weight at 90° to 100° C. Use a weighed amount of this film for analysis.

During the course of routine testing of ethyl cellulose films different ethoxyl values were given by samples from the same film. To investigate the cause, films were prepared and analysed by cutting a strip approximately 1 cm broad diametrically across the film. Suitable portions of the strips were cut off and the ethoxyl content determined. The results are shown in Table IV.

TABLE IV

ETHOXYL CONTENT OF ETHYL CELLULOSE FILMS

Sample	Alkoxyl found, %	Theory, %
Control .. .. (α-methyl-D-mannoside)	15·9 as $-OCH_3$	15·98
Film strip "A"	48·9 48·9 49·2 49·1 49·0 49·0 as $-OC_2H_5$	
Control	15·9 as $-OCH_3$	
Film strip "B"	49·2 49·1 49·1 49·5 49·2 49·0	
Thickness 0·0008 inch	as $-OC_2H_5$	
Control	16·1 as $-OCH_3$	15·98
Film strip "C"	48·4 49·8 48·7 49·3 49·6 50·2	
Thickness 0·014 inch	as $-OC_2H_5$	
Control	16·0 as $-OCH_3$	15·98
Film strip "D"	48·1 48·6 48·1 48·2 49·2 49·6	
Thickness 0·0002 inch	as $-OC_2H_5$	
Control	15·9 as $-OCH_3$	15·98

All the films referred to in Table IV were prepared from the same bulk solution of ethyl cellulose in toluene - ethanol, so that in considering the results from the individual films the question of heterogeneity of the sample did not arise. The results for samples A and B show that although fairly good agreement is obtained with some films, for others, samples C and D, the reproducibility is poor. The films used were of different thicknesses but no correlation between thickness and ethoxyl content could be found. It was disquieting to find such wide discrepancies between some films as well as between contiguous strips in the

same film and this seems to account for unwarranted suspicions of the accuracy of the method itself. Further evidence that the method of preparing the films for assay is largely responsible for erratic results is given below.

Attention was now turned to the examination of disintegrated ethyl cellulose and of films made from the same material. A comparison of the results obtained is given in Table V.

TABLE V

ETHOXYL CONTENT OF ETHYL CELLULOSE

Sample	Alkoxyl found, %						Theory, % as $-OCH_3$	Remarks
Control .. .. (α-methyl-D-mannoside)	15·90 as $-OCH_3$						15·98	
Batch A, ground sample ..	50·5	50·3	50·3	50·4	50·3 as $-OC_2H_5$	50·2		
" film	46·2				"			Film dried in a current of dry air
" "	50·6	50·2	50·6		"			Film dried in oven at 100° C
" "	49·9	50·2			"			Portion of film boiled in water and dried at 100° C
" "	49·4	49·9			"			"
" " ground sample	50·0	50·3	50·5		"			Boiled in water for 1 hour and dried in oven at 100° C
Control	15·95 as $-OCH_3$						15·98	
Batch B, ground sample ..	48·2				as $-OC_2H_5$			*Film cast from solvent*
" film	48·2	48·3			"			Toluene - butanol
Batch C, ground sample ..	50·8	50·9			"			"
" film	50·9	51·1			"			"
Batch D, film	50·9	51·0			"			Toluene - ethanol
Control	15·95 as $-OCH_3$							—
Batch E, ground sample ..	49·5	49·5			as $-OC_2H_5$			—
" film	49·0	49·6			"			Toluene - butanol
	49·0	49·9			"			Toluene - ethanol
Batch F, ground sample ..	50·1	50·2			"			—
" film	50·0	49·7			"			Toluene - butanol
" "	50·6	50·7			"			Toluene - ethanol

It will be seen from Table V that the results show greater reproducibility when the determination is carried out on the ground material. An explanation of the difference between the results obtained for ground material and film is suggested by samples A, ground and film. When the film had been dried slowly in a current of dried air, the ethoxyl value was low. With more rapid drying of the film in an oven at 100° C, good reproducibility was attained. However, on boiling a portion of the previously dried film in water as described in the method for casting films, the variation in the results became much greater. Incidentally, the physical nature of the film changed from soft, transparent and pliable after drying to hard, opaque and brittle after boiling in water. To ascertain if similar results were obtained on boiling ground material, further tests were carried out, the results of which differed but little from the previous figures for ground material. It is significant that any method involving the preparation of films from solvents, especially with alcohols as components, is liable to give erratic results; for absorbed solvents are not readily expelled from cellulose derivatives in the fibrous state or in the form of films. Variations in the films were much the same whether toluene - ethanol or toluene - butanol solvents were used. From these tests it was concluded that more consistent results are obtained on ground material than on film of the same material.

Ethyl cellulose was ground in a Raymond laboratory mill fitted with a brass screen with holes 1/64-inch diameter. Twelve samples of ethyl cellulose prepared in this way were examined by carrying out duplicate ethoxyl determinations. The results showed an average difference of 0·2 per cent.

The ground material for analysis was at first dried by exposure for half an hour at 105° C,[5] but this was later modified to drying *in vacuo* for 1 hour at 80° C, which was safer and more

efficient, because of thermal degradation of the material.[14] The extent of this is shown in the tabulated drying experiments in Table VI.

TABLE VI

LOSS IN WEIGHT OF ETHYL AND METHYL CELLULOSE ON DRYING

Test No.	Drying conditions	Ethyl cellulose		Methyl cellulose	
		Weight, mg	Loss, mg	Weight, mg	Loss, mg
1	Dried to constant weight over P_2O_5 *in vacuo* at room temperature	12·091	—	9·714	—
2	Further drying overnight at 100° C ..	12·076	0·015	9·714	Nil
3	Further drying for 5 days at 100° C ..	10·776	1·315	9·714	Nil

Table VI shows that no apparent loss in weight occurred when methyl cellulose was heated for 5 days at 100° C, but for ethyl cellulose, after drying overnight and for 5 days at 100° C, losses of approximately 0·2 and 13 per cent., respectively, were recorded. At the end of the drying treatment the ethyl cellulose had changed from a granular powder to a brittle resinous mass. A determination of the ethoxyl content of this resinous material showed that it had been considerably degraded and contained 36·3 per cent. of $-OC_2H_5$ compared with the original value of 50·6 per cent. of $-OC_2H_5$.

The deterioration of ethyl cellulose when dried at 100° C was accompanied by the liberation of aldehyde, which was detected by its odour and confirmed by reduction of ammoniacal silver nitrate solution.

Because of this deterioration of ethyl cellulose on drying, tests were made to compare the ethoxyl content after drying *in vacuo* at 80° C and in an oven at 100° C. The results are shown in Table VII.

TABLE VII

COMPARISON OF THE ETHOXYL CONTENT OF ETHYL CELLULOSE AFTER DRYING *in vacuo* AT 80° C AND IN AN OVEN AT 100° C

Test No.	Drying treatment	Ethoxyl content	Average
1	None	50·5 50·9 50·6	50·7
2	½ hour *in vacuo* at 80° C	50·7 50·2	50·5
3	1 hour *in vacuo* at 80° C	50·9 51·0	51·0
4	5 hours *in vacuo* at 80° C	50·6 51·0	50·8
5	Sample used in test 4 dried overnight in oven at 100° C	48·9 48·9	48·9

From these results it is apparent that little change in ethoxyl content occurs after 5 hours drying *in vacuo* at 80° C, but on subjecting the same material to further drying overnight at 100° C a drop in ethoxyl value of approximately 2 per cent. takes place.

As a result of this investigation all samples of cellulose esters were ground to pass a 1/64-inch circular-hole screen and dried for 1 hour at 80° C *in vacuo* before the alkoxyl content was determined as described above.

STATISTICAL ANALYSIS OF ANALYTICAL RESULTS—

Statistical analysis of the results by this method has shown that in the determination of methoxyl and ethoxyl values the order of accuracy is the same. The standard deviation of the percentage methoxyl and the percentage ethoxyl in α-methyl-D-mannoside and phenacetin, respectively, from the calculated values was 0·06 per cent. for both.

REFERENCES

1. Vieböck, F., and Brecker, G., *Ber.*, 1930, **63**, 3207.
2. Houghton, A. A., and Wilson, A. A. B., *Analyst*, 1944, **69**, 363.
3. Gibson, D. T., and Caulfield, T. H., *J. Chem. Soc.*, 1935, 1419.
4. Colson, A. F., *Analyst*, 1933, **58**, 594.
5. Samsel, E. P., and McHard, J. A., *Ind. Eng. Chem., Anal. Ed.*, 1942, **14**, 750.
6. Tribot, A., and Marsaudon, A., "Ethylation of Cellulose, Hydrolysis of Neutral Ethyl Sulphate." "Memorial des Services Chemiques de l'Etat," Imprimerie Nationale, Paris, 1947.
7. Bruckner, V., *Microchem.*, 1932, **12**, 153.

8. Furter, M., *Helv. Chem. Acta*, 1938, **21**, 1144.
9. Neumann, F., *Ber.*, 1937, **70**, 734.
10. Elek, A., *Ind. Eng. Chem., Anal. Ed.*, 1939, **11**, 175.
11. Shaw, B. M., *J. Soc. Chem. Ind.*, 1947, **66**, 147.
12. Heron, A. E., Private communication.
13. Clark, E. P., *Ind. Eng. Chem., Anal. Ed.*, 1930, **10**, 677.
14. McBurney, L. F., and Evans, E. E., *India Rubber World*, 1947, **116**, 80.

IMPERIAL CHEMICAL INDUSTRIES LTD.
NOBEL DIVISION
STEVENSTON, AYRSHIRE

November 27th, 1952

Notes

A DEPARTURE FROM BEER'S LAW AFFECTING THE SPECTROPHOTOMETRIC DETERMINATION OF DIPHENYL

THE determination of diphenyl is of importance in the citrus fruit industry, in which diphenyl-impregnated wraps and packing containers are in widespread use. Cox[1] suggested that diphenyl could be determined spectrophotometrically and the method was used by Steyn and Rosselet[2] to determine diphenyl in orange peel.

In Fig. 1 concentration of diphenyl in milligrams per 100 ml is plotted against optical density measured at 250 mμ in a 1-cm cell. The instrument used was a Beckman D.U. spectrophotometer

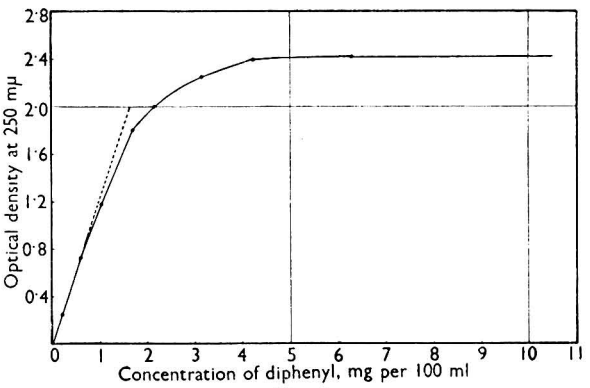

Fig. 1

and the solvent was commercial light petroleum that had been washed with a nitrating mixture and then distilled through a fractionating column to give a fraction that boiled between 55° and 62° C. At low concentrations of diphenyl the points on the graph lie on a straight line that can be extrapolated through the origin; in this region of the graph Beer's law is obeyed. However, at optical densities greater than 0·8 there is considerable departure from Beer's law and eventually the optical density becomes almost constant with increasing concentrations of diphenyl. The earlier workers[1,2] based their calculations on the molecular extinction coefficient of diphenyl, as previously determined, and recommended a concentration of 0·001 per cent. of diphenyl to ensure a convenient reading. It is evident from the graph that with our instrument this concentration would result in an error of approximately 8 per cent. In the determination of diphenyl it is, therefore, necessary to plot a calibration graph and to adjust the concentration of the test sample to give a density reading lying on the straight part of the graph.

This behaviour of diphenyl parallels that of naphthalene and anthracene as found by Braude, Fawcett and Timmons,[3] who ascribe the effect to the fluorescence of the absorbent.

REFERENCES

1. Cox, H. E., *Analyst*, 1945, **70**, 373.
2. Steyn, A. P., and Rosselet, F., *Ibid.*, 1949, **74**, 89.
3. Braude, E. A., Fawcett, J. S., and Timmons, C. J., *J. Chem. Soc.*, 1950, 1019.

COMMONWEALTH SCIENTIFIC AND
INDUSTRIAL RESEARCH ORGANISATION
DIVISION OF FOOD PRESERVATION AND TRANSPORT
HOMEBUSH, NEW SOUTH WALES
AUSTRALIA

J. B. DAVENPORT

January 12th, 1953

THE THIOCYANATE DETERMINATION OF IRON WITH HYDROGEN PEROXIDE

IT is well known that many factors influence the thiocyanate test for iron. The red ferric thiocyanate colour is deepened by an excess of the reagent and is diminished by an excess of acid; further, the colour formed fades rapidly.[1] Nevertheless, the test can form the basis of a convenient and serviceable quantitative method if conditions are rigidly standardised and the matching procedure is carried out rapidly. The method in its usual form is particularly inconvenient when several samples are to be examined, because, strictly, each sample requires its own freshly prepared standard.

STABILITY OF THE COLOUR—

Work in this laboratory has proved that the colour can be made stable for at least an hour by the addition of a few drops of a concentrated solution of hydrogen peroxide. Further, should the colour fade in the course of standing for several hours, or even days, it can be restored to its original intensity by the addition of a few more drops of hydrogen peroxide solution. It is therefore possible to bring large batches of samples to the colour stage and, at leisure and with greater accuracy, either match them against the one standard or measure their colour intensities on a photo-electric absorptiometer. The samples used in our work contained about 2 parts of iron per million and were stabilised with 3 drops of 90 to 100-volume hydrogen peroxide solution per 100 ml of sample. One millilitre of concentrated hydrochloric acid and 2 ml of 10 per cent. ammonium thiocyanate solution were used per 100 ml of sample. With these concentrations it was found that a peroxide-treated sample remained unchanged for 5 hours, during which time the colour of an untreated sample faded to less than 50 per cent. of its original intensity. This is contrary to the general conclusion of Peters, MacMasters and French,[2] who recommend the use of peroxide as an iron oxidant and as a means of intensifying a faded colour, but who state that the treatment makes the colour stable for only a few minutes under the conditions of their experiments. They however, used much greater concentrations of thiocyanate reagent, which can cause thiocyanates and hydrogen peroxide to react to develop a yellow colour. We have found that the stability can be increased by the use of thiocyanate reagent at a lower concentration. The colour does not quite obey Beer's law, so that a reference graph should be used in photo-electric determinations.

APPLICATION TO DETERMINE FERROUS IRON—

This use of hydrogen peroxide has led to a useful and simple method of determining both ferrous and ferric salts in a single solution. The sample, made acid to the extent of 1 per cent. with concentrated hydrochloric acid, is filtered if necessary; a 100-ml portion is then treated with 2 ml of a 10 per cent. solution of ammonium thiocyanate and placed immediately in a photo-electric absorptiometer or matched against a standard containing 2 parts of iron per million, which is stabilised with 3 drops of 100-volume hydrogen peroxide solution. This gives a value for the ferric iron content of the original solution. Next, the liquid is treated with 3 drops of hydrogen peroxide solution, which instantly oxidises any ferrous iron, and the colour is measured or matched to give a measure of the total iron content.

Ferrous and ferric salts in pure solutions treated in this manner have been found repeatedly to give quantitative results. When the method was used on polluted river waters, a small loss of accuracy was liable to occur. The method has proved to be extremely useful for the approximate and rapid determination of the relative quantities of ferrous and ferric iron present in waters taken from the Thames during the summer of 1952.

REFERENCES

1. Sandell, E. B., "Colorimetric Determination of Traces of Metals," First Edition, Interscience Publishers Inc., New York, 1944.
2. Peters, C. A., MacMasters, M. M., and French, C. L., *Ind. Eng. Chem., Anal. Ed.*, 1939, **11**, 502.

CHEMICAL BRANCH
PUBLIC HEALTH DEPARTMENT
L.C.C. SOUTHERN OUTFALL WORKS
CROSSNESS, ABBEY WOOD
LONDON, S.E.2

J. E. HOULIHAN
P. E. L. FARINA

January 19th, 1953

THE DETERMINATION OF NICKEL IN PLANT MATERIAL IN THE PRESENCE OF OTHER METALS

In experiments with crop plants grown in nickel-treated soils, it was necessary to determine the nickel content of the dried plant material in the presence of various amounts of iron, copper and manganese. Two colorimetric procedures both involving dimethylglyoxime were studied.

The colour produced in the method of ter Haar and Westerveld[1] is stable and the calibration graph is linear, but tests with standard solutions confirmed that copper and manganese interfere as described in the original paper. Moreover, plant digest solutions subjected to this procedure gave turbid solutions. In Sandell's method[2] only copper and nickel form complexes with dimethylglyoxime that are extractable in chloroform. The copper can be removed by shaking the chloroform extract with dilute ammonia solution, but the colour ultimately developed is unstable. By combining the methods of Sandell (for extracting the nickel) and ter Haar and Westerveld (formation of the nickel^{IV} - dimethylglyoxime complex), a procedure that is free from interferences is obtained in which the stable colour developed obeys Beer's law for 0 to 50 μg of nickel.

Method

Reagents—

Hydroxylamine hydrochloride, A.R.—A 10 per cent. w/v solution.

Sodium citrate, A.R.—A 20 per cent. w/v solution.

Concentrated ammonia solution, about 14 N—Prepared by passing ammonia gas into glass-distilled water.

Dimethylglyoxime—A 1 per cent. w/v solution in alcohol.

Chloroform—The redistilled B.P. reagent.

Hydrochloric acid, 0·33 N.

Diluted ammonia solution (1 + 50).

Sodium tartrate—A 20 per cent. w/v solution.

Potassium persulphate, A.R.—A 4 per cent. w/v solution.

Sodium hydroxide solution, 5 N.

Dimethylglyoxime—Add 1 g of dimethylglyoxime to 5 ml of 5 *N* sodium hydroxide solution and dilute with water to 100 ml.

Procedure—

Digest the dried plant material with nitric and perchloric acids, filter and make up to volume. Transfer a suitable aliquot by means of a pipette into a 125-ml pear-shaped separating funnel and add 2 ml of hydroxylamine hydrochloride solution, 5 ml of sodium citrate solution, 3 drops of phenolphthalein solution, and concentrated ammonia solution until a pink coloration is formed, and then add 4 drops more and 2 ml of dimethylglyoxime in alcohol. Adjust the volume to about 60 ml and extract the solution thrice with 5-ml portions of chloroform, shaking each time for 30 seconds. If copper is present, remove it from the chloroform extract by shaking for 1 minute with 5 ml of diluted ammonia solution (1 + 50). Then extract nickel from the chloroform layer by shaking first with 10 ml and then with 5 ml of 0·33 *N* hydrochloric acid for 1 minute. Take care to shake down the droplet of chloroform on the surface of the hydrochloric acid extract when separating the two phases, so that only a small droplet of chloroform is present in the combined hydrochloric acid extracts; collect the extracts in a 50-ml graduated flask.

To produce the red-brown nickel^{IV} - dimethylglyoxime complex, add the following reagents to the 50-ml flask: 2 ml of sodium tartrate solution, 10 ml of potassium persulphate solution, 0·6 ml of dimethylglyoxime in sodium hydroxide solution and 2·5 ml of 5 *N* sodium hydroxide solution. After making up to volume set the flask aside for 30 minutes and measure the colour within 2 hours of adding the reagents.

With a Spekker absorptiometer use Ilford No. 605 yellow-green filters, 4-cm cells and a water setting of 0·6.

Results

Tables I and II show the results of analysing standard solutions by ter Haar and Westerveld's method and the method described above, respectively.

TABLE I

DETERMINATION OF NICKEL BY THE METHOD OF TER HAAR AND WESTERVELD

Nickel taken for analysis = 50 μg

Metals added	Amount, μg	Nickel found, μg
Copper	100	51·4
"	600	41·7, 54·4
Manganese	600	53·2
Copper, iron and manganese	600 (of each)	34·0, 45·0
Aluminium, copper, iron, manganese and zinc	600 (of each)	31·2

TABLE II

DETERMINATION OF NICKEL BY THE PROPOSED METHOD

No interference was encountered when—

1. 300 or 600 μg of Cu, 600 μg of Al, Cd, Co, Mn or Zn, 600 or 1200 μg of Fe, or 10 mg of P (as KH_2PO_4) were added to solutions containing 50 μg of nickel.
2. Solutions containing 50 μg of Ni and 600 μg of Cu contained in addition—

Al, μg	Cd, μg	Co, μg	Fe, μg	Mn, μg	Zn, μg	P (as KH_2PO_4), mg
—	—	—	1200	—	—	—
—	—	—	—	600	—	—
—	—	600	—	—	—	—
—	600	600	—	600	—	—
—	—	—	600	600	—	—
600	—	600	600	600	600	—
—	—	—	600	600	—	10

Several of the determinations recorded in Table II were replicated and the mean recovery based on 26 determinations was 49·92 μg of nickel, with standard error 0·11 μg of nickel.

Table III indicates the recovery of nickel added to plant digest solutions, by the proposed method. Duplicate aliquots were taken. To one series nickel, or nickel in combination with iron, copper or manganese, was added, to the other series no addition was made. Recoveries have been corrected for the blank correction, which in all experiments was equivalent to about 0·9 μg of nickel.

TABLE III

RECOVERY OF NICKEL ADDED TO PLANT DIGEST SOLUTIONS

Digests	Metals added	Nickel recovered, μg	Other constituents of samples
Tomato	20 μg of nickel	19·3, 19·6, 19·7, 20·4, 20·0, 20·0, 20·0, 20·9	300 to 600 μg of Mn, 60 to 95 mg of K, 700 to 1150 μg of Fe, 100 to 115 mg of Ca, 40 to 50 μg of Cu, 5 to 9 mg of P
Potato	20 μg of nickel	19·9, 19·7, 19·7, 19·6, 19·6, 19·9	50 μg of Mn, 10 to 35 mg of K, 300 to 600 μg of Fe, 30 to 45 mg of Ca,
Potato	20 μg of nickel + 600 μg of manganese + 600 μg of copper + 600 μg of iron	19·9, 19·9, 20·0, 19·9, 20·1, 20·0	10 to 20 μg of Cu, 4·5 to 6·5 mg of P, 2·5 to 7 mg of Mg
		Mean—19·91 (S.E. 0·07)	

Table II shows that none of the metals aluminium, cadmium, cobalt, copper, iron, manganese or zinc, either alone or in certain combinations, interfered in the determination of 50 μg of nickel. Phosphate equivalent to 10 mg of phosphorus also did not interfere. Table III shows the recovery of 20 μg of nickel added to each of twenty plant digest solutions. The added nickel was quantitatively recovered, the mean recovery was 19·9 μg with a standard error of 0·07 μg of nickel.

The author is indebted to Dr. J. T. Martin and to Dr. D. J. D. Nicholas for their helpful criticism of this note. Thanks are also due to Mr. R. T. Dewell and Mr. D. J. Fisher, who supplied the data in the last column of Table III.

REFERENCES

1. ter Haar, K., and Westerveld, W., *Rec. Trav. Chim. Pays-Bas*, **1948**, **67**, 71.
2. Sandell, E. B., "Colorimetric Determination of Traces of Metals," First Edition, Interscience Publishers Inc., New York, 1944.

AGRICULTURAL RESEARCH COUNCIL UNIT OF PLANT NUTRITION
(MICRONUTRIENTS)
LONG ASHTON RESEARCH STATION
BRISTOL

W. A. FORSTER
January 23rd, 1953

Apparatus

A CONTINUOUS DIFFERENTIAL REFRACTOMETER FOR CHROMATOGRAPHIC ANALYSIS

THE general tendency in methods of detecting the colourless zones in chromatography is to examine the eluate leaving the column rather than the column itself. This examination can be easily effected by automatic devices based on the determination of physical properties such as conductivity, light absorption, refractive index and radioactivity.

The essential requirement of any such method is that changes in concentration of the eluate should be determined rapidly on as small a sample as possible. This ensures the maximum resolution of adsorption bands. Moreover, it is necessary that the method should be applicable to the continuous determination of the concentration of a wide range of solvents and solutes. The method that satisfies these requirements and involves the minimum of technical difficulties is based on determinations of the refractive index.[1,2,3,4,5,6,7,8,9]

In the application of refractometry to the detection of colourless bands in adsorption chromatography, the difference in the refractive index rather than its absolute value is of most interest. For dilute solutions, the difference, Δn, of the refractive index of the solution and the solvent is directly proportional to the concentration.

In view of the high cost of commercial instruments for determining the refractive index, a simple device has been devised that can be constructed at a low cost.

DESCRIPTION OF THE APPARATUS—

The optical system is best understood by reference to Fig. 1. The source of light, S, is a G.E.C. 80-watt 230-volt A.C. mercury-vapour lamp enclosed in a lamp-house provided with a slit and suitably ventilated.

The current is obtained from the mains through the appropriate choke (G.E.C. 80-volt, 50-cycle) provided with the lamp.

The light is focused on to the slit A with the aid of the lens, L_1 (of short focal length), and rendered monochromatic by the filter, B.

The slit (made from two razor blades) is placed in the focal plane of lens L_2 ($f = 50$ cm) to give a parallel beam of light, and then focused by lens L_3 ($f = 100$ cm) on to a micrometer eye-piece, P, which has a 10-mm fixed scale and a drum divided into 0·01-mm divisions. The cell, D, is placed in the parallel beam of light between L_2 and L_3. The mirror, M, is used in order to shorten the length of the apparatus.

The inner cell (Figs. 2 and 3), through which the eluate passes, has a volume of approximately 0·1 ml, and is made from metal tubing of approximately 4 mm bore. A section of the tubing 1 cm in length is cut along two planes at 45° and provided with glass windows. The cell thus formed is positioned in the optical path of the instrument. The outer cell, which contains the pure solvent, is also made of metal except for the two parallel glass windows in the optical path. The inner cell also has a B7 standard joint to enable the fixing of interchangeable columns.

The relationship between the difference in the refractive index of solution, n_1, and solvent, n, the displacement, d, and the focal distance, f, can be derived in the following manner (Fig. 4).

According to the law of refraction—

$$\frac{\sin A}{\sin (A + B)} = \frac{n}{n_1}$$

and

$$\frac{\sin C}{\sin B} = n;$$

thus

$$n_1 - n = \frac{\sin C}{\sin B}\left(\frac{\sin A \cos B + \sin B \cos A}{\sin A} - 1\right)$$

and

$$n_1 - n = \sin C\left(\operatorname{cotan} B + \operatorname{cotan} A - \frac{1}{\sin B}\right) \quad .. \quad .. \quad (1)$$

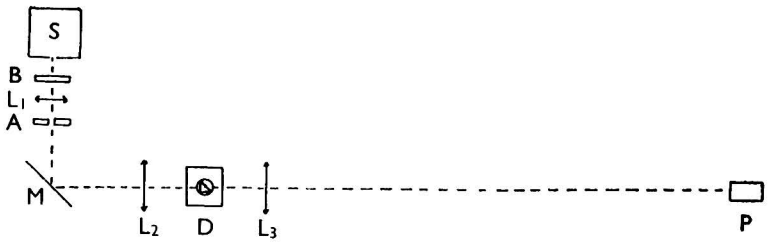

Fig. 1. Diagram of the optical arrangement

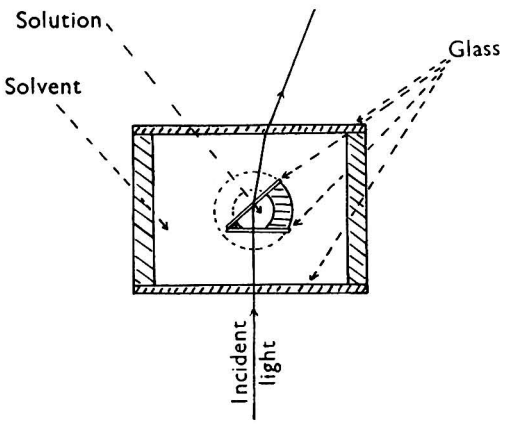

Fig. 2. Top view of cell

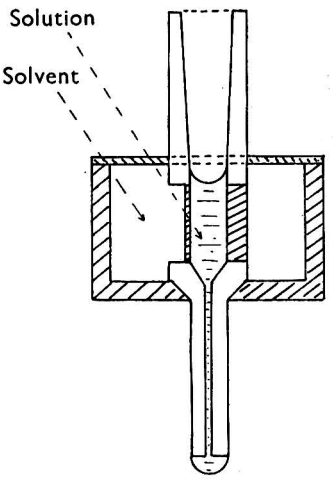

Fig. 3. Side view of cell

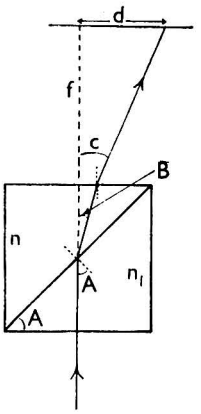

Fig. 4

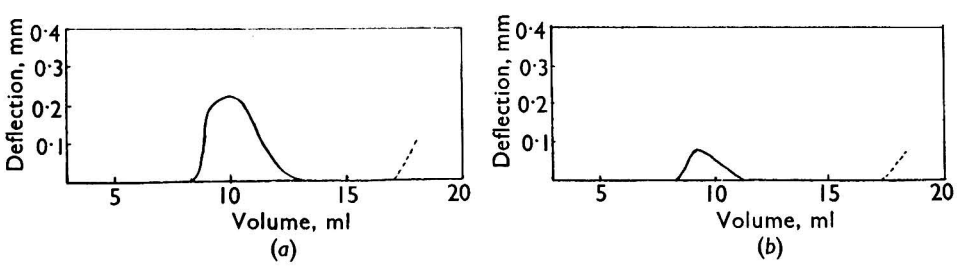

Fig. 5. Chromatographic separations of silicone oil from solutions of resins in benzene. Resins containing (*a*) 2 per cent. and (*b*) 0·4 per cent. of silicone oil

If the linear displacement, d, is small with respect to the focal length, f, so that angles B and C are small, then—

$$\sin C \simeq \tan C = d/f \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (2)$$

and $\sin B \simeq \tan B$.

Also, as angle A is 45°, expression (1) can be simplified to—

$$n_1 - n = d/f.$$

The refractive index difference is thus independent of the absolute value of the refractive index and is linear with the displacement as long as equation (2) is satisfied.

As in this instrument f is 1000 mm and d is measured to $\pm 0{\cdot}01$ mm, the differences between the refractive indices of solutions and solvent can be determined with an accuracy of $\pm 1 \times 10^{-5}$.

EXPERIMENTAL—

Fig. 5 shows typical diagrams of the separation of silicone oil of the methyl-silicone type contained in a poly-ester solution. The separation was carried out on alumina column with benzene as the eluting agent.

In Fig. 5 (*a*) the silicone oil content was 2 per cent., the amount present being 20 mg. In Fig. 5 (*b*) the silicone oil content was 0·4 per cent. and the amount initially on the column 4 mg.

The silicone oil fraction of the eluate is evaporated and identified by infra-red adsorption spectroscopy.[10]

Although the instrument is primarily intended for work on chromatography, it can also be used for other problems involving continuous measurement of refractive index.

The author acknowledges helpful discussion with Professor B. K. Johnson (Royal College of Science).

REFERENCES

1. Tiselius, A., *Trans. Faraday Soc.*, 1937, **33**, 524.
2. ——, *Arkiv. Kemi. Min. Geol., B*, 1940, **14**, No. 22.
3. ——, *Advanc. Colloid Sci.*, 1942, **1**, 81.
4. Tiselius, A., and Claesson, S., *Arkiv. Kemi. Min. Geol., B*, 1942, **15**, No. 18.
5. Tiselius, A., *Endeavour*, 1952, **11**, No. 41, 5.
6. Claesson, S., *Arkiv. Kemi. Min. Geol., A*, 1946, **23**, No. 1.
7. ——, *Ibid.*, 1949, **26**, No. 24.
8. Zaukelies, D., and Frost, A., *Anal. Chem.*, 1949, **21**, 743.
9. Holman, R. T., and Hagdall, L., *Ibid.*, 1951, **23**, 794.
10. Richards, E. R., and Thompson, H. W., *J. Chem. Soc.*, 1949, 124.

IMPERIAL CHEMICAL INDUSTRIES LTD.
PAINTS DIVISION
WEXHAM ROAD
SLOUGH, BUCKS.

H. MCCORMICK
December 29th, 1952

(The instrument described was exhibited at the International Congress on Analytical Chemistry held at Oxford, September 4th to 9th, 1952.)

THE CONSTRUCTION OF POLAROGRAPH CAPILLARIES FROM PYREX GLASS ROD

SAMPLES of Pyrex glass rod frequently contain very thin capillaries, generally about 5 to 10 cm long, which are formed presumably by the drawing out of tiny air bubbles during the manufacturing processes. These capillaries are best observed by holding the rod against a bright light. We have found the capillaries to be convenient for the construction of dropping-mercury micro-electrodes.

The Pyrex glass rod is first cut at one end of the capillary and attached at this end to a source of compressed air. The other end of the capillary is then rotated in a blow-pipe flame until it expands to form a short section of relatively wide-bore tubing. This can then be sealed on to semi-capillary Pyrex tubing with the aid of a small blow-pipe flame.[1,2]

The thickness of the Pyrex rod (we have used rod having an external diameter of 0·6 cm) makes the capillary robust. The internal diameters of the capillaries are of the order 0·01 mm. An even narrower capillary can be produced, if necessary, by drawing out the rod and cutting it at the constriction. If the tip of the capillary is ground flat, any tendency for the mercury drops

to slide off one side of the tip is precluded. Otherwise, irregular drop times may be produced. The constancy of drop-time attainable with such a capillary is illustrated in Table I.

TABLE 1

CONSTANCY OF DROP-TIME WITH PYREX GLASS CAPILLARIES

Date	Time for 20 drops, seconds
December, 1949	87·8, 87·4, 87·8
September, 1950	87·6, 87·4, 87·4, 87·8
September, 1951	87·8, 87·6, 87·6, 87·4
May, 1952	87·4, 87·4, 87·8, 87·6

Length of capillary = 1·45 cm. Mercury head = 60 cm.
Tip immersed in 0·1 *N* potassium chloride solution at 25 ± 0·02° C.
m = 1·783 mg per second.

REFERENCES

1. Stock, J. T., *Metallurgia*, 1944, **31**, 102.
2. Milton, R. F., and Waters, W. A., "Methods of Quantitative Micro-analysis," Edward Arnold & Co., London, 1949, p. 441.

INORGANIC CHEMISTRY LABORATORY
OXFORD

D. J. FERRETT
C. S. G. PHILLIPS
January 21st, 1953

Ministry of Food

STATUTORY INSTRUMENTS*

1953—No. 1125. The Feeding Stuffs (Revocation) Order, 1953. Price 2d.

This Order, which came into operation on August 1st, 1953, abolishes all controls on the manufacture, distribution and price of feeding stuffs.

Included in the Schedule of Orders Revoked is The Feeding Stuffs (Manufacture) Order, 1952 (S.I., 1952, No. 375) and the Amending Order (S.I., 1952, No. 1652).

1953—No. 1277. The Oils and Fats (No. 2) Order, 1953. Price 4d.

This Order, which came into operation on August 23rd, 1953, replaces the Oils and Fats Order, 1953 (S.I., 1953, No. 210; Analyst, 1953, 78, 187), as amended (S.I., 1953, Nos. 865 and 1155). The principal changes are—

(i) *The definition of "cooking fats" is revised to exclude all oils and fats other than mixtures containing any of the oils shown in the Second Schedule to the Order.*

(ii) *Restrictions on the sale of technical tallow and rendered pig fat other than lard are removed and the definition of "lard" is amended to exclude the fat obtained by cooking pork.*

THE SECOND SCHEDULE

Any of the following oils, in any state of manufacture whether crude, refined, deodorised, hardened, pressed or bleached:—

Coconut Oil.
Cotton Seed Oil.
Gingelly (Sesame) Oil.
Ground Nut Oil.
Herring Oil.
Kapok Seed Oil.
Maize Oil.
Palm Oil.
Palm Kernel Oil.
Pilchard Oil.
Seal Oil.
Shea Butter or Oil.
Soya Bean Oil.
Sunflower Seed Oil.
Whale Oil.

Any residue arising from the refining of any of the above oils.

Any mixture of any two or more of the above oils and residues and any mixture of any one or more of the above oils and residues with any other oil, not being margarine or cooking fats as defined in Article 2 of this Order.

* Obtainable from H.M. Stationery Office. Italics indicate changed wording.

1953—No. 1282. The Flour Order, 1953. Price 4d.

This Order, which came into operation on August 30th, 1953, revokes and replaces the Flour Order, 1952 (S.I., 1952, No. 749), as amended (S.I., 1952, No. 2041 and S.I., 1953, No. 582). The principal changes are—

(1) *The definition of National Flour is now as follows—*

"National Flour" means flour complying with the following conditions:—

(*i*) it shall contain the maximum quantity of wheat germ which, having regard to the type of milling, can be included in such flour;

(*ii*) it shall not include any coarse or added bran; and

(*iii*) it shall consist of wheat flour and shall either be of 80 per cent. extraction or shall be of substantially the same nature and contain substantially the same quantities and proportions of constituents as flour of 80 per cent. extraction.

(2) *The existing control of production and packing is abolished, as also are the controls on distribution, use and price.*

(3) *The addition of creta praeparata to all flour is now required unless it is flour which contains the whole product of the milling of wheat and no additions whatsoever.*

(4) *The restoration of certain specified nutrients is required to be made to all flours of an extraction rate of less than 80 per cent.*

(5) *Containers containing National Flour or National Brown Flour for sale otherwise than by retail must be marked or labelled to show their contents.*

COMPULSORY ADDITIONS

Type of Flour	Required Addition	Milligrams
All Flour	Creta Praeparata of granularity standard (*i*) not more than 0·1 per cent. to remain on 100 mesh sieve, and (*ii*) not more than 0·2 per cent. to remain on 240 mesh sieve. At the rate of 14 oz. per 280 lb.	
Flours of an extraction rate less than 80 per cent.	Iron .. .. .. In sufficient quantity to ensure a minimum content of ..	1·65
	Vitamin B_1	0·24
	Nicotinic Acid ..	1·60

per 100 grams of flour in a form authorised by the Minister and conforming to the standards of the British Pharmacopoeia or the British Pharmaceutical Codex.

1953—No. 1283. The Bread Order, 1953. Price 4d.

This Order, which came into operation on August 30th, 1953, revokes and replaces the Bread Order, 1952 (S.I., 1952, No. 1781). The definition of national bread is revised and a definition of national brown bread is now provided.

THE FIRST SCHEDULE

Substances other than national flour which may be included in national bread and which (in addition to national brown flour or a mixture of national flour and national brown flour) may be included in national brown bread.

Oils and fats
Water
Salt
Yeast
Improvers of the nature of yeast food
Any acid or acidic substances suitable for regulating the acidity of the dough
Barm
Any substance required by the Flour Order, 1953, to be added to national flour or national brown flour.

British Standards Institution

AMENDMENT SLIP*

A PRINTED slip bearing amendments to a British Standard has been issued by the Institution, as follows—

PD 1655—Amendment No. 3 (July, 1953) to B.S. 188:1937. Method for the determination of viscosity of liquids in absolute (C.G.S.) units.

DRAFT SPECIFICATIONS

A FEW copies of the following draft specifications, issued for comment only, are available to members of the Society, and can be obtained from the Secretary, Society of Public Analysts and Other Analytical Chemists, 7–8, Idol Lane, London, E.C.3.

Draft Specification prepared by Technical Committee LBC/16—Glass Electrodes.
CR(LBC)4106—Draft B.S. for Glass Electrodes.

Draft Specification prepared by Panel CHE/37/1/1—Analysis of Waters Used in Steam Generation (Editing).
CR(CHE)4245—Draft B.S. Methods of Test for Water Used in Industry.

Book Reviews

MÉTHODES ET RÉACTIONS DE L'ANALYSE ORGANIQUE. Volume II. MÉTHODES DE CARACTÉRISATION. By M. PESEZ and P. POIRIER. Pp. vi + 278. Paris: Masson et Cie. 1953. Price 2500 fr.

The classification adopted here is somewhat unusual in that it follows the type of reaction (*i.e.*, in effect the reagent) rather than the nature of compound to be characterised.

The first chapter deals with alkylation and arylation; these terms are used in a broad sense to include the use of chloroacetic acid for phenols, nitrobenzyl halides for phenols, acids and barbiturates, chlorodinitrobenzene for phenols and mercaptans, various phenacyl bromides for acids, xanthhydrol for amides, dinitrothiophenol for alkyl halides, and so on. The chapter opens with a plate showing four photomicrographs of appropriate compounds, then discusses briefly the various reactions concerned, gives the reagents, nearly always with adequate detail for their preparation, and the methods for their application and concludes with tabulated lists of melting points. The other chapters discuss on similar lines acylation; ureas, carbamates and allophanates; amidation; oximes and hydrazones; salts and addition compounds; miscellaneous reactions. As a result of the method of classification, the tables of derivatives of phenols, for example, are distributed throughout the book, but they can readily be traced from the quite adequate index.

A feature which is uncommon in a book of this kind is the inclusion of extensive references to original literature. The text is even easier to follow than that of the earlier volume in this series (*Analyst*, **1953, 78**, 70).

B. A. ELLIS

VISUAL LINES FOR SPECTROSCOPIC ANALYSIS. By D. M. SMITH. Second Edition. Pp. 102. London: Hilger & Watts Ltd. 1952. Price 16s.

This is a second edition of a booklet originally published in 1928, when spectroscopic analysis was at a much earlier stage of development than it is to-day. The first volume was compiled to encourage the wider adoption of visual spectroscopy by those engaged in chemical analysis, and it contained tables of the wavelengths of the most sensitive lines for 52 elements. The original form has been retained, but data for 72 elements are now given, and a second table listing the lines in decreasing order of wavelength is included. The brief introductory matter has been expanded by short notes on excitation sources, and a section dealing with the detection of the halogens, selenium and sulphur, follows the principal wavelength tables.

The advances in spectrographic techniques, and the wealth of reference literature that has become available during the past twenty-five years, have made the kind of information assembled in this book to a large extent unnecessary. Visual methods are not often adopted except in a few experimental problems, so that it is very doubtful whether those in serious practice in the spectroscopic field would ever want to consult data in this form. Those who think that the information could be helpful will find the tables clearly printed and the binding durable enough for a handy book of reference.

H. P. ROOKSBY

* Obtainable from the British Standards Institution, Sales Department, 2, Park Street, London, W.1.

A HANDBOOK OF COLORIMETRIC CHEMICAL ANALYTICAL METHODS FOR INDUSTRIAL, RESEARCH AND CLINICAL LABORATORIES. DEVELOPED FOR USE WITH THE LOVIBOND COMPARATOR. Pp. xii + 119. Salisbury: The Tintometer Ltd. 1953. Price 15s.

Many analysts will know that the Lovibond comparator consists of a viewing instrument used in conjunction with the Lovibond discs, which contain a series of permanent glass colour standards. It may be recalled that each disc is designed for a particular colorimetric determination that must be conducted according to instructions printed on an accompanying pamphlet. This handsome and well-bound book is a collection of all pamphlets so far issued, apart from those concerned with specialised commercial grading tests and with toxic gases in air. The value and popularity of this means of applying colorimetric analysis may be judged from the fact that this collection consists of 56 monographs, excluding those that apply to the measurement of pH.

Each monograph of instructions is clearly written and conveniently sub-headed while references to the original papers are given. It is known to the reviewer that the standardisation of the discs has been carried out by direct personal collaboration, either with the originators of the colorimetric methods or with experts in the particular field of analysis involved. As new standard discs are produced so further monographs are published and provision is made in the binding for adding new pages, whilst each volume is individually dated before issue.

No high degree of precision is claimed for this method of applying colorimetric procedures, but in practice it is often sufficiently accurate for process control, and it has also proved to be popular for applying many of the routine determinations involved in the day-to-day work of chemical pathologists. These different fields may be typified by such diverse determinations as carbon disulphide in benzole and bilirubin in blood-serum. N. L. ALLPORT

Publications Received

GLYCEROL. American Chemical Society Monograph Series No. 117. Edited by CARL S. MINER and N. N. DALTON. Pp. xiv + 460. New York: Reinhold Publishing Corp.; London: Chapman & Hall Ltd. 1953. Price 96s.; $12.00.

A SIMPLE GUIDE TO MODERN VALENCY THEORY. By G. I. BROWN, B.A., B.Sc. Pp. x + 174. New York and London: Longmans, Green & Co. Ltd. 1953. Price 12s. 6d.

LUMINESCENCE AND THE SCINTILLATION COUNTER. By S. C. CURRAN, F.R.S. Pp. x + 219. London: Butterworths Scientific Publications. 1953. Price 32s. 6d.

CHEMISTRY OF CARBON COMPOUNDS. Volume II, Part A. ALICYCLIC COMPOUNDS. Edited by E. H. RODD, A.C.G.I., D.I.C., D.Sc., F.R.I.C. Pp. xxiv + 488. Amsterdam, London and New York: Elsevier Publishing Co.; London: Cleaver-Hume Press Ltd. 1953. Price 84s.

STARCH: ITS SOURCES, PRODUCTION AND USES. By CHARLES ANDREW BRAUTLECT. Pp. vi + 408. New York: Reinhold Publishing Corp.; London: Chapman & Hall Ltd. 1953. Price $10.00; 80s.

BRITISH STANDARDS INSTITUTION YEARBOOK, 1953. Pp. 488. London: British Standards Institution. 1953. Price 12s. 6d.

ORGANIC CHEMISTRY. AN ADVANCED TREATIS. Volumes III and IV. Editor-in-Chief HENRY GILMAN. Pp. xxxviii + 1–580; xxxviii + 581–1246. London: Chapman & Hall Ltd.; New York: John Wiley & Sons Inc. 1953. Price 70s., $8.75; 70s., $8.75.

CRYSTAL STRUCTURES. Volume III. By RALPH W. G. WYCKOFF. Loose-leaf. With Supplement II. New York and London: Interscience Publishers Inc. 1953. Price for Volume III $14.50, 116s.; for Supplement II $4.00, 32s.

REPORTS ON THE PROGRESS OF APPLIED CHEMISTRY. Volume XXXVII. Edited by F. CLARK, B.A., B.Sc. Pp. 983. London: The Society of Chemical Industry. 1952. Price 40s.

SYNTHETIC ORGANIC CHEMISTRY. By ROMEO B. WAGNER and HARRY D. ZOOK. Pp. xii + 887. New York: John Wiley & Sons Inc.; London: Chapman & Hall Ltd. 1953. Price $11.50; 92s.

THE NATIONAL FORMULARY 1952: FIRST AMENDMENT 1953. Pp. 16. London: The British Medical Association and The Pharmaceutical Press. 1953. Price 6d.

CHROMATOGRAPHIC METHODS OF INORGANIC ANALYSIS: WITH SPECIAL REFERENCE TO PAPER CHROMATOGRAPHY. By F. H. POLLARD, B.Sc., Ph.D., and J. F. W. MCOMIE, M.A., D.Phil. Pp. viii + 192. London: Butterworths Scientific Publications Ltd. 1953. Price 30s.

GENERAL BIOCHEMISTRY. By JOSEPH S. FRUTON and SOFIA SIMMONDS. Pp. xii + 940. New York: John Wiley & Sons Inc.; London: Chapman & Hall Ltd. 1953. Price $10.00; 80s.

Volume 78. No. 930, Pages 509-568 September, 1953

THE ANALYST

THE JOURNAL OF
THE SOCIETY OF PUBLIC ANALYSTS AND OTHER ANALYTICAL CHEMISTS

CONTENTS

Printed and Published for the Society of Public Analysts and Other Analytical Chemists by W. Heffer & Sons Ltd., Cambridge, England. **Communications to be addressed to the Editor, F. L. Okell, 7-8, Idol Lane, London, E.C.3.** Enquiries about advertisements should be addressed to Walter Judd Ltd., 47, Gresham Street, London, E.C.2.

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