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Draft Specification prepared by Technical Committee LBC/5—Hydrometers.
CV(LBC)5280—Draft B.S. for Density - Composition Tables for Aqueous Solutions of Hydrochloric Acid.

Draft Specification prepared by Technical Committee FCC/4—Solvents and Allied Products.
CV(FCC)5112—Draft B.S. for Dipentene.

COMMUNICATIONS ACCEPTED FOR PUBLICATION IN *THE ANALYST*

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

"The Separation of Group IIA Elements of the Periodic Table by Paper Chromatography," by R. J. Magee and J. B. Headridge.

"The Absorptiometric Determination of Polyethyleneglycol Mono-oleate," by E. G. Brown and T. J. Hayes.

"The Separation and Quantitative Determination of Platinum, Palladium, Rhodium and Iridium on Paper Strips," by N. F. Kember and R. A. Wells.

"The Use of Lithium Hydroxide for the Qualitative Separation of the Copper and Arsenic Groups," by C. F. James and P. Woodward.

"The Analysis of Phosphating Preparations used for the Protection of Steel Surfaces," by G. H. Bush, D. G. Higgs and F. W. Box.

"The Determination of *cyclo*Pentadiene and Maleic Anhydride," by P. Unger.

"The Action of Sulphuric Acid in Colour Reactions of Organic Compounds: Detection of Monochloroacetic acid and other Compounds Splitting off Formaldehyde," by F. Feigl and R. Moscovici.

"The Volumetric Assay of some Binary Alloys of Palladium," by R. H. Atkinson, R. N. Rhoda and R. G. Lomell. (Note.)

"The Detection of Dicyanogen on the Micro Scale: a Contribution to the Chemistry of Specific, Selective and Sensitive Reactions," by F. Feigl and L. Hainberger.

"The Determination of Lead in Organic Material," by J. C. Gage.

"New Spray Reagents for Paper Chromatography of Barbiturates," by E. Hjelt, K. Leppänen and V. Tamminen. (Note.)

"An Improved Volumetric Method for Zirconium," by G. W. C. Milner and J. W. Edwards.

"A Simple Storage Burette for Air-sensitive Solutions," by A. G. Hamlin. (Apparatus.)

"Examination of an Interpolymer of Polycaproamide, Polyhexamethylenediamine-adipamide and Poly-*p*-diaminodi*cyclo*hexylmethaneadipamide (Nylon 6, Nylon 66 and Nylon PACM 6)," by M. Clasper, J. Haslam and E. F. Mooney.

"The Identification of Fuel Oils Polluting Coastal Waters," by J. K. Johannesson. (Note.)

"The Determination of Sulphur in Plain Carbon Steel," by R. Belcher, D. Gibbons and T. S. West.

"The Determination of Thiamine in Vitamin Concentrates containing Creta Preparata," by H. N. Ridyard.

"Sucrose Loss from Ice-cream on Storage," by H. J. Evans, W. Kwantes, D. C. Jenkins and J. L. Phillips.

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No. 29 July, 1955

THE SOCIETY FOR ANALYTICAL CHEMISTRY

BULLETIN

INTERNATIONAL UNION OF PURE AND APPLIED CHEMISTRY

ANALYTICAL SECTION

International Congress on Analytical Chemistry, Lisbon, September 9th to 16th, 1956

PROFESSOR I. M. Kolthoff, President of the Analytical Section of the International Union of Pure and Applied Chemistry, has announced that the Congress on Analytical Chemistry to be held in Lisbon from September 9th to 16th, 1956, will be under the patronage of the International Union of Pure and Applied Chemistry. Professor Fernando Pires de Lima, Minister of Education, has appointed Professor D. Antonio Pereira Forjaz to be President of the Organising Committee. Arrangements for the meeting are in the hands of Professor Pierre A. Laurent, Instituto Superior Tecnico, Avenida Rovisco Pais, Lisbon, Portugal. Inquiries regarding plans for the meeting should be directed to him. It is intended that the Congress should cover broadly all aspects of analytical chemistry. Further details will be published as soon as they become available.

PROFESSOR Pierre Laurent has announced that the following sections have already been selected for the Congress—

1. Microchemical Methods.
2. Biological Methods.
3. Electrical Methods.
4. Optical Methods.
5. Radiochemical Methods.
6. Organic Complexes.
7. Statistical Interpretation of Results.
8. Adsorption and Partition Methods.
9. General.
10. Standardisation of Methods and Miscellaneous Applications.

An abstract of any paper that authors in the United Kingdom and the Colonies propose to submit to this Congress should be sent to The British National Committee for Chemistry, The Royal Society, Burlington House, London, W.1.

MEETINGS FOR THE PRESENTATION OF ORIGINAL PAPERS

THE Society proposes to devote two ordinary meetings in the forthcoming session, those on Wednesday, October 5th, 1955, and Wednesday, April 4th, 1956, to the presentation by their authors of papers submitted for publication in *The Analyst*. All unpublished papers in the hands of the Editor not less than six weeks before the dates of the meetings will be considered for such presentation. Authors wishing to have an opportunity of presenting a paper at a meeting of the Society are asked to note this.

BRITISH STANDARDS INSTITUTION

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, The Society for Analytical Chemistry, 7–8 Idol Lane, London, E.C.3.

THE ANALYST

PROCEEDINGS OF THE SOCIETY FOR ANALYTICAL CHEMISTRY

HONORARY MEMBERS

THE Council is very pleased to record that—

Professor Dr. Eng. Fritz Feigl, Ministry of Agriculture, Rio de Janeiro;
Professor N. Howell Furman, Princeton University;
Professor J. Heyrovský, Central Institute of Polarography, Prague;
Professor I. M. Kolthoff, University of Minnesota;

have been elected Honorary Members of the Society.

ORDINARY MEETINGS

AN Ordinary Meeting of the Society, organised by the Physical Methods Group, was held at 7 p.m. on Wednesday, April 6th, 1955, in the meeting room of the Chemical Society, Burlington House, London, W.1. The Chair was taken by the President, Dr. K. A. Williams, F.R.I.C., A.Inst.P., M.Inst.Pet.

The subject of the meeting was "End-point Detection by Physical Methods," and the following papers were presented and discussed: "The Location of the End-point in Titrimetric Procedures," by E. Bishop, B.Sc., A.R.T.C., A.R.I.C.; "End-point Determination by High-frequency Methods," by J. P. Dowdall, B.Sc., A.R.C.S., D.I.C., A.R.I.C., D. V. Sinkinson and H. Stretch, A.R.I.C.; "Spectrophotometric Titrations," by R. A. Chalmers, B.Sc., Ph.D.; "A Short Account of the Scope and Precision of Amperometric Titration," by J. Watt, B.Sc.

AN Ordinary Meeting of the Society was held at 7 p.m. on Wednesday, May 4th, 1955, in the meeting room of the Chemical Society, Burlington House, London, W.1. The Chair was taken by the President, Dr. K. A. Williams, F.R.I.C., A.Inst.P., M.Inst.Pet.

The following papers were presented and discussed: "The Detection and Determination of Traces of Polynuclear Hydrocarbons in Industrial Effluents and Sewage. Part III. The Examination of Some Gasworks Effluents," by P. Wedgwood, M.Sc., M.Inst.Gas E., M.Inst.F., A.Inst.S.P., F.R.I.C., and R. L. Cooper, M.Sc., Ph.D., A.M.Inst.Gas E., A.R.I.C.; "The Analysis of Mixtures of Phenols by Partition Chromatography and Ultra-violet Spectrophotometry," by R. M. Pearson, A.R.I.C.; "The Determination of Traces of Benzene Hexachloride in Water and Sewage Effluents," by W. Hancock, B.Sc., and E. Q. Laws, B.Sc., F.R.I.C.

NEW MEMBERS

ORDINARY MEMBERS

Roy William Adams, B.Sc. (Lond.); Reuben Lloyd Barker, B.Sc., Ph.D.; Bernard Braithwaite, B.Sc. (Notts.); Alexander C. Bushnell, F.R.I.C.; William Alexander Dickie, B.Sc. (Lond.), F.R.I.C.; Geoffrey James Sinclair Haughton, A.M.C.T., A.R.I.C.; Kenneth John Hayden, A.R.I.C.; Norman Charles Knight, B.Sc. (Lond.), A.R.I.C.; Agnes Elizabeth McCarter, A.R.T.C., A.R.I.C.; George Frederick Smith, Ph.D.

JUNIOR MEMBERS

Jean Burn, B.Sc. (Lond.); Joseph Dawson, B.Sc. (Edin.); Robert Alexander Sutter, B.Sc. (Edin.); Clive Vickers, B.Sc. (Lond.), A.R.I.C.

NORTH OF ENGLAND SECTION

AN Ordinary Meeting of the Section was held at 2.15 p.m. on Saturday, April 30th, 1955, at the City Laboratories, Mount Pleasant, Liverpool, 3. The Chair was taken by the Chairman of the Section, Mr. J. R. Walmsley, A.M.C.T., F.R.I.C., F.P.S.

A lecture entitled "Margarine" was given by W. L. Wren, B.Sc., F.R.I.C.

SCOTTISH SECTION

AN Ordinary Meeting of the Section was held at 7.15 p.m. on Thursday, April 28th, 1955, in the George Hotel, Edinburgh, when the following original papers were presented and discussed: "The Determination of Small Amounts of Zinc in Various Materials," by J. A. Hunter, B.Sc.; "The Determination of Calcium and Magnesium in Plant Material Using Disodium Ethylenediaminetetra-acetate," by Miss E. S. R. McCallum, B.Sc., and A. M. Smith, Ph.D., D.Sc., F.H.-W.C., F.R.S.E., F.R.I.C.; "The Chromatographic Separation and Determination of Alkaline Earth and Alkali Metals," by J. B. Headridge, B.Sc., and R. J. Magee, M.Sc., Ph.D.

Comments on the Determination of Vitamin A in Natural Products and Especially Cod-liver Oils

BY R. A. MORTON AND F. BRO-RASMUSSEN

(*Presented at the meeting of the Society on Wednesday, December 1st, 1954*)

In view of the lack of official recognition of vitamin A_2 it is not perhaps desirable to express the results of spectrophotometric assays in terms of total vitamin-A activity without indicating how much is due to vitamin A_2. Liver oils from salt-water fish in general have at least 90 per cent. of the total activity supplied by vitamin A_1.

The analytical procedures of the British and United States Pharmacopoeias are discussed in relation to the historical setting and the newer problems arising out of the presence of three vitamin-A-active substances (all-*trans* and neovitamin A_1 and vitamin A_2) in fish-liver oils.

The properties of the three active substances are noted and the combined effects of their intrinsic biological activities and their absorption spectra are worked out with special reference to conversion factors.

The geometrical correction procedure by means of the fixation points for all-*trans* vitamin A_1 over-corrects the neovitamin-A contribution to the total absorption, but this is very nearly balanced by the lower biological activity of neovitamin A compared with the all-*trans* form.

Three cod-liver oils and two rich oils have been examined by the new chromatographic method of Bro-Rasmussen, Hjarde and Porotnikoff, which permits quantitative estimate of how the total vitamin-A absorption at 325 mμ is distributed between the three active substances. The same oils tested on the unsaponifiable fraction without chromatography but corrected by the "geometrical" procedure lead to much the same estimate of total vitamin-A_1 potency.

THE determination of vitamin-A activity has received a great deal of attention during the past 25 years from individuals or groups of workers and from official bodies. The analyst's legitimate goal is the "right" answer, the very concept of which is robbed of simplicity by the fact that at least three distinct substances (all-*trans* vitamin A_1, neovitamin A_1 and vitamin A_2) contribute to the biological potency. For many purposes a close approximation to the answer which would be obtained by unusually detailed study is good enough, given a convenient method reproducible from one laboratory to another and commanding a wide measure of acceptance.

It will be recalled that provitamin A (carotene) became available as a pure crystalline substance much earlier than vitamin A and that the first unit was 1 μg of carotene (a mixture

of α- and β-carotene), which was later replaced by 0·6 μg of pure β-carotene. The β-carotene standard and the International Standard Preparation played a very important part in subsequent developments. It had been recognised that in principle the ultra-violet absorption spectrum of vitamin A and the antimony trichloride colour test were both capable of replacing the tedious and not very precise bio-assays. The physico-chemical methods had perforce to be linked with biological activity by means of "conversion factors," and these were based on numerous large-scale biological tests. After much careful collaborative work in several countries it was agreed that a conversion factor of 1600 (*i.e.*, $E^{1\%}_{1cm}$ at 328 mμ, the agreed measure of vitamin-A absorption, multiplied by 1600) would give the potency in international units per gram. For a very wide range of fish-liver oils this factor gave results agreeing with the colour test and with many bio-assays.

It was known that relatively low-potency oils such as cod-liver oils exhibited at 328 mμ a substantial amount of irrelevant absorption, most of which was eliminated by preparing the unsaponifiable extract. For such oils the conversion factor of 1600 was only applied to the results obtained with the unsaponifiable portion. Some very rich oils or ester concentrates exhibited negligible irrelevant absorption ($E^{1\%}_{1cm}$ at $\lambda_{max.}$ was practically the same for the oil and for its unsaponifiable portion expressed in terms of equal weights of original oil), and it became doubtful whether a conversion factor of 1600 was high enough for such materials. Indeed bio-assays (against β-carotene) led to 1800 as the conversion factor appropriate to the best concentrates of vitamin A prepared from fish-liver oils.

In order to refine the spectrophotometric method it is necessary to eliminate irrelevant absorption. Absorbing impurities may be removed by a chromatographic separation or they may be discounted by a correction procedure, which allows for the distortion of the vitamin-A curve by impurities.

The problem of effecting without loss a complete chromatographic separation has not been solved and there are special difficulties with materials containing oxidation products and vitamin-A artefacts. The correction procedure of Morton and Stubbs,[1] which took advantage of the accuracy of measurements of absorption intensity with photo-electric spectrophotometers, rested upon two assumptions: (*a*) that the irrelevant absorption over the wavelength range 310 to 340 mμ was linear (*i.e.*, E/λ a straight line) and (*b*) that an abridged specification of the absorption spectrum of the purest vitamin A would apply to the vitamin A in the material to be assayed.

The widespread adoption of this method coincided in time with increasing availability of pure crystalline all-*trans* vitamin-A alcohol and acetate. The re-definition of the international unit as the activity of 0·3 μg of vitamin-A alcohol (or its equivalent of acetate) fixed the potency as $3{\cdot}3 \times 10^6$ i.u. per gram and the conversion factor (or better, factors) followed from the measurements of $E^{1\%}_{1cm}$ at $\lambda_{max.}$ in various solvents (*cf.* Cama, Collins and Morton[2]). The conversion factor 1900 recommended by the 1949 World Health Organisation Conference was accepted by the British and United States Pharmacopoeias.

Even at the time when pure all-*trans* vitamin-A acetate was chosen as the International Standard substance it was realised that there were further problems in the background. It was known, for instance, that natural vitamin A was a variable mixture of all-*trans* vitamin A and at least one *cis* isomer, neovitamin A. The properties of neovitamin A were less well known than those of the all-*trans* form and it was not at all clear whether the proportion of neovitamin A to all-*trans*, taken in conjunction with any differences in the ultra-violet absorption curves, could seriously influence the application of the Morton - Stubbs correction.

It was also known that, although mammalian-liver oils did not contain vitamin A_2, fish-liver oils in general contained perhaps 1 molecule of vitamin A_2 for 5 to 25 molecules of vitamin A_1.

The procedures described in the British and United States Pharmacopoeias are thus in principle very suitable for such medicinal or food products as contain synthetic all-*trans* vitamin A; they are less precise for products containing mammalian-liver oils or concentrates, because no guidance is given concerning neovitamin A, and in respect of vitamin A_2 there is official silence.

In fact, then, the determination of vitamin A in cod-liver oil, for instance, rests to-day upon an officially sanctioned convention which gives a serviceable approximation. The purposes of this paper are (*i*) to discuss the nature of the approximation and (*ii*) to compare two different ways of testing its validity.

A number of preliminary points may be noted—

(*i*) some of the less potent cod-liver oils exhibit absorption curves (measured on solutions of oil in *cyclo*hexane) with irrelevant absorption considerably more intense than the aggregate absorption due to the two forms of vitamin A_1 and the vitamin A_2, so that geometrical correction procedures are of no value in such circumstances,

(*ii*) although irrelevant absorption may be very largely removed (by preparing the unsaponifiable fraction or a chromatographic fraction containing all the vitamins A), the problem of evaluating mixtures of *cis* and *trans* vitamin A_1 and of vitamin A_2 is not thereby advanced in any way,

(*iii*) the antimony trichloride colour test has little quantitative validity when carried out on cod-liver oils (unsaponified), because it is substantially and variably inhibited. If, however, the colour test is applied to unsaponifiable matter and measured by means of a spectrophotometer, the relative proportions of vitamins A_1 and A_2 with $\lambda_{max.}$ at 620 mμ and 693 mμ, respectively, can be determined with some confidence. The absolute amount of vitamin A_1 may however be less certain, because the intensity of absorption at 620 mμ may be influenced to some extent by residual inhibition and by variations in the relative proportions of *cis* and *trans* forms,

(*iv*) the determination of relative proportions of all-*trans* and neovitamin A by the maleic anhydride procedure of Robeson and Baxter[3] is probably reasonably accurate but too complicated and tedious for routine work,

(*v*) the absorption curves of the purest specimens of all-*trans* vitamin A (or its acetate) and those of the purest neovitamin A (or its acetate), although very similar, are not identical. This lack of identity holds in all the solvents commonly used and applies both to the shape of the curves on the wavelength scale and the maximal intensities of absorption. The absorption spectrum of vitamin A_2 with maxima at 350 and 286 mμ is quite different from that of vitamin A_1,

(*vi*) the biological potencies of neovitamin A_1 and of vitamin A_2 expressed in international units (0·3 μg of all-*trans* vitamin A_1) have not been determined in many laboratories and must be regarded as known only approximately. The main properties are summarised in Table I.

TABLE I

PROPERTIES OF VITAMIN-A ALCOHOLS

	All-*trans* vitamin A_1	Neovitamin A_1	Vitamin A_2
Potency, i.u. per g × 10^6	3·33*	2·66†	1·33‡
$\lambda_{max.}$ in *cyclo*hexane, mμ	326	328	351
$E^{1\%}_{1cm}$ at $\lambda_{max.}$ in *cyclo*hexane	1745	1650	1350
$E^{1\%}_{1cm}$ at 326 mμ in *cyclo*hexane	1745	1615	1050
Conversion factor (*i.e.*, potency/$E^{1\%}_{1cm}$ at 326 mμ) ..	1908	1647	1267
$\lambda_{max.}$ in ethanol, mμ	325	328	350
$E^{1\%}_{1cm}$ at $\lambda_{max.}$ in ethanol	1800	1720	1460
$E^{1\%}_{1cm}$ at 325 mμ	1800	1685	1060
Conversion factor (*i.e.*, potency/$E^{1\%}_{1cm}$ at 325 mμ) ..	1850	1579	1255

* By definition.
† Harris *et al.*[4]
‡ Shantz and Brinkmann.[5] Spectroscopic properties of neovitamin A from Chatain and Debodard.[6] Properties of vitamin A_2 from Shantz[7] and from Cama and Morton.[8]

The proportion of vitamin A_2 in cod-liver oils varies over a narrow range, but for the purpose of illustration let it be assumed that for every molecule of A_2 there are 8 of A_1, 6 as

all-*trans* and 2 as neovitamin A. With *cyclo*hexane as solvent, the intensity of absorption for such a mixture would be—

	$E^{1\%}_{1cm}$ at $\lambda_{max.}$
All-*trans* contribution 6/9 of 1745	1163·3
Neovitamin-A contribution 2/9 of 1615	358·9
Vitamin-A_2 contribution 1/9 of 1050	116·7
	1638·9

The potency would be—

All-*trans* contribution 6/9 of $3{\cdot}33 \times 10^6$	$2{\cdot}22 \times 10^6$ i.u.
Neovitamin-A contribution 2/9 of $2{\cdot}66 \times 10^6$	$0{\cdot}593 \times 10^6$ i.u.
Vitamin-A_2 contribution 1/9 of $1{\cdot}33 \times 10^6$	$0{\cdot}148 \times 10^6$ i.u.
	$2{\cdot}961 \times 10^6$ i.u.

The over-all conversion factor would be—

$$\frac{2{\cdot}961 \times 10^6}{1638{\cdot}9} = 1807.$$

In a mixture containing no vitamin A_2 but in which 1 molecule in 4 of vitamin A_1 is neovitamin A_1 the absorption intensity would be—

$$\frac{1745 \times 3}{4} + \frac{1615}{4} = 1712{\cdot}5$$

and the potency—

$$\frac{3{\cdot}33 \times 10^6 \times 3}{4} + \frac{2{\cdot}66 \times 10^6}{4} = 3{\cdot}166 \times 10^6$$

and the conversion factor—

$$\frac{3{\cdot}166 \times 10^6}{1712{\cdot}5} = 1849.$$

Now suppose that the analyst wished in the first example to obtain the vitamin-A_1 activity from the total absorption—

A_1 activity, $(2{\cdot}222 + 0{\cdot}593) \times 10^6 = 2{\cdot}815 \times 10^6$ i.u.

Total absorption, $E^{1\%}_{1cm}$ 1638·9

Conversion factor for $A_1 = \frac{2{\cdot}815 \times 10^6}{1638{\cdot}9} = 1718.$

Thus the outcome of such considerations is that in order to estimate the total potential vitamin-A activity or the vitamin-A_1 contribution in international units the appropriate conversion factor will vary with the mixture of all-*trans* and neovitamin A_1 and A_2.

Tables II and III show the variation of the conversion factor (for solutions in ethanol or *cyclo*hexane) appropriate when all the vitamin A_1 and A_2 is in a simple mixture without irrelevant absorption and the total vitamin-A activity is required.

TABLE II

CONVERSION FACTORS TO GIVE AGGREGATE VITAMIN-A ACTIVITY FOR MIXTURES OF ALL-*trans* VITAMIN-A_1, NEOVITAMIN-A_1 AND VITAMIN-A_2 ALCOHOLS; INTENSITIES OF ABSORPTION IN *cyclo*HEXANE UNCORRECTED, NO IRRELEVANT ABSORPTION

Vitamin A_2 / Neovitamin A_1	0%	3%	6%	9%	12%	15%	18%	21%	24%	27%	30%
0%	1908	1897	1885	1872	1859	1846	1833	1819	1805	1791	1776
3%	1901	1890	1877	1864	1851	1838	1825	1811	1797	1782	1768
6%	1894	1882	1869	1857	1844	1830	1817	1802	1788	1774	1759
9%	1886	1873	1861	1849	1836	1822	1808	1793	1779	1765	1750
12%	1878	1866	1854	1840	1828	1814	1799	1785	1771	1756	1741
15%	1871	1859	1846	1833	1819	1805	1791	1777	1763	1748	1732
18%	1863	1851	1838	1825	1811	1797	1783	1769	1754	1739	1723
21%	1856	1843	1830	1816	1803	1789	1775	1760	1745	1730	1714
24%	1848	1835	1822	1809	1795	1781	1767	1752	1737	1721	1705
27%	1840	1827	1814	1801	1787	1773	1759	1744	1728	1712	1696
30%	1832	1819	1807	1793	1779	1765	1750	1735	1719	1703	1687

TABLE III

CONVERSION FACTORS TO GIVE AGGREGATE VITAMIN-A ACTIVITY FOR MIXTURES OF ALL-*trans* VITAMIN-A_1, NEOVITAMIN-A_1 AND VITAMIN-A_2 ALCOHOLS; INTENSITIES OF ABSORPTION IN ETHANOL UNCORRECTED, NO IRRELEVANT ABSORPTION

Vitamin A_2 / Neovitamin A_1	0%	3%	6%	9%	12%	15%	18%	21%	24%	27%	30%
0%	1850	1839	1828	1818	1866	1793	1781	1769	1756	1743	1729
3%	1842	1832	1821	1809	1797	1785	1772	1761	1747	1734	1720
6%	1835	1824	1812	1801	1790	1777	1764	1752	1739	1725	1712
9%	1827	1815	1804	1793	1782	1768	1756	1743	1730	1716	1703
12%	1819	1808	1796	1785	1774	1760	1747	1735	1721	1707	1693
15%	1811	1800	1788	1777	1765	1752	1739	1726	1712	1698	1684
18%	1803	1792	1780	1769	1756	1743	1730	1717	1703	1689	1674
21%	1796	1783	1772	1760	1748	1734	1722	1708	1695	1680	1665
24%	1788	1776	1764	1752	1740	1726	1713	1700	1686	1671	1656
27%	1780	1768	1756	1744	1733	1717	1704	1691	1677	1662	1647
30%	1771	1760	1747	1736	1722	1710	1696	1683	1667	1653	1637

Revised constants for all-*trans* vitamin A and its acetate have been recorded by Cama, Collins and Morton.[2]

With use of the fixation points for all-*trans* vitamin A, pure all-*trans* would obviously need no correction, but if the procedure is applied to the best curve for neovitamin-A alcohol the correction results in a reduction of intensity by 20 per cent. (*cyclo*hexane) or 25 per cent. (ethanol). Similarly when the procedure is applied to vitamin A_2 the reduction is 90 per cent. (*cyclo*hexane) or 85 per cent. (ethanol). In other words the Morton - Stubbs procedure substantially eliminates vitamin A_2 and overcorrects the vitamin A_1 by from 1/5 to 1/4 of the neovitamin-A_1 content.

The situation may be illustrated by an example worked out in detail.

EXAMPLE—

Out of each 100 "vitamin-A" molecules let 70 be all-*trans* A_1, 21 neovitamin A_1 and 9 vitamin A_2. The potency will be—

$$(0{\cdot}7 \times 3{\cdot}33 + 0{\cdot}21 \times 2{\cdot}66 + 0{\cdot}09 \times 1{\cdot}33) \times 10^6 \text{ units per g}$$
$$= 3{\cdot}013 \times 10^6 \text{ units per g} \qquad (2{\cdot}893\ A_1 + 0{\cdot}12\ A_2) \times 10^6$$

In cyclo*hexane*—$E^{1\%}_{1cm}$ at 326 mμ will be—

$$(0{\cdot}7 \times 1745) + (0{\cdot}21 \times 1615) + (0{\cdot}09 \times 1050) = 1655{\cdot}1$$

and on applying the correction procedure the expected result would be—

$$(0{\cdot}7 \times 1745) + (0{\cdot}21 \times 1615 \times 0{\cdot}80) + (1050 \times 0{\cdot}09 \times 0{\cdot}10) = 1503.$$
$$1503 \times 1900 = 2{\cdot}86 \times 10^6 \text{ units per g,}$$

which is very close to the potency for A_1 ($2{\cdot}893 \times 10^6$).

In ethanol—$E^{1\%}_{1cm}$ will be—

$$(0{\cdot}7 \times 1800) + (0{\cdot}21 \times 1685) + (0{\cdot}09 \times 1600) = 1709$$

and on applying the correction formula—

$$(0{\cdot}7 \times 1800) + (0{\cdot}21 \times 1685 \times 0{\cdot}75) + (0{\cdot}09 \times 1600 \times 0{\cdot}15) = 1539.$$
$$1539 \times 1900 = 2{\cdot}92 \times 10^6 \text{ units per g,}$$

which agrees closely with $2{\cdot}893 \times 10^6$ units per g.

The above proportions of all-*trans* and neovitamin A_1 and vitamin A_2 are not unusual. The correction procedure has eliminated the vitamin A_2 and the over-correction for neovitamin-A_1 absorption fortuitously matches its lower intrinsic potency. The B.P. and U.S.P. methods with the Morton - Stubbs correction will thus not be far from correctly expressing the A_1 potency. That of vitamin A_2 can be determined separately.

The next paper[9] describes how chromatography on dicalcium phosphate (under defined experimental conditions) permits a very good separation of neovitamin A, all-*trans* vitamin A and vitamin A_2. The loss by destruction during the chromatography is about 10 per cent., so the separation is used to establish the relative proportions of the three

TABLE IV

ANALYTICAL RESULTS ON THREE COD-LIVER OILS

OIL A

	Liverpool		Copenhagen		
$E^{1\%}_{1\,cm}$ at 326 mμ gross on unsap. in *cyclo*hexane	0·66	0·642	0·681	0·668	
Corrected	0·50(5)	0·488	0·56(5)	0·550	
Simple chromatography total vitamin-A fraction	—	—	0·65(8)	0·641	0·62(8)
$E^{1\%}_{1\,cm}$ at 693 mμ ($SbCl_3$ colour test)	0·23	—	—	—	—

Chromatographic separation: percentage of total absorption at 326 mμ due to vitamin A—

	%
due to:	
all-*trans* A_1 ..	71
neo A_1	22·5
vitamin A_2 ..	6·5

Using 0·64(2) for the total vitamin A distributed in the proportions shown above—

$E^{1\%}_{1\,cm}$ at 326 mμ

	0·65 × 1600 =	1040 u. per g	
A_1	0·50 × 1900 =	950 u. per g	
A_2	0·23 × 330 =	76 u. per g	
all-*trans* A_1	0·456 ≃	844 u. per g	
neo A_1	0·144 ≃	228 u. per g	
vitamin A_1		1072 u. per g	
vitamin A_2	0·042 ≃	53 u. per g	

OIL B

	Liverpool		Copenhagen		
$E^{1\%}_{1\,cm}$ at 326 mμ gross on unsap. in *cyclo*hexane	1·70	1·70	1·66		1·68
Correction	1·31	1·30	1·33		1·35
Simple chromatography vitamin-A fraction	—	—	1·59	1·57	1·53(6)
$E^{1\%}_{1\,cm}$ at 693 mμ ($SbCl_3$ colour test)	0·54	—	—	—	—

Chromatographic separation: percentage of total absorption at 326 mμ due to vitamin A—

	%
due to:	
all-*trans* A_1 ..	69·5
neo A_1 ..	24·5
vitamin A_2 ..	6·0

Using 1·56 for the total vitamin A distributed in the proportions shown above—

$E^{1\%}_{1\,cm}$ at 326 mμ

	1·70 × 1600 =	2720 u. per g	
A_1	1·31 × 1900 =	2490 u. per g	
A_2	0·54 × 330 =	180 u. per g	
all-*trans* A_1	1·08 ≃	2000 u. per g	
neo A_1	0·38 ≃	600 u. per g	
vitamin A_1		2600 u. per g	
vitamin A_2	0·10 ≃	125 u. per g	

OIL C

	Liverpool		Copenhagen		
$E^{1\%}_{1\,cm}$ at 326 mμ gross on unsap. in *cyclo*hexane	1·29(5)	1·27	1·250	1·215	1·275
Corrected	1·00	1·024	0·97(3)	0·97(3)	0·98(7)
Simple chromatography	—	—	1·20(9)	1·16(1)	1·16(9)
$E^{1\%}_{1\,cm}$ at 620 mμ	2·9	—	—	—	—
693 mμ ($SbCl_3$ colour test)	0·43(5)	—	—	—	—

Chromatographic separation: percentage of total absorption at 326 mμ due to vitamin A—

	%
due to:	
all-*trans* A_1 ..	69
neo A_1 ..	26
vitamin A_2 ..	5

Using 1·18 for the total vitamin A distributed in the proportions shown above—

$E^{1\%}_{1\,cm}$ at 326 mμ

	1·28(2) × 1600 =	2050 u. per g	
A_1	1·01(2) × 1900 =	1923 u. per g	
A_2	0·435 × 330 =	135 u. per g	
all-*trans* A_1	0·81(4) ≃	1506 u. per g	
neo A_1	0·306 ≃	483 u. per g	
vitamin A_1		1989 u. per g	
vitamin A_2	0·06 ≃	75 u. per g	

NOTE—The figures in brackets are the third decimal place, not known with certainty.

OILS D AND E

	Liverpool		Copenhagen	
	D	E	D	E
$E^{1\%}_{1cm}$ at 327·5 mμ on oil in *cyclo*hexane	28·9	9·05		
Corrected (B.P.)	23·5	7·7		
Colour test: $E^{1\%}_{1cm}$ at 693 mμ	5·25	2·0		
620 mμ	73·8	21·5		
620 mμ (corr.)	72·0	20·9		
Vitamin-A ester fraction after chromatography as oil (calculated on initial weight)				
$E^{1\%}_{1cm}$ at 327·5 mμ in *cyclo*hexane	25·3	7·8		
Corrected	22·6	6·7		
Colour test: $E^{1\%}_{1cm}$ at 693 mμ	3·5	2·25		
620 mμ	71·0	22·0		
620 mμ (corr.)	69·8	21·25		
Residue eluted from column by ether				
Colour test: $E^{1\%}_{1cm}$ at 603 mμ	5·0	1·6		
$E^{1\%}_{1cm}$ at 325 mμ (U.S.P. XIV procedure) on unsaponifiable fraction in *iso*propanol	29·1, 28·75	9·2, 8·97		
Corrected	25·0, 25·0	7·25, 7·27		
$E^{1\%}_{1cm}$ at 325 to 326 mμ on unsaponifiable fraction in *cyclo*hexane	27·3	8·5	27·4	8·25
	22·0	6·45	24·1	6·55
Robeson and Baxter's maleic anhydride method				
all-*trans* to neo (ratio)	70/30	76·5/23·5		
Routine chromatography of unsaponifiable $E^{1\%}_{1cm}$ at 325 mμ (in ethanol)			25·8	7·49
Percentage of total absorption at 325 mμ — all-*trans*			72·5	66·0
neo			25·0	30·0
vitamin A_2			2·5	4·0

vitamin-A-active substances and the total is obtained from a simpler chromatographic separation.

Table IV records the results of examining five oils, A, B, C, D and E, at Liverpool and Copenhagen. A, B and C are cod-liver oils and D and E are much richer oils.

The method of Bro-Rasmussen, Hjarde and Porotnikoff applied to oils D and E accepts the intensity of absorption shown at 325 mμ after a simple chromatography as the main measurement. The chromatography distributes this absorption over all-*trans* and neo-vitamin A_1 and vitamin A_2.

Oil	Absorption at 325 mμ		$E^{1\%}_{1cm}$ at 325 mμ		Contributions to vitamin activity, i.u. per g	
	D, %	E, %	D	E	D	E
all-*trans* A_1	72·5	66·0	18·7	4·94	34,600	9140
neo A_1	25·0	30·0	6·45	2·25	10,200	3550
A_2	2·5	4·0	0·65	0·30	800	380
	100·0	100·0	25·8	7·49	45,600	13,070

In interpreting the Liverpool results use will be made of the findings of Cama, Collins and Morton.[2]

The B. P. correction on the oil eliminates vitamin A_2 and the conversion factor of 1900 is appropriate because neovitamin A is less potent than vitamin A by just about the right amount to balance an over-correction—

Oil D $23·5 \times 1900 = 44{,}650$ i.u. per g (A_1)
Oil E $7·7 \times 1900 = 14{,}630$ i.u. per g (A_1).

To apply the conversion factor of 1900 to the gross values for the ester fraction after chromatography would be correct only if neovitamin A_1 and vitamin A_2 were absent, hence—

Oil D $25·3 \times 1900 = 48{,}070$
Oil E $7·8 \times 1900 = 14{,}820$.

The true ester activities must be less than the above figures. The colour test on the chromatographed oils (ester fractions), leads to—

Oil D $69{\cdot}8 \times 660 = 46{,}068\ A_1$ plus $1160\ A_2$
$(47{,}520\ A_1$ plus $1750\ A_2)$
Oil E $21{\cdot}25 \times 660 = 14{,}025\ A_1$ plus $750\ A_2$
$(13{,}794\ A_1$ plus $660\ A_2)$
(figures in brackets for whole oil).

The free vitamin-A contribution was of the order 1700 for oil D and 500 i.u. per g for oil E. The U.S.P. method leads to—

Oil D $25{\cdot}0 \times 1900 = 47{,}500$
Oil E $7{\cdot}26 \times 1900 = 13{,}794$.

In fact the true conversion factor for vitamin-A alcohol in *iso*propanol should be 1820 and not 1900 and to that extent high results tend to be obtained (although vitamin A_2 is discounted).

The corrected values for the unsaponifiable fraction in *cyclo*hexane give appreciably lower results—

Oil D $23{\cdot}0 \times 1900 = 43{,}700$ i.u. per g (A_1)
Oil E $6{\cdot}5 \times 1900 = 12{,}350$ i.u. per g (A_2).

	Oil D, i.u. per g	Oil E, i.u. per g
B.P. on oil (A_1)	44,650	14,630
U.S.P. (XIV) on unsap. (A_1)	47,500	13,794
Upper limits (including A_2)	48,070	14,820
Lower limit (including A_2)	44,500	13,650
Copenhagen results		
A_1	44,800	12,690
A_2	800	380
Total	45,600	13,070

Discussion

The method in use in the Copenhagen laboratory is the best so far described for separating the three main vitamin-A-active substances, but the recovery is only 90 per cent., judging from the sum of the extinctions compared with that of the vitamin-A fraction put on the column. By distributing the three substances in the found proportions over the total recorded after simple chromatography, it is assumed that whatever the cause of loss the three substances are affected unselectively. It is also assumed that the simple chromatography gives a fraction with no absorption whatever at 325 mμ apart from that of the three vitamin-A forms. It is possible, however, that vitamin A_2 is more readily destroyed than vitamin A_1. It seems better to risk a small over-estimate by relying on simple chromatography for the sum of the vitamin-A absorptions than to risk a larger under-estimate by using the figures for the intensities of absorption actually recovered in the final fractionation.

The Liverpool laboratory makes use of the nine point correction procedure of Cama *et al.*[2] This eliminates true irrelevant absorption and over-corrects a little for neovitamin A. By a fortunate combination of circumstances the over-correction is proportionate to the lower intrinsic potency of neovitamin A_1 compared with the all-*trans*. The antimony trichloride colour test allows the vitamin-A_2 content to be determined and the vitamin-A_1 content approximately estimated.

In the three oils A, B and C the Liverpool estimate of the vitamin-A_2 content is appreciably greater than that reached by chromatographic separation—

	Oil A		Oil B		Oil C		Oil D		Oil E	
Vitamin ..	A_1	A_2	A_1	A_2	A_1	A_2	A_1	A_2	A_1	A_2
Liverpool ..	950	76	2490	180	1923	135	45,000	1150 to 1750	14,000	500 to 700
Copenhagen ..	1072	53	2600	125	1989	75	44,800	800	12,690	380

Nevertheless, the results, particularly for oils B and C above, are in good agreement, considering the complexity of the problem. The chromatographic separation of all-*trans* and neovitamin A_1 is perhaps a better analytical procedure than the method based on maleic anhydride adduct formation, but it is possible that saponification may change the all-*trans*

to neovitamin ratio. The agreement between the two methods is not good and further work is necessary.

It is perhaps questionable whether or not analysts should at present express their results as total vitamin-A activity, because there is no official sanction for including vitamin A_2. The combination of geometrical correction and a conversion factor of 1900 leads to substantially correct results for mixtures of all-*trans* and neovitamin A_1 in which the former predominates. The new chromatographic procedure strengthens the evidence that isomers other than all-*trans* and neovitamin A do not occur to any substantial extent. Further work will be needed to account for the discrepant vitamin-A_2 estimations made by this method compared with the antimony trichloride colour test, but in fish-liver oils from sea fish the vitamin-A_2 contribution will not exceed 10 per cent. and for most species will be less than 5 per cent. of the total.

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Chromatographic Separation of Vitamin-A-active Compounds in Cod-liver Oil

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(*Presented* at the meeting of the Society on Wednesday, December 1st, 1954*)

A method is given for the separate determination of neovitamin A, all-*trans* vitamin A and vitamin A_2 in fish-liver oils. A separation adequate for ascertaining the relative proportions of the three compounds can be obtained by chromatography on dicalcium phosphate columns 300 to 600 mm long. Examples are given of two such determinations in cod-liver oil; good agreement (only shown in one case) has been found between the determined content of the three compounds and the light absorption for the total vitamin-A fraction obtained by a normal chromatographic isolation of vitamin A.

Absorption curves are given for the three substances.

The biological value of the vitamin A contained in fish-liver oil cannot be ascribed to a single substance, but (*e.g.*, in cod-liver oil) is due to at least three compounds. Previous investigations have shown that in cod-liver oil all-*trans* vitamin A, neovitamin A and vitamin A_2 are present and that all three exhibit vitamin-A activity. The three compounds do not, however, possess the same activity per unit weight. Thus, 1 g of all-*trans* vitamin A has, by definition, an activity of 3·33 million i.u., whereas the activity of neovitamin A is given as 2·4 to 2·7 million i.u. per g (Harris, Ames and Brinkmann[1]) and the activity of vitamin A_2 as about 1·3 million i.u. per g (Shantz[2]). The relative proportions in which the three vitamin-A-active compounds occur can thus significantly affect the total vitamin-A activity. The routine determination of the vitamin-A content of fish-liver oil is generally based on a measurement of the total absorption of the three compounds at 325 mμ, $E^{1\%}_{1cm}$ at 325 mμ

* The authors, who were unable to be present at the meeting, thank Mr. G. A. J. Pitt of the Department of Biochemistry, University of Liverpool, for presenting their paper.

being multiplied by a conversion factor to give the result in i.u. per g. The factor 1900 based on the properties of pure all-*trans* vitamin A must be used with caution (see Morton and Bro-Rasmussen[3]).

Geometrical correction methods frequently used in the vitamin-A calculation (Morton and Stubbs[4]; British Pharmacopoeia 1953, p. 844; U.S. Pharmacopoeia XIV) eliminate irrelevant absorption; at the same time they reduce somewhat the contribution made to the light absorption by neovitamin A and almost completely discount the absorption due to vitamin A_2. Nevertheless, these methods will not give an accurate value for the vitamin-A potency of a mixture of the three compounds.

Attempts have been made recently to determine each of the vitamin-A-active compounds separately. A reaction with maleic anhydride has been used to determine the neovitamin A content.[5,6] The determination of vitamin A_2 has been carried out on the basis of the difference in the reaction products of vitamins A_1 and A_2 with antimony trichloride, which show different light absorption.[7,8]

It is, however, also possible by means of chromatography to separate all-*trans* vitamin A_1, neovitamin A_1 and vitamin A_2 and to determine the amounts of each. An account is given below of a method that has proved suitable for this purpose.

In a previous publication, Hjarde[9] described a method for the determination of vitamin A in fish-liver oils by chromatography on short columns of dicalcium phosphate. During such chromatography the vitamin-A fraction was always observed to separate into two zones, which in ultra-violet light showed some difference in the colour of the fluorescence. There was also a difference in light absorption between the vitamin A first collected and that most strongly adsorbed; the first fraction to leave the column showed an absorption maximum at a slightly higher wavelength than that eluted later. This suggests that neovitamin A is less strongly adsorbed on dicalcium phosphate than all-*trans* vitamin A. Collection of the two different zones separately is, however, not possible, and the experiments carried out have had the aim of obtaining a better separation on the column.

Experimental

Extracts of the unsaponifiable fraction have been used for all the work described below. Saponification and extraction have been carried out as described earlier.[9]

The chromatographic procedure has, however, been modified, pressure instead of suction being used to obtain a suitable rate of flow. The shape of the chromatographic tubes used is shown in Fig. 1, and the dimensions of the column are given for each experiment.

Reagents—

Dicalcium phosphate—The sample used for adsorption was activated as described by Moore.[10] The procedure is as follows. Crystalline dicalcium phosphate (100 g) suspended in a solution of disodium phosphate (5 g of $Na_2HPO_4.2H_2O$ in 800 ml of water) is heated at the boiling point for 15 minutes. After the solution has cooled, the dicalcium phosphate is filtered off (Buchner funnel), washed with 500 ml of water and dried at 110° C for at least 2 days. It is then placed in a hygrostat with about 50 per cent. relative humidity (*e.g.*, over saturated potassium thiocyanate solution), where it remains for 24 hours. It is finally ground to powder in a mortar and sieved through 45-gauge wire mesh.

It appears, however, that not all commercial preparations of dicalcium phosphate give satisfactory adsorption, even when they are subjected to this activation. The treated dicalcium phosphate must fulfil requirements similar to those put forward in the previous publication.

Light petroleum, boiling point below 70° *C*—This grade is used as solvent for the chromatography. Spectrophotometric measurements on the solvent against an equal thickness of absolute ethanol must not show extinction higher than 0·01 at any wavelength in the region 280 to 360 mμ.

Light petroleum, with the addition of various amounts of ethyl ether, is used for elution. The latter must be peroxide-free.

Generally the collected eluates are directly measured on the spectrophotometer, but, when measurements are to be made in ethanol, evaporation of light petroleum is effected at room temperature with suction in a stream of carbon dioxide.

EXPERIMENT 1—

The material for chromatography was the unsaponifiable fraction from about 500 mg of cod-liver oil, having approximately 500 i.u. of vitamin-A activity.

Chromatographic column: $a = 200$ mm, $d = 18$ mm (see Fig. 1).

Eluent: light petroleum - ether mixture (200 to 1 v/v).

After about 400 ml of eluent had passed through the column, the vitamin A could be seen by the fluorescence in ultra-violet light at the bottom of the column. Collection of 5-ml fractions was then begun and 50 such fractions were obtained in all.

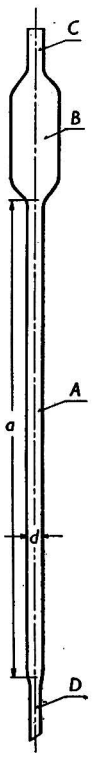

Fig. 1. Chromatographic column filled with dicalcium phosphate: A, column; B, reservoir for eluent; C, connection to pressure; D, outlet tube to retain plug of cotton-wool at base of A

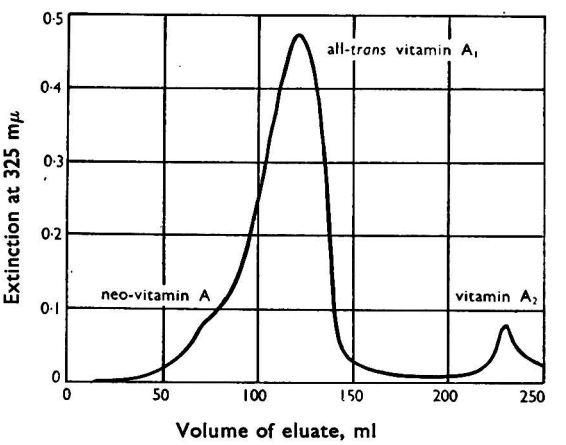

Fig. 2. Fractionation curve for cod-liver oil with a column 200 mm long

Fig. 2 shows the course of the fractionation, the extinction at 325 mμ being plotted for each fraction.

The shape of the curve indicates the presence of three different compounds, which can be identified by means of their light absorption. As previously mentioned, the neovitamin A is the least tightly bound to the adsorbent. Separation of the latter from all-*trans* vitamin A is very poor, as no peak corresponds to neovitamin A and only an inflexion can be seen. On the other hand, the separation of vitamin A_2 (the compound most strongly adsorbed) is relatively good. The three compounds can also be identified by their fluorescence. Neovitamin A shows a more yellowish-green and brighter fluorescence than the somewhat blue-green fluorescence from all-*trans* vitamin A. Vitamin A_2 has a much weaker brownish fluorescence. This can be observed both on the column and in the solutions.

EXPERIMENT 2: SIGNIFICANCE OF THE ETHER CONCENTRATION IN THE ELUENT—

The material for chromatography was the unsaponifiable fraction from about 15 mg of vitamin-A concentrate, *i.e.*, about 650 i.u. of vitamin-A activity.

Chromatographic column: $a = 200$ mm, $d = 18$ mm.

Eluent for experiment 2 (A): light petroleum - ether mixture (100 to 1 v/v); collection in 25-ml fractions.

Eluent for experiment 2 (B): light petroleum - ether mixture (12 to 1 v/v); collection in 5-ml fractions.

Fig. 3 illustrates the fractionations.

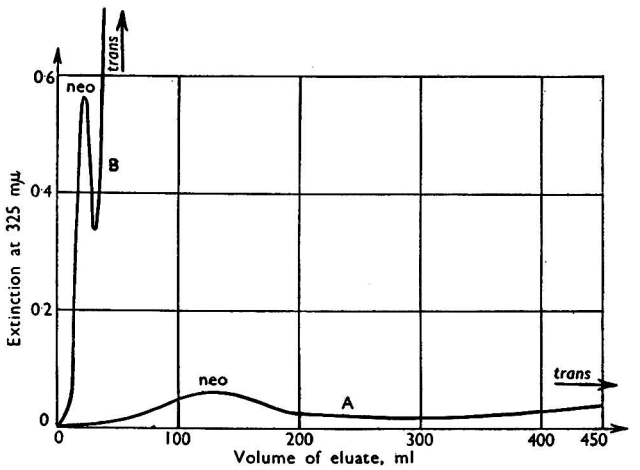

Fig. 3. Fractionation curve for unsaponifiable fraction of vitamin-A concentrate with a column 200 mm long

The experiment shows that although the ether concentration of the eluent has a decisive influence on the concentration of vitamin A in the eluates, the separation of neovitamin A and all-*trans* vitamin A is only very slightly affected. The ratio of the maximum to the minimum in curve A is only slightly greater than the ratio in curve B.

Hence it is not possible to improve the separation by the use of eluents with a low ether concentration.

EXPERIMENT 3: SIGNIFICANCE OF THE AMOUNT OF VITAMIN A—

In experiment 3 (*a*) the material was the unsaponifiable fraction from about 2 mg of vitamin-A concentrate, a total of about 1000 i.u.

In experiment 3 (*b*) the material was the unsaponifiable fraction from 0·2 mg of vitamin-A concentrate, *i.e.*, about 100 i.u.

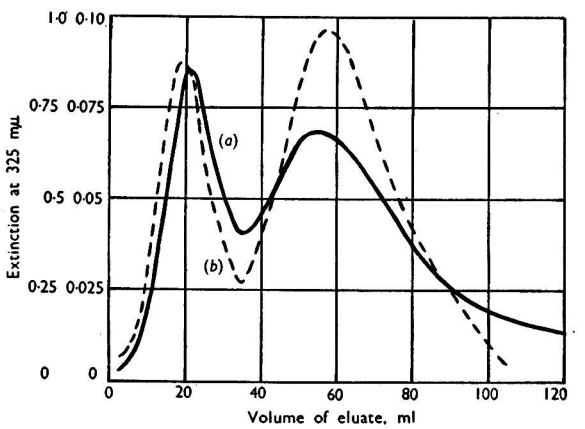

Fig. 4. Effect of amount of vitamin A on the fractionation: curve (*a*), 2 mg of vitamin-A concentrate used; curve (*b*), 0·2 mg of vitamin-A concentrate used

Chromatographic column: $a = 200$ mm, $d = 18$ mm.

Eluent: light petroleum - ether mixture (16 to 1 v/v); collection in 5-ml fractions.

Fig. 4 shows the fractionations achieved.

The experiment shows only a slight difference in separation between large and small total amounts on the column. Hence the only other possibility for obtaining better separation is to lengthen the column.

EXPERIMENT 4: CHROMATOGRAPHY OF COD-LIVER OIL—

The material was the unsaponifiable fraction from 3·03 g of cod-liver oil, a total of about 3000 i.u. of vitamin-A activity.

Chromatographic column: $a = 500$ mm, $d = 30$ mm.

Eluent: light petroleum - ether mixture (16 to 1 v/v); collection in 15-ml fractions.

The fractionation obtained is shown in Fig. 5.

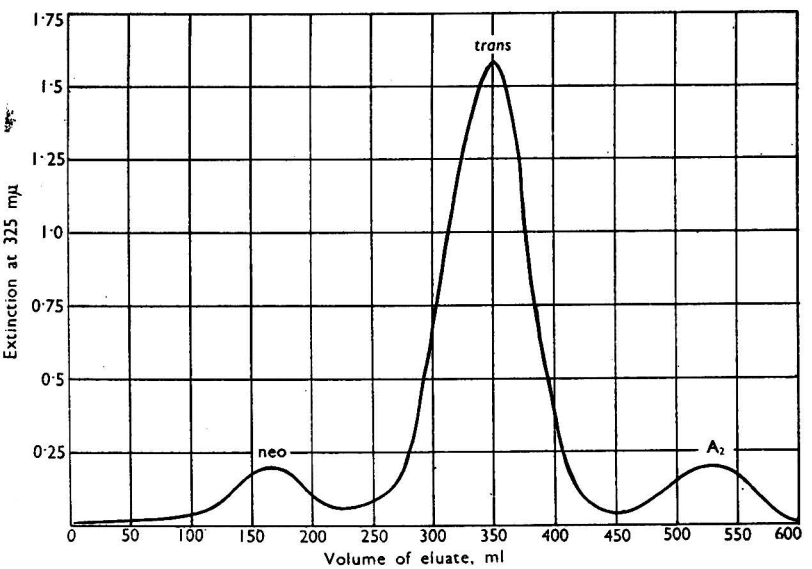

Fig. 5. Fractionation curve for cod-liver oil with a column 500 mm long

EXPERIMENT 5 (*a*): CHROMATOGRAPHIC ISOLATION OF ALL-*trans* VITAMIN A—

The material was the unsaponifiable fraction from a synthetic vitamin-A preparation, about 800 i.u. of vitamin A.

Chromatographic column: $a = 300$ mm, $d = 12$ mm.

Eluent: light petroleum - ether mixture (12 to 1 v/v); collection in 5-ml fractions.

From the fractionation curve, which is not shown here, the content of neovitamin A was about 10 per cent. and that of all-*trans* vitamin A 90 per cent.

The peak fraction for all-*trans* vitamin A dissolved in absolute ethanol showed the curve characteristic for vitamin A, with maximum at 324·5 mμ, and the following ratios were found—

$$\frac{E_{310}}{E_{325}} = 0{\cdot}856 \quad \text{and} \quad \frac{E_{350}}{E_{325}} = 0{\cdot}449.$$

EXPERIMENT 5 (*b*): CHROMATOGRAPHY OF PURE ALL-*trans* VITAMIN A—

The *trans* vitamin-A fraction from experiment 5 (*a*) was re-chromatographed.

Chromatographic column: $a = 300$ mm, $d = 12$ mm.

Eluent: light petroleum - ether mixture (11 to 1 v/v); collection in 5-ml fractions.

The fractionation curve (Fig. 6) shows the presence of only one compound with light absorption at 325 mμ and the peak fraction, dissolved in ethanol, shows a curve identical with that found in experiment 5 (*a*) for *trans* vitamin A.

EXPERIMENT 6: CHROMATOGRAPHIC ISOLATION OF PURE NEOVITAMIN A—

The material was about 100 μg of a crystalline neovitamin-A preparation.

Chromatographic column: $a = 300$ mm, $d = 18$ mm.

Eluent: light petroleum - ether mixture (25 to 1 v/v); collection in 5-ml fractions.

The fractionation curve is shown in Fig. 7. It can be seen that it is only possible to recognise the presence of one compound with light absorption about 325 mμ. This compound, dissolved in ethanol, showed an absorption curve with its maximum at 328 mμ, and the following ratios were found—

$$\frac{E_{310}}{E_{325}} = 0{\cdot}809 \quad \text{and} \quad \frac{E_{350}}{E_{325}} = 0{\cdot}543.$$

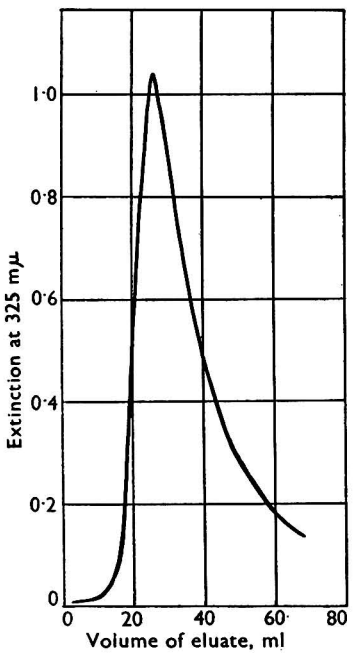

Fig. 6. Fractionation curve for all-*trans* vitamin A

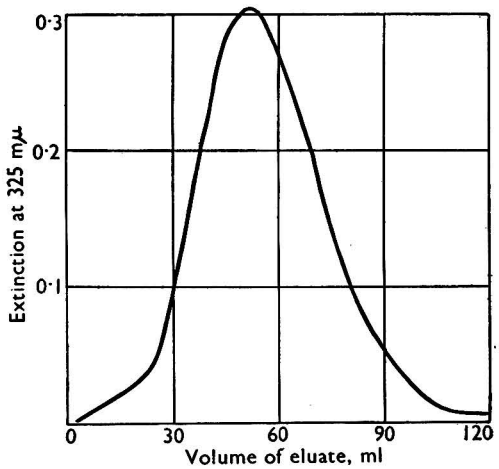

Fig. 7. Fractionation curve for neovitamin A

EXPERIMENT 7: CHROMATOGRAPHIC ISOLATION OF PURE VITAMIN A_2—

The material for chromatography was the unsaponifiable fraction from about 25 g of carp liver.

Chromatographic column: $a = 500$ mm, $d = 30$ mm.

The eluents were used in the following order—

400 ml of light petroleum - ether mixture (50 to 1 v/v),
600 ml of light petroleum - ether mixture (25 to 1 v/v),
500 ml of light petroleum - ether mixture (20 to 1 v/v),
400 ml of light petroleum - ether mixture (12 to 1 v/v),
300 ml of light petroleum - ether mixture (10 to 1 v/v);

the eluates were collected in 15-ml fractions.

The fractionation curve showed that about 60 per cent. of the total extinction at 325 mμ was due to vitamin A_2.

It must be mentioned here that in this experiment the collected all-*trans* vitamin-A fractions did not show the characteristic *trans* curve, the ratio E_{350}/E_{325} being high. This observation will be discussed later.

Fig. 8 shows the absorption curve for the "peak" fraction of vitamin A_2 in absolute ethanol.

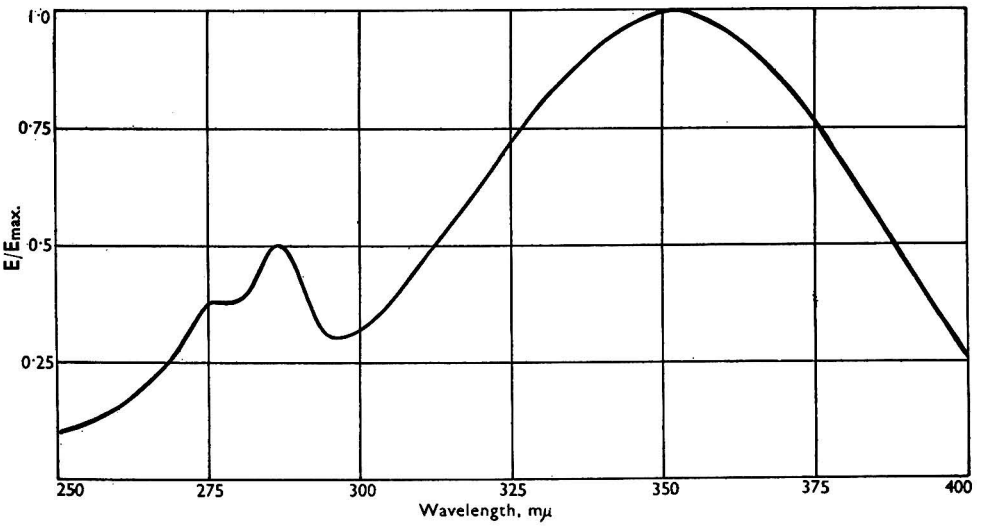

Fig. 8. Absorption curve for vitamin A_2

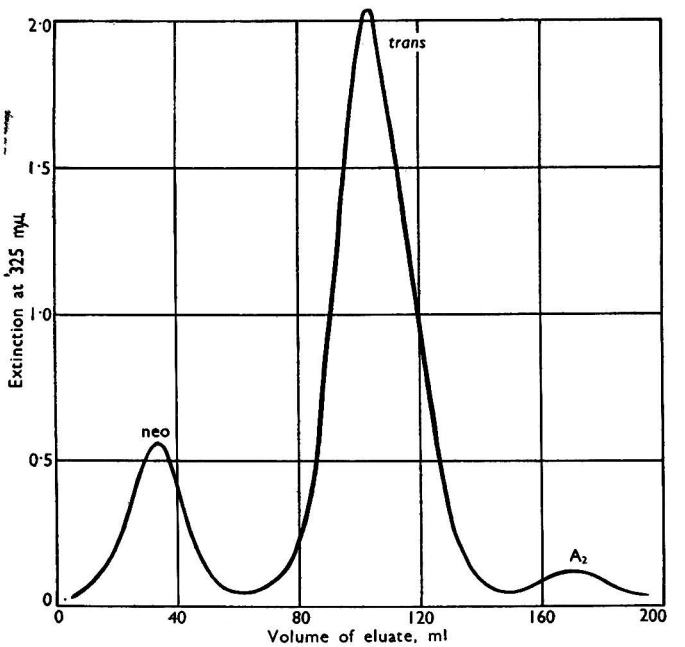

Fig. 9. Fractionation curve for cod-liver oil with a column 600 mm long

The maximum lies at 351·5 mμ and—

$$\frac{E_{310}}{E_{325}} = 0{\cdot}630 \quad \text{and} \quad \frac{E_{350}}{E_{325}} = 1{\cdot}37.$$

EXPERIMENT 8: CHROMATOGRAPHY OF COD-LIVER OIL—

The material used was the unsaponifiable fraction from 0·894 g of cod-liver oil, a total of about 1600 i.u. of vitamin A. By simple chromatographic separation on a

100-mm column and total elution of the vitamin-A-active compounds the extract used showed—

$E^{1\%}_{1cm}$ at 325 mμ = 0·970, $E^{1\%}_{1cm}$ at 310 mμ = 0·805 and $E^{1\%}_{1cm}$ at 350 mμ = 0·507

measured in absolute ethanol and expressed in terms of the actual weight of cod-liver oil.

Chromatographic column: a = 600 mm, d = 15 mm.

Eluents: 400 ml of light petroleum - ether mixture (16 to 1 v/v) and 400 ml of light petroleum - ether mixture (12 to 1 v/v); collected in 10-ml fractions.

The fractionation curve (Fig. 9) shows the presence of three compounds with light absorption at 325 mμ. For each of these fractions the absorption curve of the peak fraction was measured after dissolving it in absolute ethanol. These curves, which are all drawn in Fig. 10, show good agreement with the curves found in experiments 5, 6 and 7.

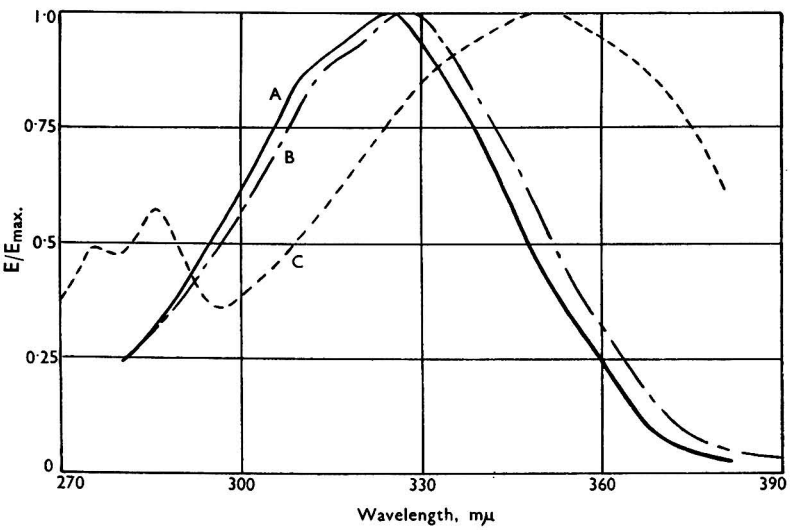

Fig. 10. Absorption curve for the three vitamin-A active materials isolated from cod-liver oil: curve (*a*), all-*trans* vitamin A; curve (*b*), neo-vitamin A; curve (*c*), vitamin A_2

Discussion

Experiments 1, 2 and 3 show that, as it is not possible to obtain a satisfactory chromatographic separation of the three vitamin-A-active compounds on a dicalcium phosphate column of length 200 mm, a longer column must be used.

It may be noted that for the cod-liver oil in experiment 1 a much smaller separation of neovitamin A and all-*trans* vitamin A is achieved than for the vitamin-A concentrates in experiments 2 and 3. It seems reasonable to assume that this difference is due to the larger amount of unsaponifiable residue which has been placed on the column in experiment 1. Probably some of the compounds in the unsaponifiable residue from the cod-liver oil act as "displacement carriers" for all-*trans* vitamin A, which presumably disturbs the separation of neo- and *trans*-vitamin A, but favours good separation of *trans*-vitamin A and vitamin A_2. This appears to be the case in experiment 1. Another indication of this may be that the tendency to "tail" formation for *trans*-vitamin A in experiment 1 (Fig. 2) is only slight.

It is obvious that the higher the ether concentration used in the eluent the more quickly will elution occur, *i.e.*, the less the amount of eluent necessary to complete the process. At low concentrations of ether the vitamin-A zone spreads out over the column, but, as shown in experiment 2, this does not result in a better separation of the zones, which overlap just as much. On the other hand, if the elution is performed with low ether concentrations, a larger number of fractions will be obtained (if they are of the same volume); although this results in a more accurate determination of the fractionation curve, the losses during chromatography will be greater.

The significance of the amount of vitamin A used for chromatography (experiment 3) can be considered in somewhat the same way. Within certain limits the amount has no decisive effect on the separation between the different zones. Larger amounts of vitamin A require increasing amounts of eluent for complete elution of the vitamin, but the amounts required are far from proportional. The concentration of vitamin A in the eluate increases considerably, and the losses decrease when the total amount is increased.

The chromatography in experiment 4 shows a separation of neovitamin A, *trans*-vitamin A and vitamin A_2 from cod-liver oil sufficiently good to allow calculation of the content of each of these three compounds in the oil with considerable accuracy.

$E^{1\%}_{1cm}$ at 325 mμ can be calculated for each of the three vitamin-A-active compounds from the extinction of the individual fraction collected—

for neovitamin A—

$E^{1\%}_{1cm}$ at 325 mμ = 0·059 $\simeq$ 26·5 μg per g of cod-liver oil

for all-*trans* vitamin A—

$E^{1\%}_{1cm}$ at 325 mμ = 0·447 $\simeq$ 248 μg per g of cod-liver oil

for vitamin A_2—

$E^{1\%}_{1cm}$ at 325 mμ = 0·060 $\simeq$ 56·5 μg per g of cod-liver oil

Total = 0·566

The values given for the amounts of the three substances are calculated from the following extinction coefficients—

For neovitamin A: the value given by Robeson and Baxter[5]—

$E^{1\%}_{1cm}$ at 328 mμ = 1645, which corresponds to

$E^{1\%}_{1cm}$ at 325 mμ = 1610.

For all-*trans* vitamin A—

$E^{1\%}_{1cm}$ at 325 mμ = 1800.

For vitamin A_2: the value given by Shantz—

$E^{1\%}_{1cm}$ at 351 mμ = 1460, which corresponds to

$E^{1\%}_{1cm}$ at 325 mμ = 1060.

With the previously noted (p. 418) biological values of the individual vitamin-A-active compounds, we find the total biological value to be 990 i.u. per g of cod-liver oil.

The total extinction 0·566 multiplied by the factor 1900 leads to 1075 i.u. per g.

The validity of the calculation of course rests on the assumption that the collected fractions correspond to the three vitamin-A-active compounds. The identification of the three compounds depends on measurements of light absorption in the region 280 to 360 mμ, but the curves vary somewhat with the solvent, so that measurements made directly on the eluates, which contain various amounts of light petroleum and ether and possibly some moisture, are not satisfactory. The measurements should be made on solutions in a pure solvent, and absolute ethanol has been chosen for this purpose. In experiments 5, 6 and 7 the curves for all-*trans* vitamin A, neovitamin A and vitamin A_2 were respectively measured on a synthetic vitamin-A preparation, a purified neovitamin-A preparation and the unsaponifiable part of liver from a fresh-water fish, each material having first been chromatographed. For the measurements, that fraction collected during chromatography which contained the highest concentration of the compound required (peak fraction) was used.

Although it was not possible to determine molecular extinction coefficients ($\epsilon_{max.}$) for the three compounds, since the solutes in the peak fractions were not isolated and weighed, it is of interest to compare the results with those obtained by other workers.

For the all-*trans* vitamin-A curve, the ratios given in experiment 5—

$$\frac{E_{310}}{E_{325}} = 0{\cdot}856 \quad \text{and} \quad \frac{E_{350}}{E_{325}} = 0{\cdot}449,$$

show a slightly "flatter" curve than that given by Cama *et al.*[7] where—

$$\frac{E_{310}}{E_{325}} = 0{\cdot}848 \quad \text{and} \quad \frac{E_{350}}{E_{325}} = 0{\cdot}443.$$

This may mean that the solution measured in experiment 5 contained other compounds absorbing in the region 310 to 350 mμ, but in fact the deviations from the curve measured by Cama *et al.*[7] scarcely exceed the errors of measurement.

For neovitamin A, the curve is in complete agreement with measurements communicated to us by Messrs. E. & O. Collett & Co. A/S, Oslo.* Chatain and Debodard[11] have published curves for neovitamin A measured in *iso*propanol and *cyclo*hexane. The curve for *iso*propanol solution corresponds fairly well to our measurements in absolute ethanol, but the curve for *cyclo*hexane shows a considerable difference. According to the measurements of Cama *et al.*,[7] the same is true of all-*trans* vitamin A.

The curve for vitamin A_2 can be compared with that measured by Shantz[2] and also with that given by Cama and Morton.[8] The characteristic maxima at 287 and 351 mμ are found, but the curve measured in experiment 7 shows a somewhat steeper slope, which indicates somewhat greater purity. Thus we find $E_{350}/E_{325} = 1{\cdot}37$, whilst Shantz only finds 1·30 and Cama and Morton 1·23. We find $E_{350}/E_{287} = 2{\cdot}0$, Shantz 1·78 and Cama and Morton 1·84. This indicates that our solution has less impurity at 325 and 287 mμ. The latter can also be seen from the fact that, whilst we find $E_{287}/E_{295} = 1{\cdot}67$, Cama and Morton's measurements show 1·38.

As mentioned previously the all-*trans* vitamin-A fractions in experiment 7 showed a ratio E_{350}/E_{325} higher than 0·5. The ratios varied from fraction to fraction in such a way that it seems reasonable to assume that the *trans*-vitamin A here is contaminated by a compound in quantities so small that a peak corresponding to this compound is not visible on the fractionating curve because of overlapping by the *trans*-vitamin A. The fractions which had the greatest content showed the ratios—

$$\frac{E_{310}}{E_{325}} = 0{\cdot}77 \quad \text{and} \quad \frac{E_{350}}{E_{325}} = 0{\cdot}83.$$

The unknown compound is obviously like vitamin A_2 and has a somewhat greater intensity of absorption at 350 mμ than at 325 mμ.

In the course of chromatographing cod-liver oil unsaponifiable matter, we always observe one or two fractions with a ratio E_{350}/E_{325} higher than 0·5. Such fractions occur at the beginning of collecting the *trans*-vitamin A. The possibility cannot be excluded that neovitamin A_2 occurs in small quantities, in addition to the three vitamin-A-active compounds already mentioned.

Experiment 8, like experiment 4, is a chromatographic separation of cod-liver oil. Its purpose has been to compare absorption curves of the three separated compounds with the reference curves measured in experiments 5, 6 and 7, and in each case the curves refer to "peak" fractions in absolute ethanol. The curves (Fig. 10) show, in the case of *trans*- and neovitamin A, close agreement with the curves found in experiments 5 and 6, as is seen in the following table—

		E at 310 mμ / E at 325 mμ	E at 350 mμ / E at 325 mμ
trans-Vitamin A	Experiment 5	0·856	0·449
	Experiment 8	0·858	0·439
Neovitamin A	Experiment 6	0·809	0·543
	Experiment 8	0·806	0·544

In the case of vitamin A_2 the agreement is not so good; thus $E_{350}/E_{325} = 1{\cdot}30$, but it must be remembered that the amount of vitamin A_2 in the oil is only very small, and from the shape of the absorption curve there is no doubt that this fraction is relatively pure vitamin A_2.

It is appropriate here to compare the results of the full separation with that obtained by chromatography on an ordinary small column,[9] so as to assess the loss incurred during separation and also to investigate the efficiency of the simple chromatographic separation, *i.e.*, whether, after such a separation, there will be appreciable amounts of other light-absorbing compounds present.

* Personal communication from Gunnar Baalsrud, chief chemist.

Summation of the extinction at 325 mμ of the three groups of fractions gives $E^{1\%}_{1cm} = 0{\cdot}887$, whereas with a simple chromatographic separation 0·970 was found—

	$E^{1\%}_{1cm}$ at 325 mμ	Relative composition from extinction at 325 mμ, %
Neovitamin A	0·150	17
trans-Vitamin A	0·688	77½
Vitamin A_2	0·048	5½
Total	0·887	

Hence the losses during full separation have been about 9 per cent. higher than during the simple chromatographic separation, *i.e.*, a total of about 10 per cent.

In order to investigate the effectiveness of the purification obtained by simple chromatography, the shape of the curve measured can be compared with the shape of the curve that can be calculated from the ratio of the mixture of neovitamin A, *trans*-vitamin A and vitamin A_2 corresponding to that found above. In this calculation, it has been assumed that the loss is evenly distributed among the three substances. Furthermore, the possible occurrence of neovitamin A_2 has not been taken into account.

	$E^{1\%}_{1cm}$		
	325 mμ	310 mμ	350 mμ
For neovitamin A	0·165	0·133	0·090
For all-*trans* vitamin A	0·753	0·644	0·339
For vitamin A_2	0·052	0·033	0·071
Total	0·970	0·810	0·500
Found by simple chromatographic separation		0·805	0·507

The calculation shows excellent agreement with values measured. Hence, in the case of the oil investigated, it can be concluded that the solution obtained by ordinary routine chromatographic separation does not contain compounds with light absorption in the region 310 to 350 mμ other than the vitamin-A-active compounds. Other investigations have shown that this is true for fish-liver oils in general.

We are much indebted to Dr. J. Matet, l'Alimentation Equilibrée, Commentry, for supplying us with the crystalline neovitamin A.

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STATENS VITAMIN-LABORATORIUM
COPENHAGEN, DENMARK

December 10th, 1954

A Modified Method for the Spectrophotometric Determination of Vitamin A in Margarine

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(*Presented at the meeting of the Society on Wednesday, December 1st, 1954*)

In principle, the modified method is similar to the Official method, but does not require specially activated adsorbents. Commercially available de-fatted bone meal of appropriate particle size is the selected adsorbent. Experiments on margarine show that it is possible to recover from the column a fraction giving spectral absorption substantially the same as that of pure vitamin A. After a correction for slight irrelevant absorption, recovery of vitamin A is near 100 per cent., compared with about 107 per cent. by the Official method. Results on commercial margarines by the Official and modified methods are for practical purposes in good agreement.

Until comparatively recently the determination of vitamin A in low-potency foods, such as margarine, had perforce to be done by using the Carr - Price test, because a method of removing completely and reliably the large amount of spectrally absorbing material accompanying the vitamin A had not been found. However, in 1951, Boldingh and Drost[1] described a method whereby this could be done by using tandem columns containing, respectively, specially activated alumina and specially prepared, but somewhat unstable, alkaline alumina. With slight modifications, the conditions and procedure described by Boldingh and Drost have been adopted in the Food Standards (Margarine) Order, 1954, as the Official method[2] for determining vitamin A in margarine.

Since its first appearance in 1951, several attempts have been made to simplify the original procedure so as to make it more convenient for routine use. For instance, Braekkan and Lambertsen[3] used both adsorbents in one column, and very recently Rosner and Kan[4] have claimed that only the alkaline alumina is really necessary. However, both these modifications depend for their success on the preparation under rigidly defined conditions of a specially activated and unstable adsorbent, which, particularly for the occasional user, is inconvenient.

Simultaneously with the above-mentioned studies, we were attempting to remove irrelevant absorption from the unsaponifiable fraction of margarine without resorting to the use of specially activated adsorbents. The adsorbent we selected for this purpose was de-fatted bone meal, which was first used by Mann[5] in the carotenoid field and recommended by Goodwin and Morton[6] for separating carotene and vitamin A when both are present in the unsaponifiable fraction of margarine. Preliminary experiments (see below) were intended at first merely to find a means of improving the precision of the Carr - Price test when applied to margarine. It was found that an improvement could be brought about because bone meal is capable of holding vitamin-A alcohol adsorbed, while pigments (whether carotene or dye) are washed through together with a good deal of other debris, amounting to about 50 per cent. by weight of the total unsaponifiable fraction. These findings encouraged us to extend our studies to see whether, by enlarging the column of bone meal and controlling experimental conditions more rigidly, it would be possible to recover a fraction exhibiting no spectral absorption in the region of 325 mμ except that attributable to vitamin A.

Experimental

The unsaponifiable fraction from 25 g of margarine is prepared according to standard practice and taken up in 10 ml of light petroleum, boiling range 40° to 60° C. The solution is transferred quantitatively to a short column of de-fatted bone meal (supplied by The British Drug Houses Ltd.) standardised according to Nelson,[7] 3 cm long by 3 cm in diameter, contained in an ordinary drip-through extraction tube supported in a Buchner flask. The column is washed with light petroleum alone until no more pigment (carotene or dye) comes through. If the eluate contains carotene, it is laid aside for determination, otherwise it is discarded. The column is then washed with light petroleum containing 5 per cent. of freshly

distilled diethyl ether until a second (fainter) pigment zone has been washed through. This eluate is discarded. Finally, the column is stripped with 75 ml of acetone, and the colourless eluate containing vitamin A is transferred to chloroform for determination.

The effect of this procedure on the precision of the Carr - Price test applied to the unsaponifiable fraction of margarine is shown by the data in Tables I and II, for which results were obtained, respectively, on a Beckman model DUV photo-electric spectrophotometer and on a Unicam photo-electric photometer,[8] a calibration curve being used.

TABLE I

EFFECT OF REMOVAL OF MARGARINE PIGMENTS ON CARR - PRICE TEST

Concentration of unsaponifiable fraction with respect to margarine	Aliquot used,* with 4 ml of Carr - Price reagent	Extinction coefficient at 620 mμ†	
		On whole unsaponifiable fraction	After bone-meal treatment
500	0·13	0·0364	0·0321
250	0·26	0·0380	0·0332
167	0·40	0·0408	0·0335
125	0·50	0·0442	0·0353

* Aliquots chosen to ensure Beckman readings in the range 0·4 to 0·6.
† Each result the mean of three individual readings.

TABLE II

RESULTS ON COMMERCIAL MARGARINE ASSAYED IN THREE LABORATORIES

	On whole unsaponifiable fraction*			After bone-meal treatment		
	Laboratory A	Laboratory B	Laboratory C	Laboratory A	Laboratory B	Laboratory C
	15·4	20·1	16·6	13·9	16·7	17·1
	14·6	17·3	16·9	16·2	17·2	16·9
	15·2	20·1	16·7	16·8	16·5	17·9
Mean		17·0			16·6	
Standard Deviation		2·0			1·1	

* Gross reading × 0·95 (to allow for interference by the dye).

The foregoing few results show that removal of the dye, and with it about half of the total unsaponifiable fraction by weight, does bring about a marked improvement in the precision of the Carr - Price test. In laboratories where of necessity the Carr - Price test may have to be used, this observation may be of some practical value. However, examination of the spectrum in the ultra-violet region of the vitamin-A-containing eluate showed that it still retained a large amount of material having general absorption which greatly distorted the vitamin-A band. It was to improve the quality of the vitamin-A fraction that the column was enlarged and conditions of elution were more rigidly controlled.

Exploratory experiments showed that the isolation from the column of a fraction having spectral absorption at 325 mμ due predominantly to vitamin A is brought about better by using light petroleum of boiling range 80° to 100° C than by light petroleum of boiling range 40° to 60° C. It was therefore necessary to establish criteria of spectral purity for vitamin-A alcohol in light petroleum of boiling range 80° to 100° C. The criteria selected as convenient for this solvent are as follows—

$$E^{1\%}_{1\,cm} \text{ at } 325\ m\mu = 1830,$$
$$E \text{ at } 315\ m\mu = E \text{ at } 335\ m\mu,$$
$$E \text{ at } 315\ m\mu / E \text{ at } 325\ m\mu = 0{\cdot}91 \pm 0{\cdot}02.$$

The separation of the vitamin-A fraction is subject to two opposing factors, namely, speed of elution and purity as measured by spectral absorption. Increasing the amount of diethyl ether in the eluting solvent accelerates the rate of elution, but the spectral absorption

of the vitamin-A fraction becomes more and more distorted, owing to the presence of irrelevant absorption, as shown by the following typical data—

Percentage of diethyl ether in light petroleum	8	12	16	20
Total volume of solution, ml	250	180	125	100
Tube numbers for vitamin-A fraction	40 to 50	25 to 37	16 to 25	12 to 20
E at 315 mμ/E at 325 mμ	0·89	0·91	0·92	0·95
Recovery, per cent.	104	104	108	113

The conditions given below (for 16 per cent. of diethyl ether) were selected because, for routine purposes, they combine acceptable spectrophotometric purity with moderate volumes of solvent and reasonable speed of working.

Before proceeding to describe the method and its application in detail, it is of interest to consider the mechanism of the separation and the sharpness with which the separation of the vitamin-A fraction is brought about by using the selected conditions.

The unsaponifiable fraction of margarine was subjected to the chromatographic procedure. The fractions preceding and accompanying the vitamin A and that adsorbed on the column (stripped with acetone) were compared on the basis of weight and of spectral absorption at 325 mμ. Typical results are as follows—

Fraction	Percentage by weight of total unsaponifiable matter	Percentage of total spectral absorption at 325 mμ
Preceding vitamin A	65	60
Vitamin A	15	35
Adsorbed on column	20	5

For the vitamin-A fraction the separation is rather better than Morgareidge[9] found by the Official method in America; 27 per cent. by weight of the total unsaponifiable fraction

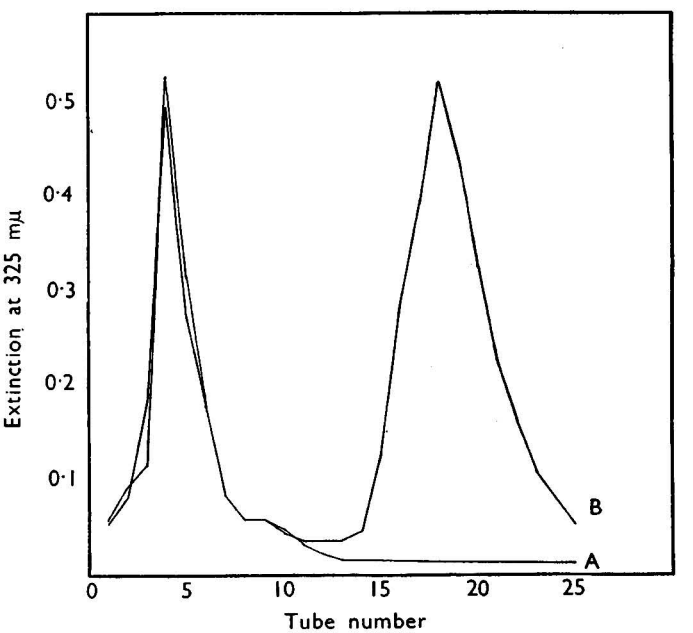

Fig. 1. Spectral absorption at 325 mμ of 5-ml portions of eluate from bone-meal columns: curve A, unvitaminised margarine; curve B, vitaminised margarine

accompanied the vitamin A. Of the spectral absorption of the vitamin-A fraction, about 95 per cent. is due to the vitamin itself.

In Fig. 1 is illustrated the sharpness with which the vitamin-A fraction is removed from the column. Unsaponifiable fractions from margarine were passed through the column, and the eluates collected in 5-ml portions. The extinction at 325 mμ was measured on each

tube. The general picture is in close agreement with that given by Rosner and Kan,[4] who used alkaline alumina.

METHOD

REAGENTS—

Light petroleum, boiling range 80° *to* 100° *C.*

Diethyl ether—Freshly distilled from sodium to remove peroxides.

Carr - Price reagent—As supplied by The British Drug Houses Ltd. for vitamin-A tests.

Bone meal—As supplied by The British Drug Houses Ltd. for carotene determinations (standardised according to Nelson[7]). A fraction passing No. 80 B.S. sieve and retained by No. 170 B.S. sieve has been used throughout our experiments. This range is not considered very critical, but to ensure a rate of flow acceptable for practical application of the method, the fines passing No. 200 B.S. sieve must be removed.

APPARATUS—

The chromatographic tube and the calibrated tubes (5 ml) for collecting the eluate are illustrated in Fig. 2.

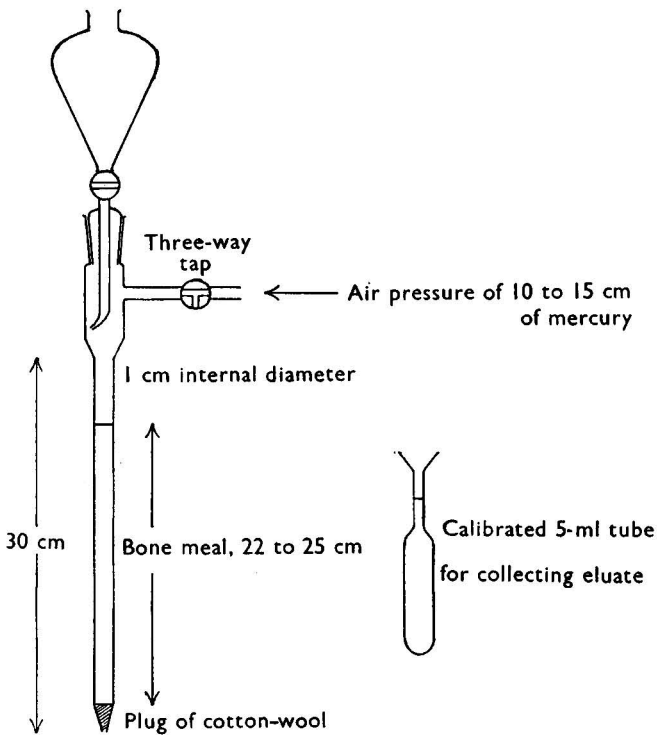

Fig. 2. Apparatus for applying the bone-meal technique to the unsaponifiable fraction of margarine

PREPARATION OF THE ADSORPTION COLUMN—

Plug the tube at the tapered end with a small wad of cotton-wool, and run in screened bone meal either dry and in suitable portions with tamping to ensure uniform packing or suspended in light petroleum. Enough bone meal is added to give a column 22 to 25 cm in length by 1 cm in diameter. Light petroleum, boiling range 80° to 100° C, is run on to the top of the column and drawn through by suction to remove air-pockets and until the whole column is full. Some light petroleum should remain above the bone meal. As a precaution against photochemical decomposition of the adsorbed vitamin A, sheath the column in black paper. In this condition the column is ready for use and is connected by its side-arm to a source of compressed air.

PROCEDURE—

Prepare the unsaponifiable fraction from 10 g of margarine, according to standard practice as described in the Official method.[2] Transfer this quantitatively to the column in light petroleum, boiling range 80° to 100° C. Apply sufficient air pressure to the column to cause a rate of flow of about 2 drops per second (10 to 15 cm of mercury has been found in practice to bring this about). Care must be taken from this point onwards always to keep some solvent above the top of the column.

Wash the column with about 50 ml of light petroleum until no more colour (carotene or dye) appears in the eluate. Change the eluting solvent to light petroleum containing 16 per cent. v/v of freshly distilled ether, and continue to wash the column. (If the mixed solvent is cloudy, it should be shaken with anhydrous sodium sulphate to remove moisture before use.) The eluate is collected in 5-ml portions. A second group of pigments (rather faint) appears in the eluate and acts as a marker for the approach of the vitamin-A fraction. This appears usually 3 or 4 tubes after the second group of pigments and extends through 7 to 12 tubes. Identify the tubes containing vitamin A by testing 0·4-ml aliquots with Carr - Price reagent and, from those giving a blue colour, unite the aliquots (2 or 3 ml) and make them up to suitable volume (usually 20 or 25 ml) in light petroleum, boiling range 80° to 100° C. Take readings at 315, 325 and 335 mμ, using a photo-electric spectrophotometer. The method of using these data to calculate vitamin-A potency is given under Results.

REGENERATION OF THE ADSORBENT—

A property of bone meal apparently not possessed by other adsorbents is that it can be used repeatedly. Throughout our experiments we have used a single column of bone meal for two or even three determinations. Between runs the column should be washed thoroughly with about 75 ml of light petroleum containing 16 per cent. of diethyl ether, drawn dry, and finally filled with light petroleum, boiling range 80° to 100° C.

Experience has shown that repeated use *in situ* cannot be continued indefinitely, partly because the adsorbent retains some of the unsaponifiable matter and partly because continued washing with solvent tends to break down the particle size and cause channelling. The result is that more and more irrelevant absorption appears in the vitamin-A fraction. When this happens (usually after not more than three determinations), the column should be emptied and the adsorbent thoroughly washed by decantation, a mixture of light petroleum, diethyl ether and acetone (3 + 1 + 1) as recommended originally by Mann[5] being used. After being dried, the bone meal should be screened again. It can then be packed into the column for further use.

In practice, we have found it most convenient to use two columns, so that when one is fresh the other is being regenerated.

RESULTS

Throughout the experimental work, the Official method has been used as a standard of comparison; for commercial margarines, aliquots of the same unsaponifiable fraction, each equivalent to 10 g of margarine, have been assayed by the two methods.

RECOVERY EXPERIMENTS—

As a preliminary, it was shown that vitamin-A alcohol in light petroleum solution could be passed through bone meal by using the selected experimental conditions and recovered completely (average 97 per cent., in agreement with the Official method) and spectrally unimpaired.

Known amounts were then added to the unsaponifiable fractions of unvitaminised margarines of various but typical composition in their fat blends. The spectral quality of the recovered vitamin-A fraction compared with pure vitamin-A alcohol is illustrated in Fig. 3, and quantitative recovery is shown in Table III, which includes figures showing the consistent spectrophotometric purity of the vitamin-A fraction.

The recovery is high, but in good agreement by the two methods, and also very close to that (107 per cent.) found recently by Morgareidge[9] in a collaborative test in which the Official method was used. Such high results can be explained only in terms of a small amount of irrelevant absorption in the vitamin-A fraction. However, from the spectral characteristics of the vitamin-A fraction it would seem that the nature of the irrelevant absorption differs

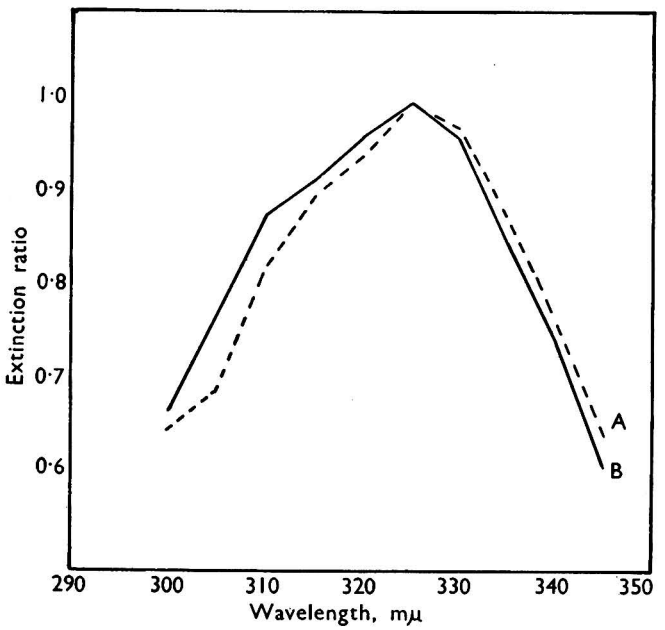

Fig. 3. Spectral absorption of vitamin-A alcohol, before (curve A) and after (curve B) recovery from the unsaponifiable fraction of margarine by the bone-meal method

TABLE III

RECOVERY OF VITAMIN-A ALCOHOL ADDED TO UNVITAMINISED UNSAPONIFIABLE PORTION OF MARGARINE

Bone-meal method

Vitamin-A alcohol added, i.u. per g	E at 315 mμ / E at 325 mμ	E at 325 mμ / E at 325 mμ	Vitamin-A alcohol recovered, i.u. per g	Vitamin-A alcohol recovered, %
21·3	0·92	0·87	25·2	118
36·1	0·91	0·85	38·1	106
34·1	0·92	0·85	37·9	111
31·6	0·93	0·85	33·4	106
24·0	0·93	0·86	25·8	108
36·2	0·92	0·85	39·4	109
		Mean recovery		109 per cent.

Official method

Vitamin-A alcohol added, i.u. per g	E at 309 mμ / E at 324 mμ	E at 334 mμ / E at 324 mμ	Vitamin-A alcohol recovered, i.u. per g	Vitamin-A alcohol recovered, %
31·2	0·87	0·86	33·3	107
29·0	0·87	0·85	30·8	106
31·0	0·89	0·85	33·2	107
26·9	0·86	0·85	28·2	105
34·3	0·86	0·85	36·6	107
33·1	0·85	0·85	37·7	114
		Mean recovery		108 per cent.

in the two methods. The spectral characteristics by the Official method conform to official standards ($0{\cdot}86 \pm 0{\cdot}02$), hence the curve must be lifted bodily. On the other hand, the spectral characteristics by the modified method imply that the curve is tilted to the short-wave side.

NATURE OF THE IRRELEVANT ABSORPTION—

Some clue as to the spectral nature of the irrelevant absorption was obtained by subtracting the "ideal" vitamin-A spectrum from the observed one. The results in Table IV show the amount of irrelevant absorption at wavelengths appropriate to the two methods.

TABLE IV

DATA SHOWING THE NATURE AND EXTENT OF THE IRRELEVANT ABSORPTION

	Bone-meal method				Official method		
	315 mμ	325 mμ	335 mμ		309 mμ	324 mμ	334 mμ
	0·045	0·043	0·031		0·028	0·029	0·023
	0·023	0·021	0·007		0·026	0·025	0·018
	0·028	0·020	Nil		0·041	0·030	0·022
	0·045	0·042	0·018		0·021	0·018	0·011
	0·013	0·017	Nil		0·028	0·032	0·020
	0·025	0·011	0·008		0·050	0·063	0·048
Means	0·030	0·026	0·010	Means	0·032	0·033	0·024

The general picture presented by these results was confirmed by using the unsaponifiable fractions from several samples of unvitaminised margarine and collecting from the adsorption columns the eluates which experience has shown would contain vitamin A if any were present.

From these experiments, the following conclusions were drawn—

(*a*) By both methods, the irrelevant absorption in the region of 325 mμ is approximately the same (compare mean values).

(*b*) The type of irrelevant absorption differs in the two methods. The tilted spectrum by the bone-meal method and the lifted spectrum by the Official method are consistent with the data on spectral properties given in Table III.

(*c*) In the bone-meal method, the irrelevant absorption at 335 mμ is relatively low, and at 325 and 315 mμ it is approximately equal. It follows that the quantity (E at 315 mμ − E at 335 mμ) gives a good estimate of the irrelevant absorption at 325 mμ.

The last observation can be made the basis of a simple correction procedure, partly experimental and partly empirical, which can be applied to the bone-meal method but not to the Official method.

CORRECTION FOR IRRELEVANT ABSORPTION—

In Table V are shown the bone-meal results from Table III corrected for irrelevant absorption by means of the formula—

E at 325 mμ (corrected) = E at 325 mμ (observed) − (E at 315 mμ − E at 335 mμ).

TABLE V

RECOVERY OF VITAMIN A AFTER CORRECTION FOR IRRELEVANT ABSORPTION

Vitamin A added, i.u. per g	$\frac{\text{E at 315 m}\mu}{\text{E at 325 m}\mu} = \frac{\text{E at 335 m}\mu}{\text{E at 325 m}\mu}$	Vitamin A recovered, i.u. per g	Vitamin A recovered, %
21·3	0·92	23·8	112
36·1	0·91	35·0	97
34·1	0·91	35·4	104
31·6	0·92	30·9	98
24·0	0·92	24·2	101
36·2	0·91	37·0	102
	Mean recovery		102 per cent.

It will be seen not only that recovery is very much nearer 100 per cent., but also that the spectral characteristics are close to those found for pure vitamin-A alcohol (0·91 ± 0·02 in light petroleum, boiling range 80° to 100° C).

APPLICATION TO COMMERCIAL MARGARINE—

Twenty-five-gram samples of commercial margarines of six different brands were saponified, and aliquots of the unsaponifiable fraction each equivalent to 10 g of margarine were submitted to assay by the bone-meal and Official methods. Typical figures are given below to illustrate the method of applying the correction procedure and calculating the vitamin-A potency in i.u. per gram of margarine.

The vitamin A from 10 g of margarine was contained in eight tubes. Aliquots (3 ml) were withdrawn from each, combined and made up to 25 ml.

Strength of solution in terms of margarine =

$$\frac{3}{5} \times 10 \times \frac{100}{25} = 24 \text{ per cent.}$$

Observed optical densities (1-cm cell) at 315, 325 and 335 mμ were 0·405, 0·440 and 0·383, respectively,

whence $\frac{\text{E at 315}}{\text{E at 325}} = 0{\cdot}92$ and $\frac{\text{E at 335}}{\text{E at 325}} = 0{\cdot}87$.

E at 325 (corrected) $= 0{\cdot}440 - (0{\cdot}405 - 0{\cdot}383)$

$= 0{\cdot}418$,

whence $\frac{\text{E at 315}}{\text{E at 325}} = \frac{\text{E at 335}}{\text{E at 325}} = 0{\cdot}916$.

Vitamin-A potency $= \frac{0{\cdot}418}{24} \times 1820 = 31{\cdot}7$ i.u. per g.

Results in Table VI illustrate the application of the bone-meal and Official methods to six brands of margarine.

TABLE VI

VITAMIN-A POTENCY OF COMMERCIAL MARGARINES

Brand	Sample*	Bone-meal method: Gross	Bone-meal method: Corrected	Bone-meal method: $\frac{\text{E at 315 mμ}}{\text{E at 325 mμ}} = \frac{\text{E at 335 mμ}}{\text{E at 325 mμ}}$	Official method
I	1	29·0	26·9	0·92	28·0
	2	28·3	26·2	0·93	26·6
II	1	32·9	30·7	0·92	32·0
	2	28·8	26·9	0·92	27·8
III	1	37·5	36·2	0·91	35·6
	2	36·1	34·5	0·92	33·6
	3	35·5	33·7	0·93	32·3
	4	32·5	31·2	0·91	32·0
	5	38·3	35·6	0·92	38·2
IV	1	30·8	28·6	0·92	30·6
	2	30·7	28·6	0·92	30·1
	3	38·5	36·3	0·91	37·3
	4	37·6	35·0	0·91	36·0
V	1	29·2	27·5	0·91	29·1
VI	1	21·9	20·6	0·94	20·0

* Packets of the various brands purchased on different dates, not replicated analyses on one packet.

The precision of the two methods was compared by using duplicate determinations by each method on each of six commercial brands of margarine. The results, together with the standard deviation of a single determination by each method, are shown in Table VII.

From the results in Table VI, it was calculated that, on average, the corrected value is about 6 per cent. lower than the gross value—a result in close agreement with that of the recovery experiments (7 per cent., Tables III and V). Moreover, the spectral characteristics after correction conform closely to those of pure vitamin-A alcohol (0·91 ± 0·02).

Comparison of the corrected bone-meal results with those by the Official method showed that the former are about 3 per cent. lower. However, since there is some evidence

(Morgareidge[9] and Table III above) that the Official method tends to give high results, it may be that corrected results by the bone-meal method are slightly more accurate.

With the limited data at present available, it is not wise to press too far the question of the precision of the two methods. It would seem from the data in Table VII, however, that the precision of the bone-meal method is comparable with that of the Official method.

TABLE VII

PRECISION OF THE BONE-MEAL AND OFFICIAL METHODS

Method	Margarine sample 1	2	3	4	5	6	Standard deviation of a single result
Bone-meal (corrected)	26·3	30·7	36·2	36·4	24·9	28·6	0·8
	26·9	29·8	36·2	34·5	26·2	28·6	
Official	26·6	32·0	35·6	33·6	24·2	26·7	1·6
	28·0	32·3	37·9	32·0	26·6	30·6	

So far, all the results presented refer to one laboratory only. In order to investigate, as far as possible at this stage, the reproducibility of the bone-meal method in more than one laboratory, a small-scale collaborative trial was arranged in which four different brands of commercial margarine were used. The results are shown in Table VIII.

TABLE VIII

OFFICIAL AND BONE-MEAL METHODS APPLIED IN TWO LABORATORIES

Brand	Bone-meal method		Official method	
	Laboratory A	Laboratory B	Laboratory A	Laboratory B
I	29·5	30·2	32·5	32·2
I	26·8	26·9	27·1	27·8
II	33·0*	35·0	35·1	36·0
III	32·5*	34·7	33·9	36·8
IV	23·4*	25·6	27·0	26·6

* Samples 11 weeks old when these three results were obtained.

Considering the delay in carrying out three of the determinations by the bone-meal method in laboratory A, the accuracy (4 per cent. less than the Official method) and precision (standard deviation of a single result in either laboratory by both methods about 1·1) are in good agreement with the data given in Tables VI and VII.

CONCLUSIONS

Neither the Official method nor the present modification of it is analytically perfect; both tend to give results 5 to 10 per cent. too high owing to irrelevant absorption in the vitamin-A fraction. High results were obtained by the Official method both in our experiments and in those of Morgareidge[9] in spite of the close conformity of the observed spectral ratios (E at 309 mμ/E at 324 = E at 334/E at 324 = 0·86) with those for pure vitamin A. It would seem that by this method the presence of a small amount of irrelevant absorption may easily be overlooked, thus giving a false sense of security. With the bone-meal method, on the other hand, the presence of irrelevant absorption is easily detectable by the tilt in the spectrum, and a simple correction, largely empirical but with some justification from experimental observations, can be applied. That such a correction is necessary is unfortunate and a little disappointing, but its validity seems fairly well established by recovery experiments and by the value of the spectral ratios (E at 315 mμ/E at 325 = E at 335/E at 325 = 0·91) after its application.

The relative convenience of the two methods will doubtless depend largely upon circumstances. When margarine is being assayed routinely, and the rate of turn-over may be rapid, the maintenance over short periods of suitable supplies of specially activated adsorbents may not be a serious impediment to the Official method. On the other hand, in the occasional assay, for which the preparation of special adsorbents would be a necessary but somewhat troublesome preliminary, the use of a commercially available adsorbent may

be attractive. There may doubtless exist some suspicion as to the uniformity of a naturally occurring adsorbent such as bone meal. We can only say that many samples de-fatted by ourselves and at least six purchased during the past 9 months have all proved satisfactory in adsorbent properties. At the present time, the possibility is being explored of making available commercially de-fatted bone meal conforming to our recommended particle size. If this proves possible at a reasonable price, one further simplification in the method will have been brought about.

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Discussion on the Foregoing Three Papers

Mr. R. J. Taylor spoke several times in the discussion, and has gathered his remarks into the following written contribution—

The papers that have been presented offer a number of points of interest. In vitamin A and its congeners the dominant group involved in chromatographic separation is that of OH and normally, when trying to disentangle secondary characteristics, one would aim to depress the OH effect by esterification. The fact that dicalcium phosphate can achieve separation without esterification suggests a different adsorption mechanism from that of alumina.

So far as natural products are concerned, it is important that the various vitamins A should be recognised and determined, but it is fair to point out that, excluding vitamin A_2, it is unlikely that any serious error in determination would arise from treating all vitamin A_1 as all-*trans* vitamin A_1. This is important in relation to a question from a later speaker about the possibility of neovitamin A being produced from the synthetic all-*trans* material at some stage in processing. This is, in fact, unlikely to happen, but even if, say, 20 per cent. of neovitamin A were to be produced, it would lead to an error of only 4 per cent. in the assayed potency.

There is just one point in the first paper on which I would disagree with Professor Morton, and that was his statement that there was always some loss of vitamin A during chromatographic separation. It has been the basis of our support for methods using alumina that one can obtain 100 per cent. recovery when pure vitamin A is treated chromatographically, and it is a standard test of the technique, both in preparing the aluminas and in manipulation, to demonstrate such a recovery.

Considering the first two papers as a whole, one might say that they present a good argument for adding only the easily assayed all-*trans* compound to a food product to which guaranteed limits are applicable.

The third paper, by Mr. Lord and Miss Bradley, almost certainly reflects a fairly widespread hope that a simpler form of assay for margarine might be found feasible, and that alone would demand some defence of the Official method and clarification of its apparent complexity. But before coming to that, I should like to deal with three points arising out of the paper itself. First, as to the use of a naturally occurring absorbent, de-fatted bone meal, which, as Mr. Lord has stated, was first introduced by Barton Mann for the estimation of carotene in grass meal. We collaborated in that work and found that when we used Barton Mann's bone meal we obtained good results, but if we changed to another source, then different results were obtained. The Americans in their early vitamin-A work used another natural product, Floridin, but soon abandoned it. It seems inevitable to me that with any natural product the chance of inconsistent behaviour will always be present, and that such materials must be viewed with suspicion.

Secondly, there is a reference to the Carr - Price test, although the final form of the method does involve measurements in the ultra-violet. This test will always attract because of its essential simplicity, but its quantitative limitations—particularly with extracts from low-potency materials—must be emphasised. It is extremely sensitive to environment, and, although it may be possible for manufacturers, knowing the composition of their own products, to use it as an internal check test, it would be unwise for analysts generally to rely on results obtained with it.

Finally, it is proposed to apply a correction procedure in order to determine the true potency. Now the question of correction procedures is one that I do not wish to enlarge on here, but I do wish to present the point that, if it is possible to isolate a substance quantitatively, then that is a sounder method of analysis than to obtain an admittedly imperfect measure and to correct it.

As to the method as a whole, it would be interesting to know if it has been applied to a variety of margarine brands and what results have been obtained.

Turning now to the Official method of analysis, we must admit that the major difficulty that has been met is in the preparation of the alumina, and I think it would help towards an understanding if I gave a brief account of the process of activation. If one takes crystalline alumina trihydrate and heats it, it will lose water and change its characteristics, and the extent of those changes is determined both by the temperature at which it is held and by the duration of the heat treatment. At temperatures between 100° and 225° C, two molecules of water are driven out of the molecule, so that, at any given temperature within that range, there is an equilibrium mixture of the mono- and trihydrates. At 225° C there is only monohydrate. Both are completely defined crystalline entities and both are completely inactive.

Above 225° C ejection of the third molecule of water begins and there is a partial conversion to active γ-alumina. Ejection is complete at 500° C, and only anhydrous γ-alumina is present. This, however, is not a completely definable entity like the mono- or trihydrate. At this stage the molecules are in a highly disordered state, and it is this state of disorder which confers the property of adsorption. As the alumina is raised to yet higher temperatures, a gradual molecular re-orientation takes place and the activity of the alumina is reduced, until finally—at a temperature of about 1200° C—the molecules have re-oriented completely, and inactive α-alumina is obtained. American workers claim to recognise several intermediate stages before α-alumina is formed, but for our purpose it is easier to think in terms of an active γ-alumina that is gradually modified in adsorption characteristics as the temperature of treatment is increased.

Curiously, the highest degree of activity is reached before complete dehydration—at about 400° C—which suggests a very high degree of disorder for the anhydrous portion then formed. It is important to note, however, that, although I talk of a gradually modified γ-alumina, for any given temperature of activation an end-product of fixed adsorption characteristics will be obtained, and that subsequent heating at any lower temperature cannot alter those characteristics; it can only drive off physically adsorbed water. It is therefore meaningless to prescribe specific heat treatment for an activated alumina whose original activation temperature is not known.

Theoretically, by the correct choice of activation conditions, one could prepare an alumina for any particular purpose; but, so far as vitamin A is concerned, one must take account also of chemisorptive effects and that is the reason why 2 per cent. of water is added in the one instance and 12 per cent. in the other. The water acts as a physical barrier between vitamin A and the alumina, and, since the adsorptive forces are now acting at a distance, the alumina itself must be more active. The activity chosen is such that with 2 per cent. of water added the material is roughly in equilibrium with normal humidity conditions and hence is reasonably stable. One must be careful in using old stocks of nominally anhydrous material, however, to see that they have not already come into equilibrium. It is preferable where uncertainty exists to drive off this adsorbed water and to re-moisten quantitatively.

The source of the trihydrate that we have used is the normal commercial production of the British Aluminium Company. It should not contain more than 25 per cent. of material passing 300 mesh. There is a need here for organisation for the supply of either hydrate or activated alumina, which might best be arranged through normal laboratory suppliers. It is important, however, in the case of alumina that activation details should be stated on the label.

The alkaline alumina is not an adsorbent in the accepted sense. It has a limited life owing to gradual chemical reaction between the alkali and the alumina. It functions more as a chemical filter holding back tocopherol-like material. It should not present any difficulty if the directions for preparation are followed carefully.

For immediate use we prefer to keep both aluminas under light petroleum in small bottles, from which they are dispensed as required, after shaking.

There is a simple check on the preparation of the aluminas, which we had hoped to see incorporated in the Official method, but it was considered better dealt with elsewhere, and this would appear to be as good an "elsewhere" as any. The check is this: that during chromatographic separation vitamin A should be detected in the eluate during development with either 20 or 25 per cent. of ether in light petroleum and it should be collected in 9 to 15 tubes. If it comes earlier and in too few tubes, then one or other of the aluminas is too moist; if it comes too late and in too many tubes, they are too dry. In the former case there is likely to be irrelevant absorption on the low-wavelength side of the curve and the result will be too high; in the latter a curve of good characteristics will be obtained, but the chances are that the result will be low. This check is quite independent of the pressure applied. Development is solely a function of the types and volumes of solvents. Pressure is used for convenience in providing suitable rates of flow.

It is not only irrelevant absorption that will distort the curve, however. Light can act similarly, and the necessity for working in subdued lighting cannot be too highly stressed. Those who saw our demonstration of the method at Oxford two years ago will recollect how well it worked; more consistently so than we ourselves expected at that time. We were, of course, demonstrating in the rather poorly lit basement laboratory, and it turned out to be a good and fortunate thing. Excessive light appears to lead to destruction in part of one of the double bonds in vitamin A and so to some absorption in the four double-bond region at 290 mμ. Elimination of this type of distortion leads generally to a higher assayed potency. The spectrophotometric limits of acceptance that have been chosen are those of the World

Health Organisation. If the assay conforms with the chromatographic behaviour and spectrophotometric characteristics referred to above, then the result will fall within a ± 5 per cent. limit of error.

A further point that has been raised is about the treatment of solvents. Good quality light petroleum can be used directly, but ethyl ether must always be rendered peroxide-free. Our method is first to distil it from solid caustic pellets, then to shake it with and stand it over a 10 per cent. solution of ferrous sulphate. Before use the ether is decanted and redistilled. It is then used directly for unsaponifiable extractions, and, after drying with anhydrous sodium sulphate, for chromatographic separation.

Finally, I would refer to the charge of complexity in the Official method. It is complex, but not more so than many well-established methods in other fields of analytical chemistry: for very often quantitative accuracy involves complexity of treatment and that is not normally considered a disadvantage. I think that the dominant factor is really one of novelty, for those unused to the ways of vitamin A must find the techniques strange at first. We have no difficulty in training laboratory assistants in their use, and I am sure that, once the sense of novelty has gone, analysts will find that much of the complexity will go with it.

MR. EDMUND HAYES said that the Robeson - Baxter method for estimating neovitamin A depended on measurement of the blue colour obtained with antimony trichloride. Blue-colour measurements on low-potency materials like cod-liver oil were known to be untrustworthy. He asked if the discrepancies between the Liverpool and Copenhagen results were in any way associated with this difficulty, and also if saponification caused any change in the proportion of neovitamin A when the whole oil contained the isomers in proportions different from those in the equilibrium mixture.

MR. R. C. SPALDING said that the paper presented by Mr. Lord was of great interest to those concerned with assessing the vitamin-A potency of margarine, as it gave, compared with the Official method, a simple and rapid test. It seemed to be generally true that the preparation of a suitable alumina for the Official method was presenting much difficulty to analysts, and in the literature on the point authors did not appear to be in agreement as to the best treatment. It would therefore be very interesting to learn if Mr. Lord had experienced any difficulty in obtaining uniformly active bone meal and if he had found it necessary to sift out the "fines," since, in the questioner's experience, their presence could block the column.

MR. H. E. MONK asked if Mr. Taylor could say if the amount of "fines" in the alumina affected its sensitivity or its purification. The Official method mentioned a mesh that had to be passed, but not one to retain the alumina. He also asked Professor Morton whether there was much difference between the various isomers of vitamin A in their sensitivity to oxidation and the effect of ultra-violet light and, in general, in their liability to decomposition.

MR. R. S. HATFULL asked whether the factor used when determining the Carr - Price blue value of natural products at 620 mμ was suitable or whether a higher factor should be used when the method was applied to margarine containing the pure synthetic all-*trans* isomer, since the biological activity, weight for weight, of this isomer was greater than that of the mixture of isomers found in natural products.

MR. E. H. HOPKINS said it seemed to him that a point to watch in chromatographic assays was the selection of criteria of purity for the vitamin-A fraction. For example, he asked if it would not be possible for a vitamin-A fraction to be obtained by the Official margarine assay that would merit the direct use of the conversion factor when judged by that assay's criteria, but required correction when judged by the World Health Organisation criteria.

PROFESSOR MORTON replied that if a method was complex it remained so even if one become familiar with it. A chromatographic analytical procedure was less than ideal if the analyst had to activate alumina himself, use two preparations—one of "limited life"—and observe great vigilance to avoid loss of vitamin A from one of several possible causes.

The first two papers did not present an argument "for adding only the easily assayed all-*trans* compound to a food product to which guaranteed limits are applicable"; this might be the policy which Mr. Taylor would advocate, but to use this work in support was illegitimate.

The antimony trichloride colour test applied to unsaponifiable matter or to rich oils was capable of considerable accuracy; in studying extracts from margarine, a calibration curve made with the aid of all-*trans* vitamin-A acetate gave good results. A conversion factor of $E^{1\%}_{1\,cm}$ at 620 mμ × 660 was justified.

MR. LORD agreed with Mr. Taylor regarding the quantitative limitations and sensitivity to environment of the Carr - Price test as normally applied to margarine. However, experience typified by data given in the earlier part of the paper showed that removal of pigments together with half or more of the unsaponifiable fraction before application of the Carr - Price reagent did bring about increased precision and substantially improved the test for routine internal checking.

As for the suspicion with which Mr. Taylor regarded bone meal as an adsorbent and in reply to Mr. Spalding's question about uniformity of activity, it could only be reiterated that over some years, during which bone meal had either been de-fatted in the laboratory or purchased already de-fatted, no material that had proved unsatisfactory had been encountered. It was true that, in a method such as that just described, the demands made of bone meal, particularly in respect of particle size, were greater than had been made hitherto. However, the possibility of supplying analytical bone meal conforming

to a given specification was being further examined by The British Drug Houses Ltd. In reply to Mr. Spalding's query about fines, Mr. Lord stated that the removal of fines (by which was meant material passing a No. 200, and preferably a No. 170, B.S. sieve) was essential to the proper working of the method.

Experience so far had shown that the chances of a determination being uncertain or invalid through the use of unsatisfactory bone meal were less than they were through the use of improperly activated alumina or unstable alkaline alumina, which had been found to be unreliable after 3 or 4 days, even in sealed ampoules. This experience was consistent with Mr. Taylor's remarks concerning the method of checking the preparation of the aluminas. Despite rigidly defined conditions of preparation and activation, it seemed that the final test of their behaviour was a purely empirical one.

In regard to Mr. Taylor's third point, Mr. Lord remarked that at the present time both methods tended to give high results, and, although the need for a correction was indeed unfortunate, the choice lay between the modified method, which appeared to be amenable to such a correction, and the Official method, which did not. Both methods had been applied to many samples representative of six different brands of margarine and, from the results given, they could be seen to be in close agreement.

The Colorimetric Determination of Silicon in the Micro-analysis of Biological Material and Mineral Dusts

By E. J. KING* and B. D. STACY*

and

P. F. HOLT,† DEIDRE M. YATES† and D. PICKLES‡

The basic ferric acetate precipitation procedure used for removing phosphate from solutions in which silica is to be determined has been critically investigated. It has been found that unless strict control of the pH is observed the method may give inaccurate results, owing to (*a*) incomplete removal of phosphate or (*b*) removal of silica with phosphate.

Previous work has been confirmed that both phosphate and silica couple with molybdate in weak acid, but that only the silicomolybdate complex is reduced in strong acid. On this principle a new procedure for determining silica in biological material has been developed, in which phosphate is left in solution.

Iron, in amounts greater than those occurring in tissue, has been found not to interfere.

In urines with high phosphate concentrations two methods have been evolved to overcome interference by precipitation of ammonium phosphomolybdate. In one, phosphate is partly or completely removed by precipitation with calcium hydroxide; in the other, ammonia and urea are removed with nitrous acid and sodium molybdate is used to couple with silica.

The new colorimetric procedure gives good agreement with gravimetric analyses, and good recoveries of added silica have been obtained with tissue, blood and urine. The procedure likewise gives results in good agreement with gravimetric methods, when applied to small samples of mineral dusts.

Silicon may be determined chemically by gravimetric, titrimetric and colorimetric procedures. All methods depend on the decomposition of siliceous material to liberate silicic acid. In the gravimetric method silicic acid is subsequently dehydrated to the oxide of silicon, and the silica, SiO_2, is isolated and weighed. The silica so isolated often contains small amounts of metallic salts, and for this reason it is usual to treat the impure silica with hydrofluoric acid, which completely volatilises the silicon as silicon tetrafluoride. The gravimetric procedure is described in most textbooks of quantitative analysis, *e.g.*, Hillebrand,[1] Treadwell and Hall[2] and Scott.[3]

Gravimetric procedures as ordinarily performed are not sufficiently sensitive for the accurate determination of less than 5 mg of silica. Micro-gravimetric procedures based on similar chemical principles to the above, but with the apparatus and techniques of Emich[4] and Pregl,[5] have been described by Morgan and King,[6] Gerstel[7] and Holt.[8]

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When soluble silica (silicic acid) and ammonium molybdate are present together in acid solution, silicomolybdic acid, which contains 1 molecule of silica to 12 molecules of MoO_3 (Parmentier[9]; Asch[10]; Copaux[11]; Pechard[12]; Miolati[13]), is formed. This acid forms insoluble compounds with organic bases such as brucine, coniine, pyridine, pyramidon, quinine, quinoline and strychnine. In the case of pyramidon and silicomolybdate 3 molecules of pyramidon are combined with 1 of silicomolybdic acid. King and Watson[14] described a method in which a weighable precipitate could be obtained in terms of this heavy salt of pyramidon silicomolybdate when the amounts of silicon were too small to determine in the micro-gravimetric method. Silicomolybdate can also be determined gravimetrically (or volumetrically) as an oxine - silicomolybdic acid complex (Volinetz[15]; Merz[16]), a hexamethylenetetramine complex (Duval[17]) and a 2:4-dimethylquinoline complex (Miller and Chalmers).[18]

A titrimetric method for determining silicon depends on the reaction (Tananaev and Babko[19])—

$$SiO_2 + 6NaF + 4HCl \rightarrow Na_2SiF_6 + 4NaCl + 2H_2O.$$

The method is rapid and has been extensively used, *e.g.*, by Korol and Koluzhskaya[20] and by Kern and Jones[21]; although it is accurate with fairly large amounts of silica, it is not in our experience trustworthy as a micro method. Another procedure involves the alkalimetric finish in which a quinoline - silicomolybdate complex is used (Wilson[22]).

Colorimetric methods form the basis of all micro-analytical procedures for silicon, when it is present in quantities of less than 1 mg. These procedures depend on the formation of silicomolybdic acid, $SiO_2.12MoO_3$, a method first advocated by Jolles and Neurath[23] and later by Schreiner[24] and Lincoln and Barker.[25] This yellow compound, which was originally given the formula $H_8Si(MoO_7)_6.H_2O$ and later assigned the formula $H_4Si(Mo_3O_{10})_4$ by Souchay,[26] can be estimated colorimetrically. The well-known Dienert and Wandenbulcke[27] procedure is based on the formation of this highly coloured yellow silicomolybdic acid (see also Winkler[28]; Thresh[29]; Atkins[30]; Steffens[31]; Thayer[32]; Thompson and Houlton[33]; Liebknecht, Gerb and Bauer,[34] who investigated natural waters; and King,[35] who used it for determining the silica content of tissues).

A blue-colour method can be based on the reduction of the yellow silicomolybdic acid complex, and this has been made the basis of other colorimetric procedures. Procedures were described by Isaacs,[36] who used sodium sulphite and heat to effect the reduction; by Oberhauser and Schormüller[37] and Woods and Mellon,[38] who used stannous chloride; by Nemec, Lanik and Koppova,[39] who used sodium thiosulphate; by King,[40] Roman,[41] Bodnar and Török[42] and Gettler and Umberger,[43] who used quinol; by Berg and Teitelbaum,[44] who used pyrrole; by Parri and Scotti[45] and Rodillon,[46] who used hydroxylamine; by King and Stantial,[47] King,[48] Straub and Grabowski,[49] Olsen, Gee, McLendon and Blue[50] and Bunting,[51] who used aminonaphtholsulphonic acid; and by Gentry and Sherrington,[52] who used ferrous sulphate. The blue colour developed from a given amount of silica is more intense than the corresponding yellow colour (Roman[41]; De Eds and Eddy[53]).

The silicomolybdic acid reaction, however, is not a simple one (Armand and Berthoux[54]). The intensity of both the silicomolybdate yellow colour and the molybdenum blue reduced silicomolybdate colour depend on the acid as well as the molybdate concentration. Optimum conditions for colour development have been extensively studied by several workers, *e.g.*, King and Stantial.[47] Two forms of silicomolybdic acid (α and β) were recognised by Strickland,[55] both having the same empirical formula but different light-absorption properties. The form produced depends mainly on the degree of acidification of the molybdate. For analytical purposes it is desirable to ensure that only the β-complex is formed; this requires between 3 and 5 equivalents of acid per gram-ion of molybdate.

Silica, moreover, must be brought into a reproducible molecular state before the silicomolybdic acid reaction can be applied. Dienert and Wandenbulcke[27] distinguished between soluble silica, which reacts, and colloidal silica, which does not react with molybdate. Weitz, Francke and Schuchard[56] demonstrated that the degree of polymerisation of the silicic acid affects the rate of its reaction with molybdate; monomers react completely in 75 seconds, dimers in about 10 minutes and higher polymers take much longer. The study was extended by Alexander.[57] Alkali treatment has a depolymerising action, but it is not always reproducible (Adams[58]). Fusion with sodium carbonate is more reliable.

The reaction is also complicated by various interfering substances, of which phosphate and iron are most important. Interference can be avoided by first isolating the silica which,

is rendered insoluble by evaporation and dehydration, as in the gravimetric procedure, when it may then be fused with sodium carbonate and re-dissolved (compare King and Stantial[47]; Holzapfel[59,60]; Ohlmeyer and Olpp[61]).

Interference by phosphate can be avoided or minimised by carrying out the reaction in acetic acid (Isaacs[36]; Brabson, Harvey, Maxwell and Schaeffer[62]), when phosphomolybdic acid formation is inhibited; or the phosphomolybdic acid may be destroyed by other organic acids, *e.g.*, tartaric (Bunting[51]) and oxalic (see Schwartz[63] for a summary of these procedures; and Straub and Grabowski[49]), or by mineral acid (Kahler[64]; Harrison and Storr[65]; Milton[66,67]).

Interference by iron, which produces a green tint in the molybdenum-blue procedures, was compensated for by Isaacs,[36] but the results are not easily reproducible (King[35]). Thayer[32] found the influence of iron and of phosphate to be complicated and difficult to eliminate. Various complexes are produced (Arnfeld[68]; Wu[69]).

Atkins and Wilson,[70] King[35,48] and King and Stantial[47] thought it advisable to remove both phosphate and iron before applying the molybdate reaction. But this is not so easily done as might at first appear. Phosphate may be precipitated by magnesium, calcium and iron, in the presence of ammonia, but in the presence of iron variable amounts of ferric silicate may be precipitated with the phosphate (Thayer[32]; King[71]). A two-stage precipitation of iron by added phosphate at an acid pH, and of phosphate by calcium chloride with ammonia, was used by King and Stantial,[47] and King[48] used the basic ferric acetate procedure of Jacobs[72] for the simultaneous elimination of phosphate and iron, phosphate being removed as ferric phosphate and excess of iron as basic ferric acetate, together with any colloidal material, such as protein, fat, pigment and debris (*e.g.*, in urine). The remaining chloride has an almost negligible effect on the colour intensity (Robinson and Spoor[73]).

Although Strickland[55] has shown that the silicomolybdic acid complex can exist in two spectrographically dissimilar forms, nevertheless it is easy to obtain reproducible results with simple silicic acid solutions under standardised conditions. It is in the elimination of the interference by phosphate and iron that uncertainty may enter. Since certain materials to be analysed (*e.g.*, water; mineral dust) may contain silicon and neither phosphate nor iron, others phosphate but no iron (*e.g.*, urine) and others (*e.g.*, blood) much iron but less phosphate, it is apparent that any procedure must be varied to suit the material being analysed. In using the ferric acetate precipitation method with animal tissues to rid them of phosphate and iron, before applying the colorimetric method for silica, we have experienced occasional uncertainty as to whether we were eliminating all the phosphate or iron or both and not losing silica, and have at times encountered inaccuracies. Attention was therefore directed not to the stage of colour development in the phosphate-free solution but rather to a re-investigation of the precipitation procedure, to determine whether errors could be introduced by the incomplete removal of phosphate from the solution or by the co-precipitation of silica with the ferric phosphate and ferric hydroxide. There is no colorimetric method alternative to the molybdate procedure for the determination of very small amounts of silica. In this procedure, phosphate and silicate give similar colours, and it is possible for losses of silicate to be obscured by the retention of phosphate in the filtrate. To examine this point some of our experiments were carried out with labelled silicon and some with labelled phosphate.

As a result of this re-investigation, we have concluded that phosphate and iron can be successfully eliminated and silica completely recovered with the basic ferric acetate method; but that this can only be achieved by very careful attention to the details of the method and particularly to concentrations of reagents and pH. By taking advantage of an observation by Milton[66,67] that both silicate and phosphate couple with molybdate in weak acid solution, but that only silicomolybdate is reduced to the blue complex if the solution is first made strongly acid, we have been able to simplify the procedure greatly and to get rid of the troublesome precipitation of phosphate and iron. Likewise, amounts of iron likely to be present in animal tissues do not interfere. This paper presents the results of our critical study of the ferric acetate precipitation method, and of the development and testing of the new modified colorimetric procedure.

Experimental

Previous blue colour method—

An amount of the filtrate that may be expected to contain from 0·02 to 0·12 mg of silica is transferred to a 25-ml calibrated flask. Then 2, 5 and 10-ml portions of the weak standard

(equivalent to 0·02, 0·05 and 0·1 mg of silica) are put by pipette into similar flasks. Water is added to about 20 ml, and then 2·0 ml of acid ammonium molybdate and 0·5 ml of reducing agent. The flasks are shaken, and kept for 10 minutes for the colours to develop. Then 1 ml of 10 *N* sulphuric acid is added to each flask to stabilise the blue colours, the volumes are adjusted to 25 ml and the contents of the flasks thoroughly mixed. Comparison of the test is then made against the appropriate standard.

INVESTIGATION OF THE BASIC FERRIC ACETATE PROCEDURE FOR REMOVING PHOSPHATE AND IRON—

Adsorption of silica on the ferric hydroxide and phosphate precipitate—To 5 ml of standard silicate solution (0·142 mg of SiO_2 per ml) were added 20 ml of the ferric chloride reagent and 20 ml of sodium acetate reagent. The pH was adjusted to one of the several values given in Fig. 1 by the addition of sodium hydroxide to the acetate reagent. The solution was diluted to 100 ml, boiled, filtered and cooled. Part of the filtrate (5 ml) was transferred to a calibrated flask and the blue colour was developed by the above method. The optical densities of the solutions were determined photo-electrically.

In Fig. 1, curve A, the amount of silica lost with the precipitate is plotted against the

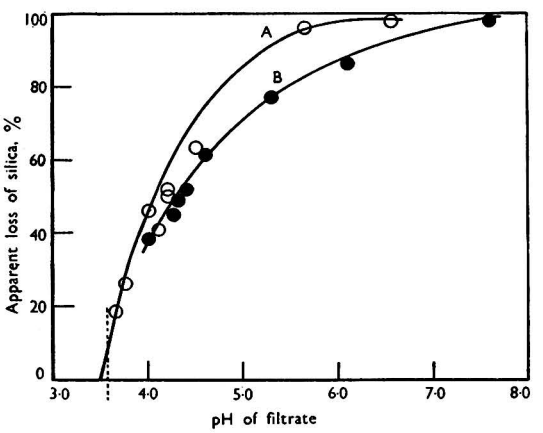

Fig. 1. Loss of silica by co-precipitation at different pH values: curve A, colorimetric measurements; curve B, radiometric measurements

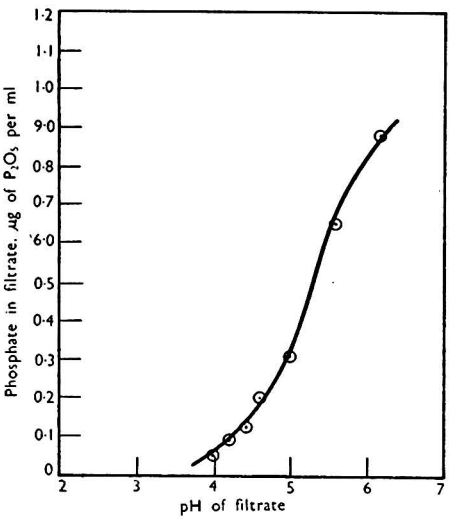

Fig. 2. Retention of phosphate by the filtrate at different pH values

pH of the solution. At pH 3·5, apparently, no silica is lost from the filtrate, but there is loss with increasing pH and at pH 5·5 practically all of the silica is retained by the precipitate. In fact, at pH 3·5 the recovery value is apparently slightly greater than the amount of silica added.

The experiment was repeated with ^{31}Si silicate. In this case the ferric phosphate - ferric hydroxide precipitate was rapidly washed to remove contaminating solution, dissolved in dilute hydrochloric acid and assayed with a Geiger counter.

The curve showing the silica loss at different pH values (Fig. 1, curve B) follows that obtained by the colorimetric method. But the values are slightly lower. Having regard to an apparent small gain in silica at low pH values shown colorimetrically, it appears that some other substance present in the reagent may give a slight blue colour with the molybdate reagents.

Efficiency of the procedure for phosphate precipitation—To each of several 10-ml samples of sodium silicate solution (1 mg of SiO_2) were added 10 ml of a solution of $NH_4H_2{}^{32}PO_4$ (24 mg of $^{32}P_2O_5$; specific activity 2·5 microcuries per ml). In each case the pH of the mixed solution was brought to one of the values shown in Fig. 2 by adding sodium hydroxide to the acetate reagent. The ferric chloride reagent (20 ml) and sodium acetate reagent (20 ml)

were added. The solutions were diluted to 100 ml, boiled, cooled and filtered. The filtrates were assayed with a Geiger counter.

The phosphate concentrations of the filtrates decreased with decreasing pH down to 0·05 μg of P_2O_5 per ml in a filtrate of pH 3·9 (Fig. 2). At a lower pH it is impossible to precipitate the ferric hydroxide completely.

Effect of phosphate concentration on the pH of the filtrate—To each of several 5-ml portions of silicate solution (0·5 mg of SiO_2) was added one of the volumes of phosphate shown in Table I and the solutions were neutralised to Congo red with dilute sulphuric acid. The 20-ml portions of the ferric chloride and sodium acetate reagents were added to each. The mixture was boiled, cooled and filtered. The pH of each filtrate was measured.

The pH of the several filtrates is shown in Table I. Although the pH is but little affected by small quantities of phosphate, larger amounts have an appreciable effect; 30 mg of phosphate may alter the pH by 0·3 units.

Buffering power of the acetate reagent—The acetate reagent was made up with a number of different concentrations of sodium hydroxide. The pH of each solution was determined by using a glass electrode.

At the concentration used, the acetate is operative as a buffer between pH 3·5 and 5·5 (Fig. 3), but even in this range small changes in the amount of alkali added produce appreciable changes in pH.

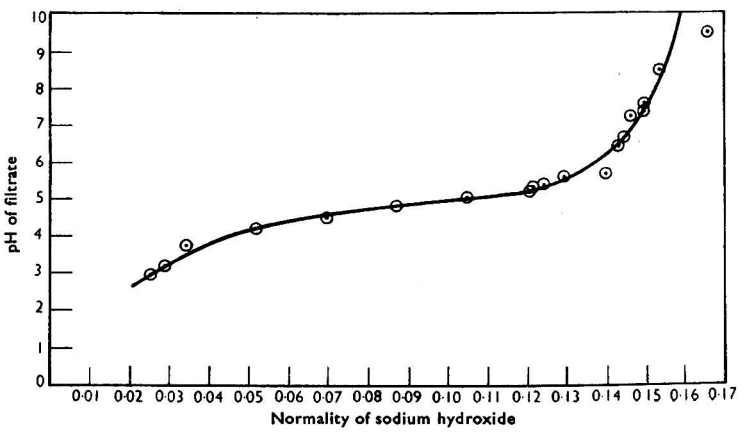

Fig. 3. Efficiency of the acetate buffer reagent

Discussion—The method of King[48] for the determination of silica in biological materials relies on the removal of phosphate, which interferes with the colorimetric determination of silica, as insoluble ferric phosphate in the so-called "basic ferric acetate precipitation." Excess of ferric chloride reagent, which also has a slight effect on the reduced molybdate colour, is hydrolysed to insoluble ferric hydroxide. The investigation described in this paper shows that phosphate is almost completely removed from the solution by this procedure if the pH is less than 3·9, and that silicate remains mainly in solution. It is equally apparent that, if the pH is even slightly higher than 3·9, phosphate appears in the filtrate and, at the same time, silica is co-precipitated with the ferric phosphate and hydroxide.

The efficiency of this method of analysis depends, therefore, on careful control of the pH. If substances that affect the pH of the solution are absent, the buffer used in the procedure will give a pH of 3·6 (indicated by the dotted line in Fig. 1). At this pH the loss of silica is small, but some iron remains in the filtrate as a ferric hydroxide sol. In the absence of other substances that affect the pH, it appears that the procedure as described will give satisfactory silica recoveries.

The sodium acetate used to buffer the solution may allow some variation in pH when small quantities of acid or alkali are added as may be seen from Fig. 3, in which is shown the effect on the pH of the filtrate of changing the sodium hydroxide concentration of the sodium acetate reagent, and in most biological materials substances that act as buffers are present. Because of this, it is possible for the buffering action of sodium acetate to be inadequate for accurate pH control. Even the precipitation of phosphate may have some

effect on the pH. Whilst small amounts of phosphate have little effect, 30 mg of phosphate, a quantity that may be present in 10 ml of urine, may alter the pH by 0·3 units. This pH shift is sufficient to cause a considerable error in the silica determination. The molybdate reagent is sensitive to many anions and this fact rules out the use of most of the usual buffer solutions. It is apparent that without careful adjustment of the pH to within 0·1 unit, which is difficult and probably impracticable with large numbers of samples, erroneous results may occur.

NEW METHOD—

In an investigation of the reduced molybdenum-blue method for silica, Milton[66,67] studied the formation of silicomolybdate and phosphomolybdate, and their reduction. Several workers (*e.g.*, Dienert and Wandenbulcke[27]; Armand and Berthoux[54]) showed that silicomolybdic acid will form only in weakly acid solution and that it is quite stable in strong acid. Phosphomolybdic acid, on the other hand, will form over a wide range of acidity, and is stable in strongly acid solution. But although phosphomolybdic acid forms over a wide range of acidity, it will not reduce to phosphomolybdenum blue in strong acid. This is in contrast to silicomolybdic acid, which can be reduced even at high acidities. Milton found that silicomolybdate forms in 0·1 *N* sulphuric acid, and that if the acidity is then raised to 2 *N* in sulphuric acid it will reduce to silicomolybdenum blue without any phosphomolybdenum blue being formed, even when considerable amounts of phosphate are present. There is no reduction of phosphomolybdate, and therefore no increased colour due to the presence of phosphate, if the solution is more than 1·5 *N* in sulphuric acid.

We have confirmed that silicate and phosphate both couple with molybdate in weak acid, but that only silicomolybdate is reduced if the solution is first made strongly acid. And we have likewise shown that small amounts of iron also do not interfere. This makes it possible to determine silica in the presence of phosphate and iron, with greater accuracy, and without the chance of loss or interference, which exists when it is necessary to remove these substances by precipitation procedures.

Development of blue colour at various acidities—

(*a*) *Silicate*—Five-millilitre portions of dilute silica standard (0·05 mg of silica) were diluted to 15 ml with water in 25-ml flasks. Then 2·0 ml of 5 per cent. ammonium molybdate in *N* sulphuric acid were added, mixed, and left for 10 minutes. Various amounts of 10 *N* sulphuric acid were added, mixed, and 0·5 ml of aminonaphtholsulphonic acid reducing agent. The solutions were made to volume, mixed, and the intensities of the colours measured in a Spekker absorptiometer after 10 minutes. Fig. 4 illustrates the results. The final acidity in the absence of any additional sulphuric acid is 0·08 *N* (2 ml of *N* acid diluted to 25 ml), and this mixture had slightly more colour than was obtained when extra sulphuric acid was added. But between 1 and 10 ml of added 10 *N* sulphuric acid, the intensity of colour was constant, showing that silicomolybdate blue is unaffected by acidity within the limits 0·5 to 4 *N*.

(*b*) *Phosphate*—One-millilitre portions of standard potassium dihydrogen phosphate solution (1·0 mg of phosphorus) were similarly treated. This amount of phosphate gave a very intense blue colour when the final acidity was kept at or below *N*. (The acidity usually maintained in phosphorus methods is 0·5 *N* to *N*.) Above this the phosphomolybdate blue colour decreased sharply, until at 1·5 *N* there was almost no colour, and at 2 *N* (5 ml of 10 *N* sulphuric acid) only the faintest tinge of blue, possibly due to a trace of silica, could be detected (Fig. 4). In another experiment larger amounts of phosphate, up to 5 mg of phosphorus, failed to give any appreciable colour.

(*c*) *Silicate and phosphate*—Five millilitres of silica standard (0·05 mg of silica) and 1 ml of phosphate (1·0 mg of phosphorus) were diluted to 15 ml, and similarly treated. With increasing acidity up to 1·5 *N*, the colour of the mixed solution was dominated by that contributed by the phosphate. Above 1·5 *N* it gave colours similar to the silicate solution with no phosphate. At 2 *N* sulphuric acid (5 ml extra of 10 *N*) and above, phosphate appeared to contribute no colour and does not interfere with the silicomolybdate blue (Fig. 4).

Under the conditions adopted for the test (volume of 25 ml, 2·0 ml of 5 per cent. ammonium molybdate and 5 ml of 10 *N* sulphuric acid), however, there is a limit to the amount of phosphate that can be tolerated. If phosphorus is present in an amount greater

than 1·2 mg of phosphorus with ammonium molybdate, or 1·3 mg with sodium molybdate, interference will occur (see Fig. 5). The intensity of the silicomolybdate blue colour decreases with increasing phosphorus concentration above this level, presumably owing to phosphate monopolising more and more molybdate, leaving insufficient for the formation of silicomolybdate. An attempt to counteract this was made by doubling the amount of molybdate.

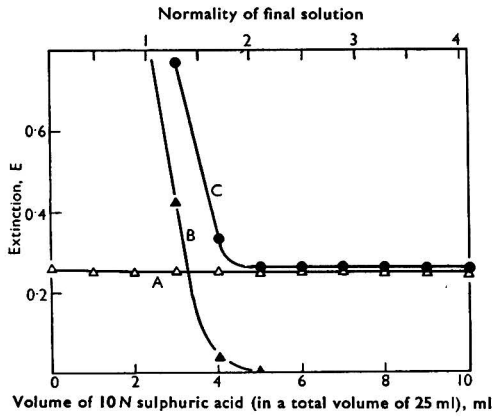

Fig. 4. Effect of acidity on the molybdenum blue colour formed by silica and phosphate: curve A, 0·05 mg of silica alone; curve B, 1·0 mg of phosphorus alone; curve C, 0·05 mg of silica plus 1·0 mg of phosphorus

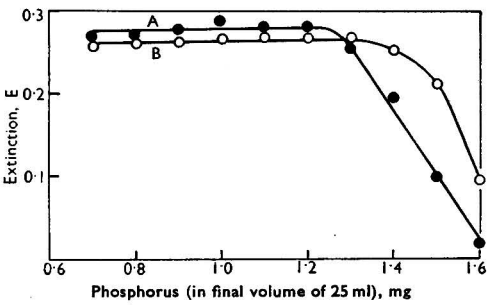

Fig. 5. Effect of phosphate on the molybdenum blue colour formed by silica: curve A, with 5 per cent. ammonium molybdate; curve B, with 7 per cent. sodium molybdate

Although higher levels of phosphate could then be tolerated, a marked colour progression was produced and there was a pronounced deviation from Beer's law.

In the molybdic acid complexes ratios of 12 molybdate to 1 phosphate or 1 silica have been found, *i.e.*—

$$\frac{MoO_3}{PO_4} = \frac{12}{1} \text{ (Schwartz[74])} \quad \text{and} \quad \frac{MoO_3}{SiO_2} = \frac{12}{1} \text{ (Snell and Snell[75])},$$

and it has been claimed that only a slight excess of molybdate is necessary for complete reaction. This does not appear to conform with the fact that 1·5 mg of phosphorus (≡4·5 mg of phosphate) interferes with the silicomolybdate formation even though excess (84 mg) of molybdate (MoO_3) is present. This may be due however to a change in the nature of the phosphomolybdate complex at these relatively high levels of phosphorus, which could alter the MoO_3 to PO_4''' ratio. Certainly the complex shows marked visible change with increasing phosphorus concentrations, changing from intense yellow at 1·2 mg to colourless at 2·0 mg of phosphorus.

Effect of iron—Small amounts of iron (up to 1 mg) (Fig. 6) have no effect on the silicomolybdate colour, either in the absence or presence of phosphate. This is more iron than is present in any sample of animal tissue that is likely to be taken for analysis, *i.e.*, it is more than is contained in 1 ml of blood, the highest iron-containing tissue. Phosphate is always in excess in animal tissue, *e.g.*, there are 40 to 50 mg of phosphorus (atomic weight 31) and 50 to 55 mg of iron (atomic weight 56) in blood (per 100 ml), and iron is unlikely to cause interference when such material is being analysed.

Method

Reagents—

All water used must be distilled and silica-free.

Silica standard: *sodium silicofluoride*—This salt is a light anhydrous non-hygroscopic powder that is hydrolysed in solution to give silicic acid. Hopkin and Williams' sodium fluosilicate (pure) and Schering - Kahlbaum's natrium silicofluoride are satisfactory. Other commercial preparations may be only partly soluble in water. A solution of 314 mg of the solid in 1 litre of water gives a concentration equivalent to 1 mg of silica in 10 ml (strong

standard). A weak standard (0·1 mg of silica per 10 ml) is made by diluting the strong standard 1 in 10. It has been found advisable to make fresh solutions each week.

Silicate solutions—Standard solutions were prepared by fusing pure silica, sometimes after irradiation in the Harwell pile (BEPO), with sodium carbonate (Holt and Yates[76]). Solutions are preserved in wax or polythene bottles.

Sulphuric acid, 10 N—278 ml of concentrated acid diluted to 1 litre with water.

Sulphuric acid, N—A 1 in 10 dilution of the 10 *N* acid.

Sodium hydroxide, N—The sodium hydroxide, to be silica-free, must be prepared from the metal. Hence 2·3 g of metallic sodium are dissolved in 100 ml of water in a nickel crucible, and the solution is preserved in a wax or polythene bottle. Sodium hydroxide pure (from sodium) as supplied by the British Drug Houses Ltd. is satisfactory. Glass pipettes should be brought into the sodium hydroxide solution only for the briefest possible times.

Phenolphthalein indicator—A 0·5 per cent. solution in ethanol.

Congo red indicator—A 0·1 per cent. solution in ethanol.

Sodium carbonate—AnalaR, and silica-free.

Acid ammonium molybdate—A 5 per cent. solution of ammonium molybdate in *N* sulphuric acid.

Acid sodium molybdate—A 7 per cent. solution of sodium molybdate in *N* sulphuric acid.

Reducing agent—A 0·2 per cent. solution of 1:2:4-aminonaphtholsulphonic acid in a solution of 2·4 per cent. of sodium sulphite, $Na_2SO_3.7H_2O$, and 12 per cent. of sodium metabisulphite in water.

Ammonium phosphate—A 2 per cent. solution of ammonium dihydrogen phosphate.

Standard phosphate solutions—Standard solutions of inactive phosphate were made from ammonium dihydrogen phosphate. These solutions were labelled by adding small quantities of $H_3{}^{32}PO_4$ (specific activity 2·0 millicuries per ml) to give a specific activity of about 2 microcuries per ml.

Potassium phosphate solution, 1 mg of phosphorus per ml—0·4388 g of AnalaR silica-free potassium dihydrogen phosphate dissolved in 100 ml of water.

Calcium chloride—A 0·5 per cent. solution of AnalaR calcium chloride, $CaCl_2.6H_2O$.

Calcium hydroxide—A saturated solution of the AnalaR solid, preserved in a wax or polythene bottle.

Sodium nitrite—A 5 per cent. solution of AnalaR sodium nitrite in water.

Trichloroacetic acid—A 20 per cent. solution in water.

Active carbon—Kahlbaum's Carbo Activ, or Neo Spectro M II, Columbian Carbon Co., Brooklyn 32, N.Y.

Ferric chloride in hydrochloric acid—A 1 per cent. solution of ferric chloride, $FeCl_3.6H_2O$, in 0·02 *N* hydrochloric acid.

Sodium acetate in sodium hydroxide—A 1·5 per cent. solution of sodium acetate, $C_2H_3O_2Na.3H_2O$, in 0·028 *N* sodium hydroxide.

Acetic acid—Glacial and 10 per cent.

TISSUE ANALYSIS—

Depending on the silica content, mix 0·05 to 0·5 g of dried powdered tissue with six times its weight of sodium carbonate in a 25 to 50-ml platinum crucible. Carefully ignite to remove organic matter and fuse to a clear melt (1 to 2 hours). Dissolve the melt in water by warming, cool, neutralise to Congo red with 10 *N* sulphuric acid and add a 0·2-ml excess of acid. Transfer to a calibrated flask and dilute to 250 ml (or to 100 ml if the tissue silica be very low). Transfer an amount of the solution containing 0·02 to 0·01 mg of silica to a 25-ml calibrated flask. To similar flasks add 2, 5 and 10-ml amounts (corresponding to 0·02, 0·05 and 0·10 mg of silica) of the weak standard and prepare a blank of water. Add water to about 15 ml and then 2·0 ml of 5 per cent. ammonium molybdate in *N* sulphuric acid. After 10 minutes add 5 ml of 10 *N* sulphuric acid and then 0·5 ml of aminonaphthol-sulphonic acid reducing agent. Make the solutions to volume, mix, and measure the intensities of the colours after 10 minutes.

METHODS FOR URINE—

Some urines may not be amenable to the colorimetric silica determination by the above silicomolybdate method. With rat urine, precipitation may occur following the addition

of the acid molybdate reagent. To obviate this difficulty modifications of the method have been investigated.

In determining silica directly by the addition of acid molybdate to diluted urine, various factors may interfere. Phosphorus forms phosphomolybdate complexes, and ammonia in the presence of phosphorus may form a precipitate of ammonium phosphomolybdate, an interference that can be overcome by the initial removal of ammonia and urea with nitrous acid (sodium molybdate being used subsequently to develop the colour), or by removal of all or part of the phosphate. Proteins and organic bases may form precipitates or turbid solutions. This can be effectively prevented by treating the urine with trichloroacetic acid and finely divided carbon.

(*a*) *By removal of phosphate*—In a Lusteroid or plastic test tube treat 0·3 ml of rat (or other herbivore) urine high in silica, or 3 ml of human (or carnivore) urine low in silica with 10 ml of 0·5 per cent. calcium chloride solution and saturated calcium hydroxide solution until pink to phenolphthalein and dilute with water to 15 ml. Add about 50 mg of carbon (Neo Spectro M II or Kahlbaum's Carbo Activ), shake the mixture, warm at 70° C for 15 minutes, cool and filter through a Whatman No. 42 filter-paper.

In 25-ml calibrated flasks place 10-ml portions of the filtrate (≡0·2 or 2 ml of urine), and 0·02, 0·05 and 0·10 mg of silica (2, 5 and 10 ml of weak standard). Add water to about 15 ml, and then 2·0 ml of 5 per cent. ammonium molybdate in *N* sulphuric acid. Mix, and after 10 minutes add 5 ml of 10 *N* sulphuric acid. After mixing, add 0·5 ml of aminonaphtholsulphonic acid reducing agent. Make the solutions to volume, mix, and measure the intensities of the colours after 10 minutes.

(*b*) *By removal of ammonia (and urea)*—Shake 2 ml of urine with 1 ml of 5 per cent. sodium nitrite solution, 2 ml of 20 per cent. trichloroacetic acid, 50 mg of carbon and 1 drop of caprylic alcohol for 5 minutes and filter. Transfer, for rat urine, 0·5 ml of filtrate (≡0·2 ml of urine), and for human urine 3 or 4 ml of filtrate (≡1·2 or 1·6 ml of urine), to a 25-ml calibrated flask and prepare standards as above. Add water to about 15 ml and 2·0 ml of 7 per cent. sodium molybdate in *N* sulphuric acid. After 10 minutes add 5 ml of 10 *N* sulphuric acid, mix and then add 0·5 ml of reducing agent. Make the solutions to volume and measure the intensities of the colours as before.

BLOOD ANALYSIS: SOLUBLE SILICA—

After deproteinising whole blood, serum or plasma with trichloroacetic acid, silica can be determined in the filtrate by the silicomolybdate method. The sample of the filtrate is heated in a boiling-water bath, before treatment with ammonium molybdate, to destroy most of the trichloroacetic acid, and so allow the silicomolybdate complex to form at the optimum pH.

Procedure—Mix 2 ml of whole blood, serum or plasma with 20 ml of 5 per cent. trichloroacetic acid and filter after 5 minutes. Transfer 15 ml of the filtrate to a 25-ml calibrated flask and heat for 15 minutes in a boiling-water bath. Cautiously shake the contents of the flask while hot to expel carbon dioxide. After cooling, add ammonium molybdate, 10 *N* sulphuric acid and aminonaphtholsulphonic acid as described above. Prepare a 0·02-mg silica standard.

DETERMINATION OF SILICA IN MINERAL DUSTS—

The total silica content of mineral dusts may be rapidly determined by the colorimetric silicomolybdate procedure. The method, although certainly not as accurate as the gravimetric, has been found to give acceptable results when samples of dust containing about 5 mg of silica are fused with 1 g of sodium carbonate. The fused melt is taken up in water, added rapidly to a known excess of acid, and shaken vigorously to expel carbon dioxide and dissolve any remaining particles of the melt. This rapid neutralisation, by preventing polymerisation of silicic acid (Holt and Osborne[77]), ensures complete reaction on subsequent treatment with ammonium molybdate. The tendency of iron to form insoluble fusion products can usually be overcome by initially roasting the sample in the platinum crucible and then carrying out the sodium carbonate fusion in an oxidising atmosphere (Hillebrand[1]).

Procedure—In a platinum crucible fuse with sodium carbonate (1 g) for 1 hour an amount of the dust containing about 5 mg of silica. Add water (20 ml) and warm the crucible for 30 minutes on a boiling-water bath, disintegrating the melt during this time with a plastic

stirring rod. Rapidly wash the contents of the crucible with water into a 250-ml calibrated flask containing 19 ml of *N* sulphuric acid (roughly 1 milli-equivalent of acid in excess of that required for the neutralisation of the sodium carbonate). Vigorously shake the flask until solution of the last particles is complete. Add water to the mark and mix the solution.

Transfer a sample of the solution containing about 0·05 mg of silica to a 25-ml calibrated flask and add water to 15 ml. Add 2·0 ml of acid ammonium molybdate, 5 ml of 10 *N* sulphuric acid and 0·5 ml of aminonaphtholsulphonic acid as described in the method for tissue analysis. In a photo-electric absorptiometer compare the intensity of the colour with that obtained from a 0·05-mg silica standard.

Results

Tissue—

Agreement between this colorimetric modification and gravimetric results on normal and pneumoconiotic lungs has been good. Mean results and range for 22 samples by gravimetric and colorimetric methods were 1·37 and 1·29 per cent. (4·2 to 0·1 per cent. and 4·6 to 0·1 per cent.), respectively, of silica. Analysis of variance showed no significant difference; residual standard deviation was 0·24 per cent.

Recoveries of pure powdered quartz added to dried, ground, foetal, human lung were 5·0 to 5·3 mg of silica when 5 mg were added to 100 mg of lung, and 9·8 to 10·0 mg of silica when 10 mg were added.

Urine—

Good recoveries (90 to 100 per cent.) have been obtained with the two modified colorimetric methods (Table II).

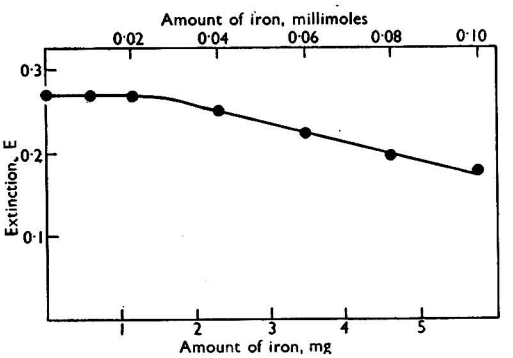

Fig. 6. Effect of iron on the molybdenum blue colour formed by silica

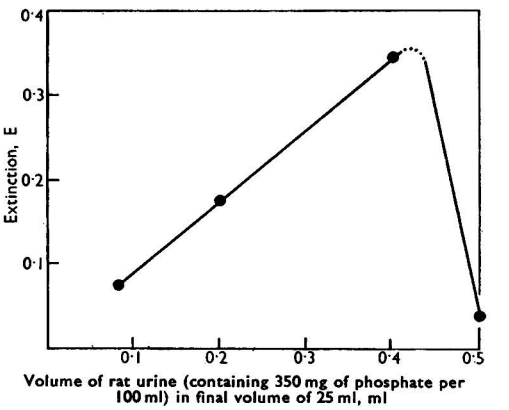

Fig. 7. Effect of increasing volume of rat urine on silica blue colour

In the sodium nitrite method not more than 1·4 mg of phosphorus should be present in the final volume (25 ml) in which the colours are developed. This method is therefore limited with human urines (with 1·4-ml samples) to those containing less than 100 mg of phosphorus per 100 ml of urine. At the risk of obtaining low absorptiometer readings, the method may be extended by using smaller samples. With rat urine, 0·2-ml samples being used in the final volume, the method should tolerate concentrations up to 700 mg of phosphorus per 100 ml of urine. This was tested by noting the effect on the silica determination of increasing the volume of rat urine. As shown in Fig. 7, there was a marked interference in colour formation when 0·5 ml of urine was used, suggesting that 0·5 ml contained an amount of phosphorus greater than 1·4 mg, *i.e.*, that the amount of phosphorus in the urine was greater than 280 mg per 100 ml. On determination it was found that the concentration of phosphorus was 350 mg per 100 ml of urine.

Blood analysis: soluble silica—

Recoveries of added silica and the silica concentration in several samples of human blood are shown in Table III.

MINERAL DUSTS—

The results (Table IV) obtained on analysing mineral dusts by the colorimetric procedure compare favourably with those determined by the standard gravimetric procedure.

TABLE I

pH OF FILTRATE AFTER THE PRECIPITATION OF PHOSPHATE

Weight of phosphate, mg of P_2O_5	0	0·5	2·0	5·0	10·0	20·0	30·0
pH of filtrate	3·77	3·74	3·76	3·78	3·85	3·98	4·09

TABLE II

COLORIMETRIC ANALYSIS OF URINE: RECOVERY OF ADDED SILICA AND COMPARISON OF SILICA ANALYSES BY DIFFERENT METHODS

	Calcium chloride precipitation method				Nitrous acid method				
Urine	Silica in urine, mg per 100 ml	Silica added, mg per 100 ml	Total silica found, mg per 100 ml	Silica recovered, mg	Urine used for analysis, ml	Silica in urine, mg per 100 ml	Silica added, mg per 100 ml	Total silica found, mg per 100 ml	Silica recovered, mg
Human 1	1·4	1·7	2·9	1·5	1·0	1·5	5·0	6·2	4·7
	1·3	1·7	2·8	1·5	1·0	1·5	5·0	6·2	4·7
					2·0	1·4	2·5	3·9	2·5
Human 2	1·2	1·7	2·9	1·7	1·0	1·1	2·5	3·4	2·3
	1·2	1·7	2·7	1·5					
Human 3	0·72				1·0	0·85	5·0	5·5	4·7
	0·74				1·0	0·95	5·0	5·5	4·6
Human 4					1·0	2·3	5·0	7·1	4·8
					2·0	2·2	1·5	3·6	1·4
Rat 1					0·2	9·2	25·0	35·0	26·0
Rat 2	10·0	10·0	20·0	10·0	0·2	10·0	25·0	35·0	25·0
					0·2	9·0	25·0	35·0	26·0

TABLE III

COLORIMETRIC ANALYSES OF BLOOD, AND RECOVERY OF ADDED SILICA

		Silica in blood, mg per 100 ml	Silica added, mg per 100 ml	Total silica found, mg per 100 ml	Silica recovered, mg
Defibrinated horse blood		0·78	1·00	1·73	0·95
		0·78	2·00	2·72	1·94
		0·78	4·00	4·67	3·89
		0·78	5·00	5·97	5·19
Mixed heparinised blood		0·33	1·00	1·23	0·90
		0·33	2·00	2·32	1·99
		0·33	4·00	4·45	4·12
		0·33	5·00	5·50	5·17
Mixed human serum		0·30	1·00	1·27	0·97
		0·30	2·00	2·40	2·10
		0·30	4·00	4·23	3·93
		0·30	5·00	5·36	5·06
Individual, heparinised human bloods	(1)	0·37			
	(2)	0·19			
	(3)	0·24			
	(4)	0·27			
	(5)	0·31			

TABLE IV

ANALYSIS OF MINERAL DUSTS

	Silica found by colorimetric analysis, %	Silica found by gravimetric analysis, %
Quartz	99·4	99·7
Kaolin	47·1	45·6
Mica	43·8	43·0
Quartz, kaolin and mica: mixture 1	45·1	44·0
Quartz, kaolin and mica: mixture 2	51·8	52·0
Quartz, kaolin and mica: mixture 3	69·9	70·5
Coal-mine dust (Ammanford B)	61·0	61·3
Coal-mine dust (Ammanford C)	53·7	54·2
*Standard granite G–1	71·5	72·6
*Diabase W–1	50·8	52·7

* U.S. Geological Survey samples obtained from American Bureau of Standards.

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Separation and Determination of Acetic and Lactic Acids by Paper Partition Chromatography and its Application to Silages*

By YEHUDITH BIRK and A. BONDI

A brief and simple paper-chromatographic method of determining and separating lactic acid from acetic acid in a mixture of volatile fatty acids and lactic acid is described. Spots of the ammonium salts of these acids were applied to two filter-papers. One filter-paper was immediately transferred to the chromatographic chamber, the spots obtained after development giving the volatile fatty acids and the sum of acetic and lactic acids. The other filter-paper was left at room temperature for several hours in order to permit the evaporation of the ammonium salts of the volatile fatty acids and then transferred to the chromatographic chamber. The spot obtained after development was due to lactic acid only. This method has been satisfactorily applied to silages.

A brief and accurate method for the determination of lower fatty acids and lactic acid in silages was sought for routine analysis. The older and still widely used techniques for determining volatile fatty acids in silage based on the distillation constants of acetic and butyric acids (Wiegner and Magasanik[1]) were found to be tedious and not very accurate. The numerous column methods summarised by Elsden[2] and the gas-phase chromatographic column by James and Martin[3] gave accurate results and are undoubtedly good for research work, but they are too involved for routine work. The paper chromatographic methods seemed to offer a solution to this problem. Of the methods available only the methods of Reid and Lederer[4] and Duncan and Porteous[5] were of quantitative nature. The method of Duncan and Porteous, which involves producing paper chromatograms in *n*-butanol - ammonia mixtures and spraying with a mixture of methyl red - bromothymol blue indicators in formalin, was most satisfactory, as it was rather sensitive and gave good colour contrast and well defined spots. However, it was not sufficient for our purpose, as lactic and acetic acids, main constituents of the silage, gave spots of the same R_F value. According to Barnett and Duncan,[6] this method could be applied to silages only after a lengthy procedure involving steam-distillation, neutralisation of the distillate and solution of the residue, with subsequent adsorption and elution. The lactic acid content of the silage could be determined colorimetrically (Barnett[7]).

This paper presents a method for the determination and separation of lactic acid from acetic acid in mixtures and in silages by the same paper-chromatographic method. The volatility of the lower fatty acids and of their ammonium salts, which has been the cause of one of the difficulties in the elaboration of a chromatographic technique, served as a means of removing ammonium acetate from its mixture with non-volatile ammonium lactate.

Method

Reagents—

Solvent system (according to Duncan and Porteous[5])—Redistilled *n*-butanol (fraction boiling at 116° to 118° C) was saturated with an equal volume of 1·5 *N* redistilled ammonium hydroxide.

Indicator solution (according to Duncan and Porteous[5])—A 0·08 per cent. w/v solution of methyl red and bromothymol blue (1 + 1) in a 1 + 5 v/v dilution of B.P. formalin in redistilled ethanol was titrated to pH 5·2 (pH meter) with 0·1 *N* sodium hydroxide.

Fatty acid solutions—Solutions of 1 to 3 per cent. w/v of analytical grade acetic acid, analytical grade propionic acid, purified *n*-butyric acid and purified lactic acid were prepared, and their pH values were adjusted to 8·0 with 1·5 *N* ammonium hydroxide.

Silage samples—200 g of the fresh silage were weighed into a 1-litre Erlenmeyer flask and covered with water to the 500-ml mark, and some drops of toluene were added. After

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being kept at room temperature for 10 to 20 hours, the contents were filtered. An aliquot of the filtrate was adjusted to pH 8·0 with 1·5 *N* ammonium hydroxide.

PROCEDURE—

Preparation of the chromatographic paper (according to Duncan and Porteous[5])—Sheets of Whatman No. 1 filter-paper, 25 cm × 20 cm, were washed for 20 minutes by transfer through two enamel dishes each containing 300 ml of the butanol layer (see solvent system) to which 5 per cent. v/v of ethanol had been added. The papers were hung individually in a fume cupboard out of contact with any metal until dry and odourless.

Preparing the chromatogram—Samples of 5 μl of standard and unknown solutions were placed 3·5 cm apart along a line 3 cm from the bottom of the paper. The spots were applied to eight similar papers; four papers were put immediately into the chromatographic chamber, at the bottom of which were placed the butanol layer (0·8 cm) and a beaker containing an equal volume of the aqueous ammonia layer to maintain phase equilibrium. The four other papers were kept at room temperature, 26° to 30° C, for 5 to 7 hours (in order to evaporate the ammonium salts of the volatile fatty acids) and then placed in the chromatographic chamber. The chromatograms were prepared by upward displacement until the solvent reached the top of the paper (about 10 hours at 26° to 30° C).

Drying and spraying technique and development of acid spots—The papers were removed from the chamber at 5-minute intervals and hung to dry at room temperature (10 to 15 minutes). Then each paper was evenly sprayed with a fine spray of the indicator solution from an all-glass sprayer; about 20 ml of indicator solution are required for each paper. The paper was then immediately introduced into a tall wide cylinder at the bottom of which was a layer of 3 per cent. v/v aqueous ammonium hydroxide. The paper was exposed several times to the ammonia vapour for 1 to 15 seconds at intervals of about 1 minute. As a result, intense red acid spots persisted on a green background. The acid spots were then marked by pencil and their area was determined either with transparent graph paper or with a planimeter.

RESULTS

The linear relationship between spot area and the acid content of the spot found by Duncan and Porteous[5] when Whatman No. 54 filter-paper and the mixed indicator spray of methyl red and bromothymol blue were used has been confirmed also for Whatman No. 1 filter-paper.

TABLE I

THE MEAN AREAS AND STANDARD DEVIATIONS OF DEVELOPED SPOTS OBTAINED BY APPLICATION OF VARIOUS MIXTURES OF ACETIC ACID, A, AND LACTIC ACID, L, WITH AND WITHOUT PRELIMINARY AERATION OF THE APPLIED SPOTS

Content of solution examined (5 μl)			Non-aerated			Aerated					
			Mean spot area of	Standard deviation		Mean spot area of	Standard deviation		Equivalent concentration of	Deviation of mean from true value	
L, μg	A, μg	Number of spots averaged	A+L, mm²	mm²	%	L, mm²	mm²	%	L, μg per 5 μl	μg per 5 μl	%
0	100	6	505	30·8	6·1	—	—	—	—	—	—
20	80	6	503	28·0	5·6	125	8·9	7·1	24·8	+4·8	24·0
40	60	6	475	35·7	7·5	215	20·7	9·6	45·2	+5·2	13·0
60	40	6	480	26·8	5·6	287	17·0	5·9	59·8	−0·2	0·3
80	20	6	517	38·4	7·4	390	24·2	6·2	75·5	−4·5	5·6
100	0	6	482	44·2	9·2	487	29·8	6·1	101·0	+1·0	1·0

The quantitative determinations were most satisfactory with solutions in which the acid concentrations ranged from 40 to 120 μg per 5 μl. At these concentrations the spots were most consistent. Acetic acid and lactic acid have the same R_F value and are thus quantitatively indistinguishable on the chromatogram. The paper-chromatographic separation and quantitative determination of acetic and lactic acids reported in the present paper was due to the volatility of ammonium acetate and the non-volatility of ammonium lactate; 5 to 7 hours of aeration at room temperature before chromatography were found sufficient

for the complete evaporation of ammonium acetate. Thus the total amount of acetic acid and lactic acid was determined from the spot areas of the papers which were placed in the chromatographic chamber immediately after application of the spots, whereas lactic acid alone was determined from the spot areas of the papers which were placed in the chamber after proper aeration.

Experiments to shorten the time of aeration by placing the papers in an incubator at 40° C for 1 to 2 hours were not successful, as the applied spot was spread on the paper. Neither was the use of a fan found advisable, as it did not shorten the time of aeration. The relationship between spot area and the acid content of the spots of various mixtures of acetic acid, A, and lactic acid, L, with and without aeration of the papers, is given in Table I.

It can be seen from Table I that the values found for L (except the case of 20 μg of L) do not deviate from their respective true values by much more than the standard deviation of different spot areas obtained from the same solution. It can also be seen that the prescribed method works almost equally well for the different amounts of L (lactic acid) examined. Thus we may summarise our results over the whole range of L from 0 to 100 μg in Table II.

Table II

Deviations of determined L (lactic acid) concentrations from actual L concentrations (A + L = 100 μg per 5 μl)

Range of $\frac{L}{A+L}$	Number of $\frac{L}{A+L}$ variations tested	Mean deviation, μg per 5 μl	Mean deviation taking account of sign (bias)	Standard deviation, μg per 5 μl	Error at mid-point of range, %
0 to 1	6 (each variation is an average of 6 experiments)	2·61	+1·05	3·8	7·6

Table III

The average amounts and standard deviations of A + L (acetic acid and lactic acid), L and B (butyric acid) found by the paper-chromatographic method and by the distillation method* in three silages

		Paper-chromatographic method							
Kind of silage	pH	Number of spots averaged	Average amount of A+L (found), μg per 2000 μg of silage	Standard deviation of A+L, μg per 2000 μg of silage	Average amount of L (found), μg per 2000 μg of silage	Standard deviation of L, μg per 2000 μg of silage	Average amount of A (calculated), μg per 2000 μg of silage	Average amount of B (found), μg per 2000 μg of silage	Standard deviation of B, μg per 2000 μg of silage
Orange pulp	3·2	4	98·5	4·7	81·6	6·6	16·9	—	—
Green maize	3·7	4	99·4	7·1	89·4	6·1	10·0	—	—
Peas and oats	4·9	4	47·0	3·6	21·9	3·4	25·1	23·0	3·1

		Distillation method						
Kind of silage	pH	Number of distillations averaged	Average amount of A (found), μg per 2000 μg of silage	Standard deviation of A, μg per 2000 μg of silage	Average amount of L (found), μg per 2000 μg of silage	Standard deviation of L, μg per 2000 μg of silage	Average amount of B (found), μg per 2000 μg of silage	Standard deviation of B, μg per 2000 μg of silage
Orange pulp	3·2	4	23·6	5·2	87·3	4·9	—	—
Green maize	3·7	4	8·7	1·3	94·7	7·4	—	—
Peas and oats	4·9	4	34·2	6·2	19·9	2·0	30·5	3·9

* Acetic acid and butyric acid were determined by the Wiegner and Magasanik[1] method, and lactic acid was determined by oxidation of the residue with chromic acid according to Flieg.[8]

The above-mentioned technique was successfully applied to silages, so permitting the determination by the same paper-chromatographic method of both the volatile fatty acids and lactic acid in the silage.

In Table III are given the results of determinations made on three different silages; 100 g of each silage were extracted with 500 ml of distilled water, and 10-μl aliquots of the extracts were chromatographed. Solutions of pure acetic acid, lactic acid and butyric acid in concentrations of 100 μg per 5 μl served as standards, yielding spot areas of about 500 sq. mm, and their respective standard deviations did not exceed 7·9 per cent.

It can be seen from Table III that the average amounts of the various acids determined by the two different methods do not differ much for L but differ more for A.

The standard deviations of A values obtained by the distillation method from the mean values of A are quite high. A disadvantage of the distillation method is that small differences in size and shape of different distillation apparatus have considerable effect on the results.

As the R_F values were 0·20 for propionic acid and 0·32 for butyric acid, no difficulty was found in identifying and determining these acids quantitatively.

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A Method for the Determination of Total Sulphur in Soils

By A. Steinbergs

A convenient method for determining total sulphur in soils has been developed. It consists of the fusion of 0·500 g soil with 1·4 g of sodium peroxide, followed by the determination of sulphate by turbidimetric measurement after interfering ions have been removed.

Reproducible turbidity readings are obtained by using a "seeded" barium chloride suspension. Preparation and examination of "seeded" suspensions are described.

The determination of total sulphur in soils by fusion with sodium peroxide followed by its gravimetric determination as barium sulphate is lengthy and not well suited to the routine analysis of large numbers of samples. The relatively small quantities of sulphur in soils require the fusion of at least 10 g of soil in order to obtain precipitates sufficient for accurate gravimetric analysis. If, however, a turbidimetric procedure were used for the final determination, smaller quantities of sulphate could be determined, so permitting a simpler and more rapid analysis.

A rapid method has been developed in which 0·500 g of soil is fused with 1·4 g of sodium peroxide and the final determination of sulphate is made by a turbidimetric procedure.

Experimental

The fusion of soil with sodium peroxide—

The procedure for the fusion of soils with sodium peroxide for the determination of total sulphur has been investigated by Hart and Peterson.[1] In this method 10 g of soil are fused with 20 g of sodium peroxide, the melt is dissolved in hydrochloric acid and the interfering

cations are removed as hydroxides before precipitation of sulphate as barium sulphate. This fusion is difficult, the filtration lengthy and subsequent evaporation of large amounts of filtrate time-consuming. In the method described it was found that fusion of 0·500 g of soil with 1·4 g of sodium peroxide, or, in the case of a fresh (newly opened) bottle of peroxide, 1·0 g of peroxide and 0·4 g of sodium carbonate, gave the most satisfactory melt.

Although only 0·500 g of soil is used, it was still found that the subsequent separations of interfering ions as hydroxides yielded bulky precipitates. To avoid filtration the hydroxides were separated and washed by centrifuging. To test for the possible loss of sulphate by adsorption and occlusion, the residues remaining in the centrifuge tube after the washing were re-dissolved in hydrochloric acid and again precipitated and washed in the same manner. The supernatant liquid was then examined and found to be free from sulphate.

TURBIDIMETRIC DETERMINATION OF SULPHATE—

Steinbergs[2] found that the sensitivity and reproducibility of turbidimetric sulphate determination depended upon the presence of insoluble impurities in the barium chloride solution at the time of precipitation of the barium sulphate, these impurities acting as nuclei which facilitate the uniform growth of crystals. Barium chloride reagents, however, do not always contain sufficient of such impurities to ensure satisfactory results. Further investigations have been made into the importance of these "seed crystals."

Twenty-three grams of barium chloride of a reagent grade containing 0·005 per cent. of heavy metals were dissolved in 50 ml of water and the solution was allowed to stand for 30 minutes in a test tube. Examination under the microscope revealed a number of different-sized tabular and acicular crystals among amorphous particles (see Fig. 1*a*).

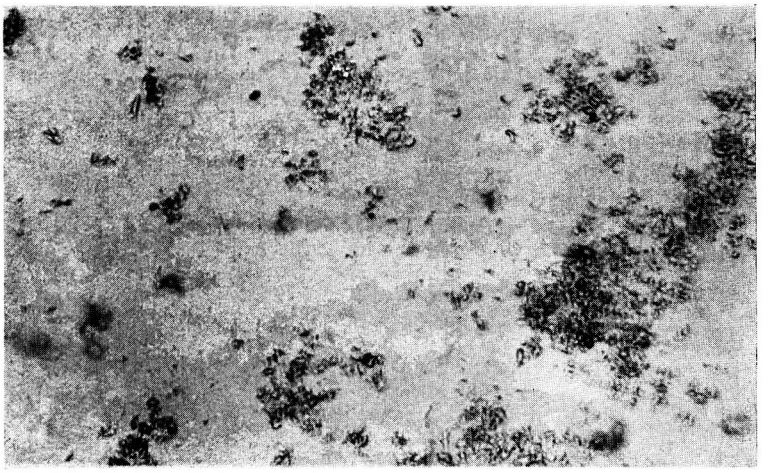
To 23·0 g of the solid barium chloride reagent in a 150-ml Erlenmeyer flask were added 4·0 ml of standard sulphate solution (0·5435 g of potassium sulphate per litre) and 46·0 ml of water. This was heated over a yellow gas flame until all the barium chloride was dissolved, cooled rapidly to room temperature, and the suspension was allowed to stand in a test tube for 30 minutes. The suspension was then siphoned into a 150-ml Erlenmeyer flask, about 2 ml of liquid being left at the bottom of the tube. Examination of the sediment under the microscope revealed differently sized barium sulphate crystals, some of them well developed tabular crystals up to 10 μ in size and some of them acicular (see Fig. 1*b*). In this suspension, crystals ranging in size up to 5 μ were observed.

To this suspension were added a further 4·0 ml of the standard sulphate solution and, after being shaken, it was allowed to stand as before for 45 minutes. No sediment formed, but after this period the turbidity at the bottom of the tube appeared greater than that at the top. The top portion of the suspension was siphoned off. Examination under the microscope of samples taken from the top and bottom portions of the suspension revealed many small crystals ranging in size up to 5 μ in the bottom layer (see Fig. 1*c*) and, in the upper layer, numerous very small crystals about 1 μ in size together with a few larger crystals up to 3 to 4 μ in size.

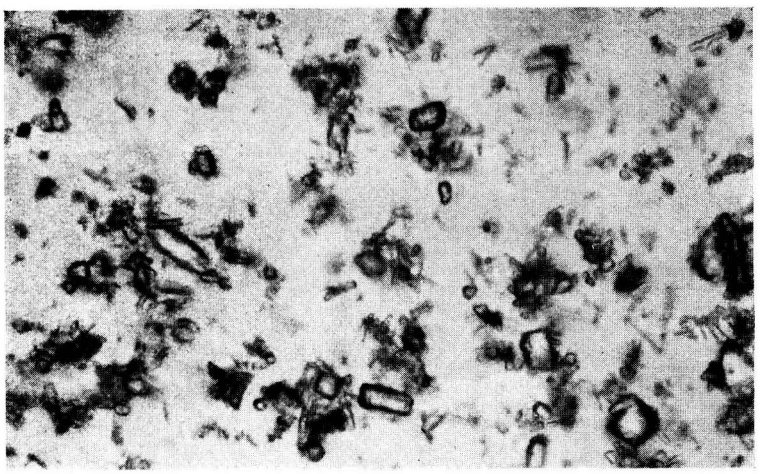
Four millilitres of standard sulphate solution were added to the top portion and allowed to stand as before for 60 minutes and again separated into upper and lower portions. In the lower portion there were now only a few crystals of a size up to 3 μ together with increased numbers of crystals of a 1-μ size (see Fig. 1*d*). In the upper portion small crystals 1 μ in size predominated.

The previous procedure was repeated and, after standing for 75 minutes, no difference was apparent under the microscope between the upper and lower contents of the tube. The crystals in both were mainly 1 to 1·5 μ in size (see Fig. 1*e*). Repetition of this procedure did not further affect the crystal size. The last suspension, on standing for periods exceeding 1 hour, began to form a clear zone at the top, but the density of the turbid lower part showed no observable change over its height, which indicated that the crystals present were approximately uniform in size.

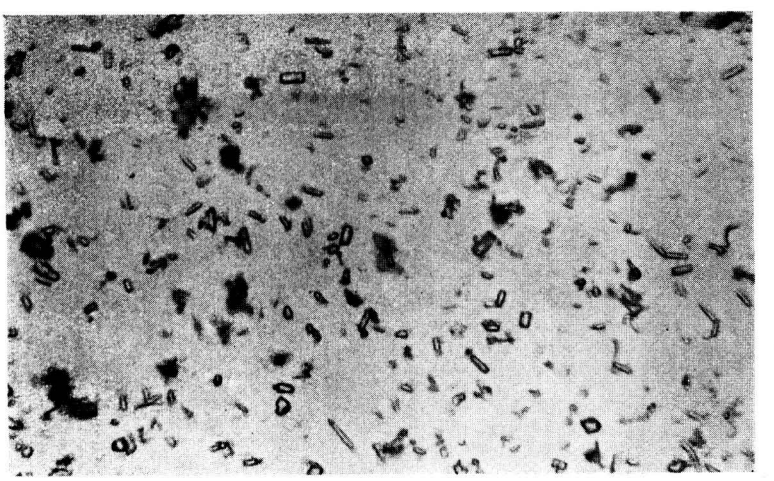
Examination at 2000 times magnification under the phase contrast microscope of the last suspensions did not reveal any of the long needles or prisms (acicular crystals) found in the original barium chloride solution and in the suspensions formed by the first two additions of the sulphate solution, but small tabular crystals resembling cubes or squares, which showed double refraction under the crossed nicols, appeared. X-ray examination proved these crystals to be normal rhombic barium sulphate crystals of size about 1 μ.



(*a*)



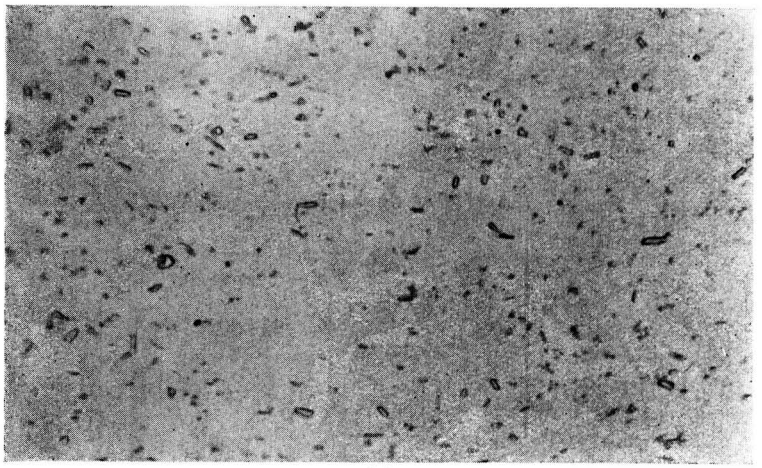
(*b*)



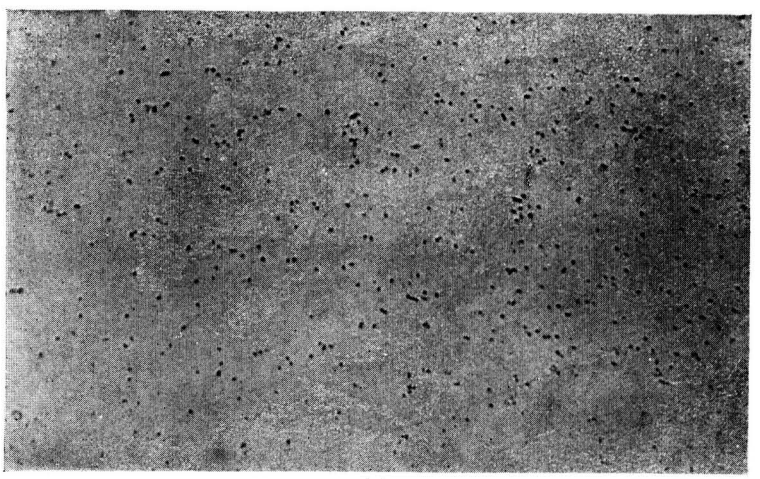
(*c*)

Fig. 1. Photomicrographs of barium sulphate crystals (× 500)

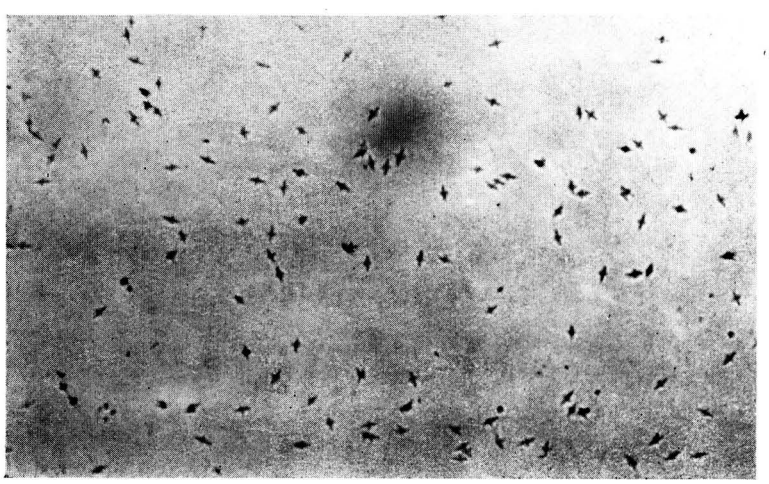
[To face p. 458



(*d*)



(e)



(*f*)

Fig. 1 (*continued*). Photomicrographs of barium sulphate crystals (× 500)

The examination of barium chloride from different sources in the manner described above yielded results similar to those described for all except the first two suspensions prepared in each case. The first two suspensions differed considerably with different sources of the reagent.

It is clear that the presence of impurities in the barium chloride reagent can greatly influence the crystal size and form of the barium sulphate produced. To investigate this a solution of 23·0 g of barium chloride in 50 ml of water was prepared and twice filtered through an Eaton Dikeman No. 850 filter-paper. Standard barium sulphate suspensions were prepared following the procedure finally adopted, this solution being used in place of the proposed "seed" suspension. Standard curves constructed from turbidity readings on these suspensions showed erratic and non-reproducible readings, particularly at the higher sulphate concentrations. Examination of these suspensions under the microscope showed that in some cases crystals of the same size and shape as those finally obtained with barium chloride solution containing suspended impurities were formed, but in others crystals of a larger size and different shape were obtained (see Fig. 1*f*). These larger cross-shaped crystals appeared to form aggregates more readily.

On the other hand, when the prepared barium chloride solution containing the "seed" suspension was used, uniform and reproducible standard curves were obtained and the suspensions always contained small uniformly sized crystals of the same crystal type as seen in Fig. 1*e*.

From experience it has been found that stability of turbidity is enhanced if the "seed" suspension is placed in the calibrated flasks and allowed to stand for at least 2½ hours or overnight before precipitation of the barium sulphate.

Recovery of the added sulphate—

As a test of the accuracy of the method, determinations were made of the recovery of sulphur added to soils. To a series of 0·500-g samples of soils, 0·3 mg of sulphur was added as sulphate or as methionine, and total sulphur was determined by the recommended method. Sulphur was added as 3 ml of a solution of 0·5435 g of potassium sulphate per litre or as 0·1 ml of a solution of 0·1396 g of methionine in 10·0 ml to the 0·500-g soil samples in nickel crucibles. The samples were dried before proceeding with the determination. The results are shown in Table I.

Table I

Recovery of sulphur added as potassium sulphate and methionine to soils

Soil	Sulphur in sample, mg	Sulphur added as potassium sulphate: Sulphur added, mg	Sulphur found, mg	Sulphur recovered, mg	Recovery, %	Sulphur added as methionine: Sulphur found, mg	Sulphur recovered, mg	Recovery, %
Sand	0·008	0·30	0·307	0·299	99·7	0·308	0·300	100·0
	0·008	0·30	0·310	0·302	100·7	0·303	0·295	98·3
A	0·038	0·30	0·335	0·297	99·0	0·343	0·305	101·7
	0·038	0·30	0·335	0·297	99·0	0·338	0·300	100·0
B	0·190	0·30	0·493	0·303	101·0	0·483	0·293	97·7
	0·190	0·30	0·488	0·298	99·3	0·478	0·288	96·0
C	0·060	0·30	0·365	0·305	101·7	0·368	0·308	102·7
	0·060	0·30	0·359	0·299	99·7	0·373	0·313	104·3

Method

Reagents—

All reagents should be of analytical-reagent grade.

Sodium peroxide.
Sodium carbonate.
Hydrochloric acid, concentrated.
Ammonium hydroxide, sp.gr. 0·880.
Sodium chloride.
Barium chloride.

Standard sulphate solution—0·5435 g of potassium sulphate in 1000 ml of water.

Acidified glycerol solution—A freshly prepared solution of 5·0 ml of glycerol and 10·0 ml of 10 per cent. hydrochloric acid in 100 ml of water.

"Seed" suspension—To a 150-ml Erlenmeyer flask add 23·0 g of barium chloride, $BaCl_2.2H_2O$, 4·0 ml of standard potassium sulphate solution and 46·0 ml of water in the order given. Dissolve over a gas flame and cool rapidly. Transfer to a test tube and allow to stand for 30 minutes. Siphon approximately 48 ml of this suspension into an Erlenmeyer flask, keeping the end of the siphon about 1 cm from the bottom of the tube and away from the sides. Discard the residue. Add another 4·0 ml of standard sulphate solution, shake for 1 minute and allow to stand for 45 minutes. Siphon off the bulk of the suspension in the manner described, add 4·0 ml of standard sulphate solution to it, shake for 1 minute and allow to stand for 1 hour. Siphon off as before, discarding the residue, and dilute to 50 ml in a measuring cylinder. Transfer to an Erlenmeyer flask and keep away from direct sunlight.

PROCEDURE—

Fusion—To 0·500 g of air-dry soil in a 40-ml nickel crucible add just sufficient water (3 or 4 drops) to moisten thoroughly. Add 0·7 g of sodium peroxide (or 0·5 g of peroxide and 0·2 g of sodium carbonate when using a newly opened bottle of sodium peroxide) and mix thoroughly with the soil, using a nickel rod or nichrome wire. If necessary, add more water to give, upon mixing, a creamy paste. Leave the crucible and the rod to stand overnight in a drying oven at 100° C. Add a further 0·7 g of sodium peroxide (or 0·5 g of peroxide and 0·2 g of sodium carbonate), cautiously spread and mix with the previous mixture. Fuse with constant stirring over a gas blow-lamp, applying the flame around the top of the walls of the crucible downwards. The temperature should reach approximately 800° C (bright red colour) and the fusion should take 5 to 7 minutes. Overheating or heating for an unnecessarily long period must be avoided as this may introduce contamination from impurities in the crucible. Cool, wash the outside of the crucible by means of a jet of hot water from a wash-bottle and submerge the crucible in approximately 100 ml of hot water in a 200-ml evaporating basin. Heat the basin on a boiling-water bath for approximately ½ hour, allowing about two-thirds of the solution in the basin to evaporate. Remove the crucible and suspend it upside down over the basin by means of a hooked Nichrome wire and wash both the inside and outside of the crucible thoroughly with hot water into the basin. Cover the basin with a watch-glass and cautiously add 10 ml of concentrated hydrochloric acid. Remove the watch-glass and evaporate to dryness on the water-bath. Break up the surface of the dry matter and heat for a further 15 minutes on the water-bath. Add 20 ml of boiling water, stir thoroughly, add 10 ml of ammonium hydroxide and leave for ½ hour at reduced heat on the water-bath.

In the event of the blue colour of the nickel - ammonium complex appearing, keep the basin on the water-bath until the excess of ammonium hydroxide has evaporated and nickel hydroxide has been precipitated. If necessary, add a little hot water during this procedure.

Separation of sulphate—Transfer the contents of the basin into a 100-ml centrifuge tube and rinse the basin until the washings amount to 50 to 60 ml; cool and centrifuge for 15 minutes at 2500 r.p.m. Decant the supernatant liquid into a clean 200-ml evaporating basin and place it on the water-bath. Add approximately 0·5 ml of ammonium hydroxide and 30 ml of water to the residue in the centrifuge tube, stir well and centrifuge for 20 minutes at 2700 r.p.m. and 27·5 cm radius. Pour off the supernatant liquid into the same basin. Repeat this procedure using 0·5 ml of ammonium hydroxide and 20 ml of water and centrifuge for 25 minutes at 2900 r.p.m. and 27·5 cm radius. Again decant the supernatant liquid into the basin. Repeat this washing a third time using 0·5 ml of ammonium hydroxide and 15·0 ml of water and centrifuge for 30 minutes at 3100 r.p.m. and 27·5 cm radius, adding the washings to the basin as before.

Evaporate all the washings to complete dryness. Transfer the dry residues carefully into a 25-ml calibrated flask using several small amounts of water, add 2·0 ml of acidified glycerol solution and adjust to volume. Add by a pipette a further 2·0 ml of water, shake thoroughly and transfer the contents of the flask into a centrifuge tube. Centrifuge for 15 minutes at 3000 r.p.m. and 27·5 cm radius, decant most of the upper part into a 50-ml Erlenmeyer flask, but filter the rest through a Whatman No. 42 filter-paper into the same flask, and set this aside for the sulphate determination.

A blank prepared in the same manner by fusing 0·5 g of purified quartz sand with 1·4 g of sodium peroxide or by dissolving 1·4 g of sodium peroxide in water is necessary as a check on all reagents. Use of quartz sand is desirable, as peroxide fused alone will attack the crucible severely.

Preparation of standards—Prepare a calibration curve by taking suitable aliquots of standard sulphate solution in 50-ml calibrated flasks, each containing 3·9 g of sodium chloride to increase the ionic concentration to a value similar to those in the samples. Add 3·7 ml of acidified glycerol solution and dilute to the mark.

Turbidimetric determination of sulphate—Shake the Erlenmeyer flask containing the freshly prepared "seed" suspension for about half a minute and with a pipette place 1 ml in 25-ml calibrated flasks. Use only calibrated flasks with smooth unscratched walls. After standing for 2½ hours or overnight, pour samples and standards into these flasks to give a final volume of 25 ml. Avoid disturbances of the flasks containing "seed" solution until the samples and standards have been added; then shake them immediately for half a minute. Allow to stand for 60 to 90 minutes away from direct sunlight before commencing readings.

Shake the flask thoroughly before transferring the contents to the cuvette. Apply the same technique of shaking to all samples and standards.

Determine the turbidity in a photo-electric absorptiometer, using a 1-cm cuvette and a Hilger 4508 filter.

Total sulphur has been determined by this method on a wide range of soil types, ranging in total sulphur content from 0·002 to 0·078 per cent.

The author desires to express his appreciation to Mr. C. H. Williams for his kind advice and help throughout this work, to Mr. A. D. Haldane for carrying out an X-ray examination of the barium sulphate crystals and to Mr. K. F. Mayer for help in the preparation of this paper.

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COMMONWEALTH SCIENTIFIC AND INDUSTRIAL
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The Determination of Sodium Bicarbonate in Self-raising Flours Containing Chalk B.P.

BY W. H. STEPHENSON AND A. W. HARTLEY

A procedure has been developed whereby it is possible to decompose selectively the sodium bicarbonate in a self-raising flour containing Chalk, B.P., by means of sodium acid pyrophosphate. The method is shown to be sufficiently accurate for routine purposes, at all levels of sodium bicarbonate and chalk content likely to be encountered in practice.

Some observations are made on the method for calculating total carbon dioxide content when the Chittick apparatus is used.

THE Flour Order, 1953, requires that Creta Praeparata (Chalk, B.P.) shall be added to all flours at the level of 14 ounces per 280 pounds.

The Self-Raising Flour Order, 1946, requires that a self-raising flour shall yield not less than 0·40 per cent. of available carbon dioxide, the available carbon dioxide being determined from the difference between total carbon dioxide and residual carbon dioxide.

When chalk is present in a self-raising flour, it can be shown by determinations of residual carbon dioxide that the chalk is not completely decomposed by the acid ingredient and does not contribute significantly to the aerating power of the self-raising flour during baking. For a true assessment of sodium bicarbonate content, or of potential aerating power, assuming the flour is of balanced composition, it is desirable to have a method of determining

"sodium bicarbonate carbon dioxide," which excludes the carbon dioxide derived from the chalk.

The situation facing analysts dealing with self-raising flour has been well described by Amos,[1] whose comments on this matter are as follows.

> "Unfortunately the presence of the Creta is a serious handicap to the rapid routine control of formulation on the self-raising flour plant. It has been customary for the cereal chemist to check the correctness of the sodium bicarbonate addition to self-raising flour by determining the total carbon dioxide content, a determination that can be performed in about 10 minutes. This is no longer possible because the carbon dioxide of the Creta is released in the test and since the distribution of Creta is not uniform, the total carbon dioxide of a self-raising flour provides no check upon the amount of sodium bicarbonate present.
>
> "Where the plain flour used in the manufacture of the self-raising flour is available a determination of Creta content can be made, but reliable conclusions could be drawn from such a test only if the distribution of the Creta were the same in each small sub-sample taken for the tests. The public analyst, and often the consultant who is called upon to analyse self-raising flour, is precluded from using even this doubtful line of approach, because the original plain flour from which the self-raising flour was made is not available to him."

The most commonly used method for the determination of total carbon dioxide in self-raising flours is that of Chittick, which is the basis of the official method of the Association of Official Agricultural Chemists in the U.S.A.[2] This method involves the volumetric measurement of the carbon dioxide liberated from the flour by diluted sulphuric acid (1 + 5). With this reagent the carbon dioxide from both the sodium bicarbonate and chalk is measured. It is stated in the official A.O.A.C. method that it is not applicable to flours containing added calcium carbonate.

In 1951 it was observed by one of us (W. H. S.) that if a 2·5 per cent. solution of sodium acid pyrophosphate is substituted for the dilute sulphuric acid reagent in the Chittick method, the sodium bicarbonate in a self-raising flour is decomposed with the liberation of its carbon dioxide, whereas chalk appears to be undecomposed under these conditions. Since that date, this reagent has been used with success in several of our laboratories for routine control of self-raising flour production.

A critical assessment of the selectivity of the reagent has now been made with self-raising flours of widely different compositions, and its usefulness has been confirmed for routine testing.

A.O.A.C. METHOD FOR CALCULATING RESULTS FROM THE CHITTICK METHOD—

A correction is made by analysing "a flour of known composition and like ingredients in the same apparatus." This presumably is to allow for gas remaining in solution, and unspecified effects due to various atmospheric conditions.

The corrected carbon dioxide content is obtained as follows—

$$\text{Corrected percentage of total carbon dioxide in unknown sample} = \frac{\text{apparent percentage of total carbon dioxide}}{x},$$

where

$$x = \frac{\text{weight of carbon dioxide found in synthetic sample}}{\text{weight of carbon dioxide present in synthetic sample}}.$$

During our experiments with flours of various sodium bicarbonate contents, with and without chalk, and with control flours containing chalk only, it was discovered that the synthetic flour used for making the above correction has to be not only of "known composition and like ingredients," but of "like composition and like ingredients."

In other words, if the sodium bicarbonate content is lower than normal, high results will be obtained unless the synthetic flour used for the correction has a similar low sodium bicarbonate content.

This can be explained by the fact that when the acid reagent is run from the burette into an empty flask connected to the Chittick gas burette, there is an apparent increase in volume measured on the burette over and above that of the volume of acid used. This

volume is variable between 1·5 and 4·0 ml, depending on atmospheric conditions and the acid reagents used, and is due to the vapour pressure above the liquid. If this blank volume, which is usually about 3 ml, represents a large proportion of the total volume being measured, it is obvious that the quotient must be obtained under similar conditions in order to allow for it.

The presence of this blank reading with an empty flask has already been noted by Hertwig and Hicks,[3] who attempted to show that greater accuracy was obtained by allowing for this effect in the calculation. Their criticism was answered by Chittick, Dunlap and Richards,[4] who showed that the technique of Hertwig and Hicks was at fault and that the official method of calculation was sound. They presented results only for baking powder. They did not deny that the vapour tension effect occurs, but claimed that "whatever vapour tension effect is produced by the liquid in the reaction flask is closely compensated for (in those working temperatures generally experienced in the laboratory) by the carbon dioxide dissolved in the liquid."

Whereas we do not dispute the accuracy of the present A.O.A.C. method of calculation for self-raising flours of normal composition, nevertheless we found that a more accurate value for the total carbon dioxide content of flours of low sodium bicarbonate content was obtained by using a modified method of calculation which employs the blank vapour-pressure correction mentioned by Hertwig and Hicks. Furthermore, the necessity for making synthetic flours of known composition for the calculation of daily correction factors can be dispensed with. All the figures presented are calculated by this modified method.

Modified method of calculating Chittick results—

A sample weight of 17 g is normally taken in the Chittick test so that the calculation is simplified, and it becomes a matter of dividing the corrected gas volume by 100.

In the proposed modification the method of calculation is as follows—

Let V = apparent volume of gas from flour of unknown composition,

V_A = apparent volume of gas from acid reagent (known as reagent blank), and

V_F = volume of carbon dioxide furnished by the flour.

Then $V_F = V - V_A$ and

$$\text{True percentage of carbon dioxide in flour} = V_F \times \left\{\begin{matrix}\text{factor from} \\ \text{A.O.A.C. tables}\end{matrix}\right\} \times \frac{K}{100},$$

where K is a constant determined by tests on mixtures of known composition. In our laboratory K has been found to be 1·1 under varying atmospheric conditions, and with different operators, but the reagent blank varies with temperature and pressure, and is determined at intervals during a series of tests.

Experimental

Reagents—

Sodium bicarbonate, B.P.—The purity of this reagent was shown to be between 99·5 and 100·5 per cent. by three methods of assay, *viz.*, decomposition by heat at 500° C; absorption of carbon dioxide in standard barium hydroxide after decomposition by acid; and by back-titration after decomposition with excess of standard acid. For purposes of calculating theoretical carbon dioxide values it was taken to be 100 per cent. pure.

Chalk, B.P.—This was shown to be of 98·5 per cent. purity by absorption of evolved carbon dioxide in standard barium hydroxide solution after decomposition with acid, and also by back-titration after decomposition with excess of standard acid.

Sodium acid pyrophosphate—Assay, 98·0 per cent. of $Na_2H_2P_2O_7$. A 2·5 per cent. w/v solution has a pH of 4·0 to 4·2.

Acid calcium phosphate—Assay, 82·5 per cent. of monocalcium phosphate, $Ca(H_2PO_4)_2$.

Acetic acid, glacial—Analytical-reagent grade.

Sodium acetate, $CH_3COONa.3H_2O$—Analytical-reagent grade.

Sulphuric acid—Analytical-reagent grade.

Procedure—

In all the tests, self-raising flours of known composition were made by adding the necessary amount of ingredients to each individual 17 g of basis flour immediately before

carrying out a determination. This procedure avoids errors due to loss of carbon dioxide, which can occur on storage of self-raising flours, and ensures that the exact amount of sodium bicarbonate intended is present in the sub-sample, which cannot always be guaranteed with bulk mixed self-raising flours.

The basis flours containing chalk at levels of 7, 14 and 21 ounces per sack were, however, made in bulk by thorough blending and sieving. The even distribution of the chalk was checked by the method of Hartley and Green.[5] The flours used were—

	Percentage of carbon dioxide
Flour A—Soft flour with no added chalk	Nil
Flour B—Flour A with 7 ounces of chalk per sack..	0·067
Flour C—Flour A with 14 ounces of chalk per sack	0·134
Flour D—Flour A with 21 ounces of chalk per sack	0·202

COMPARISON OF 1 PER CENT. SULPHURIC ACID AND 2·5 PER CENT. SODIUM ACID PYROPHOSPHATE SOLUTION AS ACID REAGENTS IN THE CHITTICK APPARATUS—

Table I shows results when 1 per cent. sulphuric acid is used as the acid reagent in the Chittick apparatus with self-raising flours of various compositions; 1 per cent. w/v sulphuric acid was used instead of the (1 + 5) concentration used in the A.O.A.C. method, because it was shown to give the same results and is a safer and less corrosive reagent for use in a factory laboratory. The range of composition covers all types of flour likely to be encountered on a self-raising flour plant, *i.e.*, the chalk content is from 1 to 21 ounces per sack, and excessive amounts of sodium bicarbonate and acid calcium phosphate are included, as well as deficiencies in these ingredients.

This reagent estimates the total carbon dioxide from both the sodium bicarbonate and the chalk, with an accuracy at all levels within ± 5 per cent. of the true value and in many cases with an accuracy of ± 2 per cent.

Table II shows similar results when 2·5 per cent. sodium acid pyrophosphate is substituted for 1 per cent. sulphuric acid as the acid reagent.

With this reagent the sodium bicarbonate is decomposed to the extent of 93 to 101 per cent., whereas the chalk is entirely undecomposed except in two cases in which 5 and 13 per cent. are decomposed. In the flours containing chalk only, less than 20 per cent. of it is decomposed.

A typical example which demonstrates the selectivity of the sodium pyrophosphate reagent is flour C containing 14 ounces of chalk per sack and 1·07 per cent. of sodium bicarbonate and 1·43 per cent. of acid calcium phosphate.

If the means of the appropriate data of Tables I and II are calculated, it is found that with 2·5 per cent. sodium acid pyrophosphate solution, the carbon dioxide found is 96 per cent. of that expected from the sodium bicarbonate present, whereas with 1 per cent. sulphuric acid, the value is 125 per cent. of that expected from the sodium bicarbonate, owing to the simultaneous decomposition of the chalk.

COMPARISON OF SODIUM ACETATE - ACETIC ACID BUFFER SOLUTIONS OF pH 5·0, 5·5 AND 6·0 AS ACID REAGENTS—

It was observed that with 1 per cent. sulphuric acid as the acid reagent, the pH of the flour suspension after the test is never greater than 2·0.

With 2·5 per cent. sodium acid pyrophosphate as the acid reagent, the pH of the suspension falls within the range 4·9 to 6·2.

It was considered possible that the selective action of sodium acid pyrophosphate solution might be due purely to a pH effect, in that it keeps the pH within this range and that at such a pH calcium carbonate is not decomposed. To test this point a comparison was made of the following three buffer solutions as acid reagents—

pH 5·0 *buffer solution*—22·75 ml of glacial acetic acid and 81·65 g of sodium acetate per litre.

pH 5·5 *buffer solution*—11·35 ml of glacial acetic acid and 108·85 g of sodium acetate per litre.

pH 6·0 *buffer solution*—2·85 ml of glacial acetic acid and 129·3 g of sodium acetate per litre.

TABLE I

RECOVERIES OF CARBON DIOXIDE WITH 1 PER CENT. SULPHURIC ACID AS ACID REAGENT (45 ml)

Sample	Total volume	Total volume *minus* 45 *minus* reagent blank = V_F	S.T.P. factor from A.O.A.C. tables	Carbon dioxide found, %	Total carbon dioxide present, %	Carbon dioxide found as percentage of that present	pH of suspension after test
Flour A	48·5	0·5	1·051	0·006	Nil	—	1·3
Flour B	54·0	6·0	1·048	0·069	0·067	103	1·3
Flour C	60·0	12·0	1·048	0·138	0·134	103	1·3
Flour D	66·5	18·5	1·045	0·213	0·202	105	1·3
Flour A + 1·07% $NaHCO_3$ + 1·43% A.C.P.*	95·5	47·5	1·043	0·545	0·560	97	1·4
Flour B + 1·07% $NaHCO_3$ + 1·43% A.C.P.	102·0	54·0	1·043	0·620	0·627	99	1·6
Flour C + 1·07% $NaHCO_3$ + 1·43% A.C.P.	108·5	60·5	1·040	0·692	0·694	100	1·5
Flour D + 1·07% $NaHCO_3$ + 1·43% A.C.P.	114·0	66·0	1·040	0·755	0·762	99	1·6
Flour A + 1·07% $NaHCO_3$ + 0·715% A.C.P.	97·0	48·5	1·036	0·553	0·560	99	1·4
Flour A + 1·60% $NaHCO_3$ + 1·43% A.C.P.	121·0	72·5	1·033	0·824	0·841	98	1·6
Flour A + 0·53% $NaHCO_3$ + 1·43% A.C.P.	74·0	25·5	1·033	0·290	0·280	104	1·4
Flour A + 1·07% $NaHCO_3$ + 2·14% A.C.P.	97·5	49·0	1·033	0·557	0·560	99	1·5
Flour C + 1·07% $NaHCO_3$ + 0·715% A.C.P.	109·5	61·0	1·033	0·693	0·694	100	1·5
Flour C + 1·60% $NaHCO_3$ + 1·43% A.C.P.	133·0	84·5	1·033	0·960	0·975	98	1·7
Flour C + 0·53% $NaHCO_3$ + 1·43% A.C.P.	85·5	37·0	1·033	0·420	0·414	104	1·5
Flour C + 1·07% $NaHCO_3$ + 2·14% A.C.P.	109·5	61·0	1·033	0·693	0·694	99	1·6
Flour A + 1·07% $NaHCO_3$ + 1·43% S.A.P.†	96·0	49·5	1·052	0·573	0·560	102	1·5
Flour C + 1·07% $NaHCO_3$ + 1·43% S.A.P.	107·5	61·0	1·052	0·706	0·694	102	1·6

* Acid calcium phosphate. † Sodium acid pyrophosphate.

TABLE II

RECOVERIES OF CARBON DIOXIDE WITH 2·5 PER CENT. SODIUM ACID PYROPHOSPHATE AS ACID REAGENT (45 ml)

Sample	Total volume	Total volume *minus* 45 *minus* reagent blank = V_F	S.T.P. factor from A.O.A.C. tables	Carbon dioxide found, %	Total carbon dioxide present, %	Carbon dioxide present from sodium bicarbonate, %	Percentage decomposition of sodium bicarbonate	Percentage decomposition of chalk	pH of suspension after test
Flour A	48·5	1·0	1·041	0·011	Nil	Nil	—	—	4·9
Flour B	48·5	1·0	1·041	0·011	0·067	Nil	—	16	5·05
Flour C	49·0	1·5	1·044	0·017	0·134	Nil	—	13	5·0
Flour D	49·0	1·5	1·041	0·017	0·202	Nil	—	8	5·0
Flour A + 1·07% $NaHCO_3$ + 1·43% A.C.P.	94·0	46·5	1·041	0·532	0·560	0·560	95	—	5·5
Flour B + 1·07% $NaHCO_3$ + 1·43% A.C.P.	95·0	47·5	1·041	0·544	0·627	0·560	97	Nil	5·5
Flour C + 1·07% $NaHCO_3$ + 1·43% A.C.P.	94·5	47·0	1·041	0·538	0·694	0·560	96	Nil	5·5
Flour D + 1·07% $NaHCO_3$ + 1·43% A.C.P.	95·0	47·5	1·041	0·544	0·762	0·560	97	Nil	5·5
Flour A + 1·07% $NaHCO_3$ + 0·715% A.C.P.	94·0	46·0	1·048	0·530	0·560	0·560	95	—	6·05
Flour A + 1·60% $NaHCO_3$ + 1·43% A.C.P.	115·5	67·5	1·048	0·778	0·841	0·841	93	—	6·2
Flour A + 0·53% $NaHCO_3$ + 1·43% A.C.P.	72·5	24·5	1·048	0·282	0·280	0·280	101	—	5·5
Flour A + 1·07% $NaHCO_3$ + 2·14% A.C.P.	95·5	47·5	1·045	0·546	0·560	0·560	98	—	5·75
Flour C + 1·07% $NaHCO_3$ + 0·715% A.C.P.	95·0	47·0	1·042	0·539	0·694	0·560	96	Nil	6·0
Flour C + 1·60% $NaHCO_3$ + 1·43% A.C.P.	116·5	68·5	1·042	0·785	0·975	0·841	93	Nil	6·2
Flour C + 0·53% $NaHCO_3$ + 1·43% A.C.P.	73·0	25·0	1·042	0·287	0·414	0·280	100	5	5·5
Flour C + 1·07% $NaHCO_3$ + 2·14% A.C.P.	95·0	47·0	1·042	0·539	0·694	0·560	96	Nil	5·75
Flour A + 1·07% $NaHCO_3$ + 1·43% S.A.P.	95·0	49·0	1·050	0·566	0·560	0·560	101	—	5·7
Flour C + 1·07% $NaHCO_3$ + 1·43% S.A.P.	96·0	50·0	1·050	0·578	0·694	0·560	100	13	5·7

The results given in Table III indicate that a buffer solution of pH 5·0 gives a selective decomposition of the sodium bicarbonate in balanced self-raising flours containing chalk, but it decomposes some of the chalk when the basis flours containing chalk only are tested.

A buffer solution of pH 5·5 gives results very similar to those with 2·5 per cent. of sodium acid pyrophosphate, *i.e.*, a selective decomposition of the bicarbonate in balanced self-raising flours, and less than 10 per cent. decomposition of the chalk when the basis flours themselves are tested.

A buffer solution of pH 6·0 gives an incomplete decomposition of the sodium bicarbonate.

In view of the promising results with the pH 5·5 buffer, some further tests were done with this reagent, the results being shown in Table IV. The reagent gives a good selectivity, but there is a slightly greater decomposition of chalk if 14 ounces of chalk per sack are present than is the case when 2·5 per cent. sodium acid pyrophosphate is used as the acid reagent.

From the above results it appears that a satisfactory buffer solution within the pH range mentioned might be found. It is probable that a phosphate or citrate buffer might be preferable. It is believed, however, that 2·5 per cent. sodium acid pyrophosphate solution has certain advantages over a conventional buffer solution, for if chalk is suspended in 2·5 per cent. sodium acid pyrophosphate solution it is practically insoluble, and the particles of chalk become protected by a coating of insoluble calcium pyrophosphate with very little apparent evolution of carbon dioxide.

A 2·5 per cent. solution of sodium acid pyrophosphate gives the required selectivity under all the conditions tested; it is cheap, simple to prepare and is readily available in cereal laboratories.

METHOD OF DETERMINING SODIUM BICARBONATE IN SELF-RAISING FLOURS CONTAINING CHALK—

Place 17 g of self-raising flour in a 250-ml wide-mouthed decomposition flask and connect to the Chittick apparatus. Care must be taken to avoid warming the flask with the hands. Open the stopcock, and by means of the levelling bulb bring the solution in the gas burette to the zero mark. Wait 2 minutes to equalise temperature and pressure, and then close the stopcock. Lower the levelling bulb, and run in 45 ml of 2·5 per cent. w/v sodium acid pyrophosphate solution of the same temperature as the apparatus. At all times during the decomposition the level in the bulb must be below that in the gas burette.

Shake the flask continuously and vigorously for 2 minutes, the flask being protected by a cloth. Allow to stand for 5 minutes with the levelling bulb 10 ml below the burette reading.

Equalise the pressure in the gas burette by means of the levelling bulb and read the total volume of gas. Observe the temperature and pressure for correction purposes.

Repeat the operations using an empty flask, to obtain the reagent blank. Use the method given previously for calculation of the true percentage of carbon dioxide derived from the sodium bicarbonate.

NOTES—

1. The gas is evolved more slowly with sodium acid pyrophosphate solution than with sulphuric acid, and it is essential to agitate the flask vigorously for 2 minutes, by which time the burette reading will have reached a constant level.

2. The sodium acid pyrophosphate reagent should be prepared afresh every week, as there is a tendency for mould to develop after storage for long periods.

3. As there is a slight rise in temperature during agitation of the flask, it is advisable to check the temperature in the flask by means of a thermometer passing through the bung.

The period of waiting to secure equilibrium can be reduced by starting with the reagent at a temperature 1° to 2° C below that of the room.

4. Factor K should be determined initially in any laboratory where the method is adopted, by analysing a number of synthetic flours of known carbon dioxide content, and calculating a mean value of K from the recoveries obtained.

DISCUSSION OF RESULTS

The method described has been found to give concordant results with different operators, but it is possible that the factor of 1·1 may need some adjustment in different laboratories,

TABLE III

RECOVERIES OF CARBON DIOXIDE WITH pH 5·0, 5·5 AND 6·0 BUFFER SOLUTIONS AS ACID REAGENTS (45 ml)

Sample	pH of buffer solution	Total volume	Total volume *minus* 45 *minus* reagent blank = V_F	S.T.P. factor from A.O.A.C. tables	Carbon dioxide found, %	Total carbon dioxide present, %	Carbon dioxide present from sodium bicarbonate, %	Percentage decomposition of sodium bicarbonate	Percentage decomposition of chalk	pH of suspension after test
Flour A	5·0	48·5	Nil	1·038	Nil	Nil	Nil	—	—	4·85
Flour B		50·0	1·5	1·036	0·017	0·067	Nil	—	25	4·9
Flour C		52·0	3·5	1·033	0·040	0·134	Nil	—	30	4·9
Flour D		53·0	4·5	1·033	0·051	0·202	Nil	—	25	4·9
Flour A + 1·07% $NaHCO_3$ + 1·43% A.C.P.		97·0	48·5	1·033	0·551	0·560	0·560	98	—	4·95
Flour B + 1·07% $NaHCO_3$ + 1·43% A.C.P.		97·0	48·5	1·033	0·551	0·627	0·560	98	Nil	4·95
Flour C + 1·07% $NaHCO_3$ + 1·43% A.C.P.		97·0	48·5	1·033	0·551	0·694	0·560	98	Nil	4·95
Flour D + 1·07% $NaHCO_3$ + 1·43% A.C.P.		97·0	48·5	1·033	0·551	0·762	0·560	98	Nil	4·95
Flour A	5·5	49·5	Nil	1·025	Nil	Nil	Nil	—	—	5·35
Flour B		50·0	0·5	1·022	0·006	0·067	Nil	—	9	5·35
Flour C		50·0	0·5	1·022	0·006	0·134	Nil	—	4	5·35
Flour D		51·0	1·5	1·022	0·017	0·202	Nil	—	8	5·4
Flour A + 1·07% $NaHCO_3$ + 1·43% A.C.P.		98·0	48·5	1·022	0·545	0·560	0·560	97	—	5·5
Flour B + 1·07% $NaHCO_3$ + 1·43% A.C.P.		97·5	48·0	1·022	0·540	0·627	0·560	96	Nil	5·5
Flour C + 1·07% $NaHCO_3$ + 1·43% A.C.P.		97·5	48·0	1·022	0·540	0·694	0·560	96	Nil	5·5
Flour D + 1·07% $NaHCO_3$ + 1·43% A.C.P.		97·5	48·0	1·022	0·540	0·762	0·560	96	Nil	5·5
Flour A	6·0	48·0	Nil	1·045	Nil	Nil	Nil	—	—	5·95
Flour B		48·0	Nil	1·045	Nil	0·067	Nil	—	Nil	5·95
Flour C		49·0	1·0	1·043	0·011	0·134	Nil	—	8	6·0
Flour D		48·5	0·5	1·040	0·006	0·202	Nil	—	3	6·0
Flour A + 1·07% $NaHCO_3$ + 1·43% A.C.P.		90·0	42·0	1·046	0·480	0·560	0·560	86	—	6·15
Flour B + 1·07% $NaHCO_3$ + 1·43% A.C.P.		88·0	40·0	1·040	0·458	0·627	0·560	82	Nil	6·15
Flour C + 1·07% $NaHCO_3$ + 1·43% A.C.P.		88·0	40·0	1·037	0·456	0·694	0·560	81	Nil	6·15
Flour D + 1·07% $NaHCO_3$ + 1·43% A.C.P.		89·5	41·5	1·037	0·473	0·762	0·560	84	Nil	6·15

TABLE IV

RECOVERIES OF CARBON DIOXIDE WITH A pH 5·5 ACETATE BUFFER AS ACID REAGENT (45 ml

Sample	Total volume	Total volume *minus* 45 *minus* reagent blank = V_F	S.T.P. factor from A.O.A.C. tables	Carbon dioxide found, %	Total carbon dioxide present, %	Carbon dioxide present from sodium bicarbonate, %	Percentage decomposition of sodium bicarbonate	Percentage decomposition of chalk	pH of suspension after test
Flour A + 1·07% $NaHCO_3$ + 0·715% A.C.P.	94·0	47·0	1·061	0·549	0·560	0·560	98	—	5·5
Flour A + 1·6% $NaHCO_3$ + 1·43% A.C.P.	117·0	70·0	1·059	0·815	0·841	0·841	97	—	5·55
Flour A + 0·53% $NaHCO_3$ + 1·43% A.C.P.	72·0	25·0	1·059	0·291	0·280	0·280	104	—	5·4
Flour A + 1·07% $NaHCO_3$ + 2·14% A.C.P.	94·5	47·5	1·056	0·551	0·560	0·560	98	—	5·45
Flour C + 1·07% $NaHCO_3$ + 0·715% A.C.P.	97·0	50·0	1·050	0·578	0·694	0·560	100	13	5·55
Flour C + 1·6% $NaHCO_3$ + 1·43% A.C.P.	119·0	72·0	1·050	0·832	0·975	0·841	99	Nil	5·6
Flour C + 0·53% $NaHCO_3$ + 1·43% A.C.P.	72·5	25·5	1·050	0·295	0·414	0·280	100	11	5·45
Flour C + 1·07% $NaHCO_3$ + 2·14% A.C.P.	96·0	49·0	1·050	0·566	0·694	0·560	100	4	5·5
Flour A + 1·07% $NaHCO_3$ + 1·43% S.A.P.	95·0	48·0	1·050	0·554	0·560	0·560	99	—	5·5
Flour C + 1·07% $NaHCO_3$ + 1·43% S.A.P.	95·0	48·0	1·050	0·554	0·694	0·560	99	Nil	5·5

depending on how vigorously the flask is shaken. Some experiments were done with a mechanical vibratory flask shaker to see whether the results could be improved by this means, but the reproducibility is not improved.

The main requirement for good reproducibility with the Chittick apparatus is the maintenance of the whole apparatus, flour and solutions at a constant temperature while a test is being carried out.

It is considered that the recoveries shown indicate that the method is sufficiently accurate and selective for the routine testing of self-raising flours on the plant, and that with special precautions such as the checking of synthetic samples of known composition, the method can be applied to samples of unknown origin.

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The Use of Adsorption Columns in the Analysis of Oil-in-Water Emulsions

By T. Green, R. P. Harker and F. O. Howitt

A general method for the analysis of the constituents of emulsions stabilised by different types of detergents has been developed. It involves adsorption of the constituents of the emulsion on an ion-exchange resin and sequential elution of the oil or grease and detergent constituents.

Methods at present available for the determination of the total materials in aqueous emulsions involve "cracking" the emulsion, separating the non-aqueous phase by solvent extraction or filtration and weighing before analytical investigation. Such methods can be applied successfully to soap-stabilised emulsions, but present difficulties with emulsions stabilised by different types of synthetic detergent, *e.g.*, anionic, cationic or non-ionic. Even with soap-stabilised systems the above procedure is rather tedious, and the need for a rapid means of isolating and estimating the components of such systems has led to the development of a technique employing ion-exchange resins, or adsorbents containing an ion-exchange resin, whereby the separation of an oil-in-water emulsion, *e.g.*, wool scouring liquors, can be achieved. This has been suggested previously[1] as being applicable to large-scale cracking and recovery of grease from industrial effluents.

In the first instance, using columns of Zeo-Karb 225 in bead form mixed with powdered animal charcoal, percolation of soap solutions and soap-stabilised emulsions gave clear liquors from which organic matter had been removed.[2] The presence of charcoal diminishes the rate of percolation that occurs with resin alone and increases the extent of adsorption; independent observations with charcoal columns indicate that the charcoal functions as an additional adsorptive material. Percolation of the emulsion and subsequent elution with a series of solvents resulted in the isolation of the separate components.

This method was extended to the analysis of emulsions stabilised by an anionic detergent (sodium cetyl sulphate) with successful results.

The analysis of an emulsion stabilised by a cationic detergent (cetyltrimethylammonium bromide) was achieved by adsorption on a column of De-Acidite FF.

The analysis of emulsions stabilised by non-ionic detergents has hitherto been restricted by the lack of a satisfactory method of quantitative separation. By an extension of the above methods, a separation has been achieved and shown to be quantitative.[3] By using columns of Biodeminrolit (an intimate mixture of Zeo-Karb 225 and De-Acidite FF), percolation of lanolin - Lissapol N emulsions containing up to 1 per cent. of sodium chloride gave clear liquors, which on evaporation left no residue. Columns containing powdered animal charcoal were equally effective but, although the lanolin could be removed quantitatively, the Lissapol N was held too firmly for successful solvent elution and subsequently charcoal was omitted from the columns. By using various solvents, the pure components could then be isolated and estimated separately.

By a combination of the above methods it has been possible to estimate accurately the separate components of Lissapol N - soap - lanolin emulsions.

Experimental

Materials—

Lanolin—Specially prepared acid-free sample supplied by Messrs. Croda Ltd.

Mineral oil—Shell clavus oil 27.

Olive oil—Normal pharmaceutical grade.

Soaps—Sodium soaps were prepared from pure 99 per cent. stearic, palmitic and myristic acids supplied by Messrs. Unilever Ltd.

Sodium cetyl sulphate—Purified sample prepared in these laboratories.

Cetyltrimethylammonium bromide—Obtainable from The British Drug Houses Ltd.

Lissapol N—(Octa-ethyleneglycol *p*-octylphenyl ether.) An extremely pure sample of definite chain length supplied by Imperial Chemical Industries Ltd.

Preparation of adsorption columns—

The columns were constructed from Pyrex-glass tubing of 2·5 cm bore and filter-paper plugs inserted so that particles of charcoal were held in the columns. The experimental set-up was such that pressure could be applied when a higher rate of percolation was required (see Fig. 1). The length of column varied; with Zeo-Karb - charcoal columns, a length of 5 cm was satisfactory, whilst with columns of De-Acidite FF and Biodeminrolit, lengths of 7 to 8 and 10 cm, respectively, were used.

Preparation of the columns involved regeneration of the resins by the Permutit procedure, *e.g.*, Zeo-Karb 225 was regenerated by percolation of dilute hydrochloric acid, followed by thorough washing with distilled water until the washings were acid-free and Soxhlet extraction with the solvents to be used for elution. The particle size of resin was usually 40 to 60 mesh, but with Biodeminrolit columns, 60 to 80 mesh was more suitable; for the Zeo-Karb 225 - charcoal columns, equal weights of the two components were taken.

The columns were made up from an aqueous slurry of the adsorbent material; this was preferred to the method of making the column with dry adsorbent, since in the latter case there is a much greater possibility of entrapping air bubbles in the column.

The elution techniques varied with the different systems investigated.

(*i*) Soap-stabilised emulsions—

The emulsion of lanolin dispersed in soap solution was percolated through a Zeo-Karb 225 - charcoal column. Sodium ions were exchanged, the soap being converted into fatty acid, and the emulsion was effectively broken down: lanolin and fatty acid were adsorbed on the column and the column was then dried in a stream of air. The fatty acid was eluted with hot ethanol (approximately 20 ml per determination), this procedure being carried out very slowly over a period of about 1 hour. If elution is carried out too quickly, there is a tendency for the ethanol to remove small amounts of adsorbed grease in addition to fatty acid. The elution time can be decreased by eluting fatty acids with hot 90 per cent. aqueous ethanol (*i.e.*, ethanol with 10 per cent. by volume of added water); this solvent shows no tendency to dissolve adsorbed grease from the column. The fatty acid was then estimated

by weight or by titration against standard alkali. The lanolin component was eluted subsequently with hot trichloroethylene and estimated by weight. Both fractions were shown to be identical with the original pure materials by comparison of their infra-red adsorption spectra, which are sufficiently characteristic for this purpose.

The determination of the soap content of an emulsion stabilised by an unsaturated soap, *e.g.*, sodium oleate, is not possible by this method, since the charcoal irreversibly adsorbs the unsaturated fatty acid. In this case, a column of Zeo-Karb 225 of finer mesh

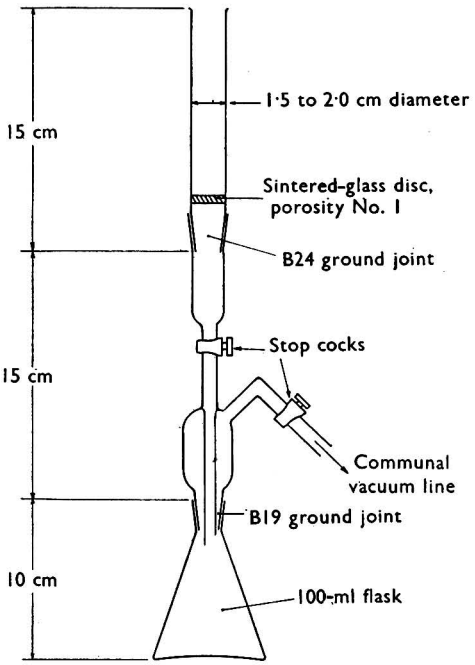

Fig. 1. Assembly of adsorption column

size (100 to 200) and of length 15 to 20 cm can be substituted for the Zeo-Karb 225 - charcoal column.

A more rapid variation in the method consists of eluting the column with hot benzene - methanol (azeotrope, 2 to 3 parts by volume) and estimating the fatty acid by titration with alkali. From the total weight of the extract, the grease can be determined by difference.

(*ii*) EMULSIONS STABILISED BY AN ANIONIC DETERGENT—

Emulsions stabilised by sodium cetyl sulphate were estimated on a Zeo-Karb 225 - charcoal column in a similar fashion to (*i*), but with the following modifications.

On percolation of the emulsion, the detergent was converted into cetyl hydrogen sulphate, which was strongly adsorbed on the column and proved most difficult to remove. Eventually, a technique was evolved whereby the lanolin was first removed by elution with trichloroethylene, and the cetyl hydrogen sulphate was subsequently eluted with ethylene glycol and estimated by titration with aqueous sodium hydroxide. The cetyl hydrogen sulphate was identified as cetyl alcohol after hydrolysis with hydrochloric acid.

(*iii*) EMULSIONS STABILISED BY A CATIONIC DETERGENT—

Emulsions stabilised by cetyltrimethylammonium bromide were percolated through columns of De-Acidite FF, which is a strong anion-exchange resin.

It was expected that the detergent would be converted into the quaternary ammonium hydroxide, which after elution could be estimated by titration with hydrochloric acid. After several experiments, it was apparent that, although the detergent was completely adsorbed on the column, the exchange of bromide ions for hydroxyl ions was incomplete and the

eluate consisted of a mixture of unchanged cetyltrimethylammonium bromide and cetyltrimethylammonium hydroxide. Consequently a method of estimation was required for the detergent which was independent of the nature of the anion; this is discussed below.

On percolation of a lanolin - cetyltrimethylammonium bromide emulsion, all organic matter was adsorbed on the column. The detergent was most conveniently eluted with 50 per cent. aqueous ethanol or with hot distilled water. Two methods of estimation were investigated, *viz.*—

(*a*) A modification of the Hartley - Runnicles titration method.[4] This involved adding an excess of a standard solution of an anionic detergent to the eluate from the column (if aqueous ethanol has been used for elution, most of the ethanol must be removed) and titrating the unused detergent with a standard solution of cetyltrimethylammonium bromide, bromophenol blue being used as indicator. This method is applicable to dilute solutions of cationic detergents, but the end-point, unless constantly practised, is difficult to judge accurately.*

(*b*) Addition of a known excess of potassium dichromate to precipitate cetyltrimethylammonium dichromate, filtration and estimation of the excess of potassium dichromate iodimetrically. This method has given very poor results on all but very concentrated solutions of the cationic detergent and consequently was not suitable for estimating the detergent in eluates from the column, since the concentration was approximately 0·001 *M*. Accordingly, method (*a*) was adopted, but it was not entirely satisfactory for the reasons outlined.

After elution of the detergent, the grease was eluted with hot trichloroethylene.

(*iv*) EMULSIONS STABILISED BY A NON-IONIC DETERGENT—

Emulsions stabilised by Lissapol N were estimated on columns of Biodeminrolit. The emulsions contained up to 1 per cent. of sodium chloride, which was removed by the ion-exchange resin, this being followed by adsorption of the lanolin and detergent. Elution with 100 ml of 50 per cent. aqueous ethanol gave quantitative yields of Lissapol N, estimated by the method of Oliver and Preston.[5] The lanolin was then eluted with trichloroethylene and estimated by weighing.

The method was further extended to the analysis of a Lissapol N - soap - lanolin emulsion by using two separate columns. Lissapol N was separated and estimated as above on a column of Biodeminrolit. Soap and lanolin were separated and estimated on a column of Zeo-Karb 225 - charcoal as described under (*i*) (p. 471). Lissapol N is strongly adsorbed by the charcoal and is not removed when the fatty acid and lanolin constituents are eluted from the column.

The Lissapol N and lanolin fractions were identified by their characteristic infra-red spectra.

RESULTS

(*i*) SOAP-STABILISED EMULSIONS—

Most of the work has been concerned with lanolin - soap emulsions, but the method is equally effective for the analysis of soap-stabilised emulsions of mineral oil, *e.g.*, Shell clavus oil 27, and vegetable oil, *e.g.*, olive oil.

With pure soaps over the concentration range 0·05 to 0·4 per cent., elution with ethanol gave a 98 to 100 per cent. recovery of fatty acid (calculated on the soap used). Over a similar concentration range, elution with trichloroethylene gave an average yield of 99 per cent. for lanolin. Table I shows some typical results from lanolin - sodium stearate emulsions at various concentrations of soap and lanolin.

(*ii*) EMULSIONS STABILISED BY SODIUM CETYL SULPHATE—

The average recovery figure for lanolin was 98 per cent., whilst recoveries of cetyl hydrogen sulphate from the column were 100 per cent.

* Since this paper was submitted for publication, it has been found that the addition of chloroform to the solution to be titrated makes the end-point much sharper and hence improves the accuracy of the titration.

(*iii*) EMULSIONS STABILISED BY CETYLTRIMETHYLAMMONIUM BROMIDE—

Reference has already been made to the difficulties in the estimation of the cationic detergent in eluates from the column. The modified Hartley - Runnicles titration method was adopted, being the only reasonable method available. After a certain amount of practice,

TABLE I

DETERMINATION OF LANOLIN AND SODIUM STEARATE IN EMULSIONS

Lanolin		Sodium stearate	
Present, mg	Found, mg	Present, mg	Found, mg
10·1	9·9	49·2	49·2
17·8	17·8	7·3	7·2
49·2	48·2	52·0	51·3
49·9	49·0	28·9	28·4
53·6	48·8	49·2	49·2
63·2	63·0	21·2	19·9
99·4	99·3	25·4	25·4
206·2	203·0	25·3	25·3

TABLE II

DETERMINATION OF LANOLIN AND CETYLTRIMETHYLAMMONIUM BROMIDE IN SYNTHETIC EMULSIONS

Lanolin		Cetyltrimethylammonium bromide	
Present, mg	Found, mg	Present, mg	Found, mg
7·8	7·9	12·1	11·9
13·0	12·8	12·1	10·8
13·0	13·0	20·2	20·0

TABLE III

DETERMINATION OF THE CONSTITUENTS OF VARIOUS LISSAPOL N EMULSIONS, (*a*) WITH LANOLIN, (*b*) WITH SHELL CLAVUS OIL 27 AND (*c*) WITH OLIVE OIL

(*a*)

Lanolin		Lissapol N	
Present, mg	Found, mg	Present, mg	Found, mg
6·0	5·8	8·3	8·1
12·0	11·9	16·6	16·4
18·0	17·0	24·9	24·7

(*b*)

Aliquot of emulsion,* ml	Mineral oil (Shell clavus oil)		Lissapol N	
	Found, mg	Ratio	Present, mg	Found, mg
10	2·6	0·99	8·0	8·0
20	5·3	2·02	16·0	16·0
30	7·9	3·0	24·0	23·9

(*c*)

Aliquot of emulsion,* ml	Vegetable oil (olive oil)		Lissapol N	
	Found, mg	Ratio	Present, mg	Found, mg
10	2·8	1·01	7·54	7·57
20	5·3	1·92	15·08	15·1
30	8·3	3·0	22·62	22·2

* The contents of mineral oil and vegetable oil of the emulsions were not accurately known, but, as can be seen from the table, the ratio of the weights obtained from 10, 20 and 30-ml aliquots were 1:2:3.

the end-point could be judged with fair accuracy, the recovery figures being 90 to 100 per cent. for the detergent. The average recovery figure for lanolin was 98 per cent. Some typical results are shown in Table II.

(*iv*) EMULSIONS STABILISED BY LISSAPOL N—

Emulsions of lanolin, Shell clavus oil 27 and olive oil with Lissapol N were analysed, and some typical results are given in Table III.

(*v*) LISSAPOL N - SOAP - LANOLIN EMULSIONS—

Several determinations of the constituents of such emulsions have been carried out, and some results are shown in Table IV.

TABLE IV

DETERMINATION OF LANOLIN, SODIUM STEARATE AND LISSAPOL N IN AN EMULSION

Lanolin		Sodium stearate		Lissapol N	
Present, mg	Found, mg	Present, mg	Found, mg	Present, mg	Found, mg
7·4	7·5	8·1	8·2	10·5	10·5
14·8	14·9	16·2	15·2	21·0	20·8

DISCUSSION OF RESULTS

It can be seen that the techniques outlined are applicable to the estimation of many types of emulsion and are capable of results of an accuracy equal to, or greater than, that afforded by existing methods. In the case of soap-stabilised emulsions (*e.g.*, wool-scouring liquors), transference errors are not present and the method is much more rapid.

The adsorption column method is particularly valuable in the estimation of emulsions stabilised by non-ionic detergents, as it is very difficult to separate waxy materials such as lanolin from non-ionic detergents by other methods, *e.g.*, solvent extraction. It would appear that the first step in breaking such emulsions is the removal of stabilising electrolyte by the mixed resin followed by the adsorption of lanolin and detergent. The Lissapol N is adsorbed in a definite band on the column: this was shown by the fact that fractional elution gave practically all the Lissapol N in one fraction.

In order to speed up estimations of this kind, an apparatus incorporating six adsorption columns and a communal vacuum line has been constructed: a single unit is shown in Fig. 1.

Work is now being carried out by the adsorption column method in the analysis of soap - soda scouring liquors containing up to 5 per cent. of wool grease: the results so far obtained have agreed quite well with those obtained by more traditional methods.

It is hoped to extend the work to the analysis of various scouring liquors incorporating Lissapol N, Lensex and other industrial detergents.

The authors are indebted to Dr. J. L. Horner and Mr. J. M. Heaps for collaboration in the early part of the work, to Messrs. Croda, Imperial Chemical Industries and Lever Bros. for the supply of materials and to the Director and Council of the Wool Industries Research Association for permission to publish this paper.

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WOOL INDUSTRIES RESEARCH ASSOCIATION
TORRIDON, HEADINGLEY, LEEDS, 6

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Determination of Small Amounts of Bismuth in Lead and Antimonial Lead Alloys

By S. J. FIANDER

A rapid method for the determination of small amounts of bismuth in lead and antimonial lead alloys has been developed, 9-methyl-2:3:7-trihydroxy-6-fluorone being used as precipitant. Antimony and tin when present are removed in the course of the assay; copper, silver and zinc in small amounts (up to 1 per cent.) cause no interference, and that due to tellurium, if present, can be readily overcome.

The method is therefore suitable for the assay of lead and antimonial lead alloys, as well as type metal, battery-grid metal and similar alloys. In the range 0·01 to 0·11 per cent. of bismuth, the method is capable of 95 per cent. or better recoveries, and this precision can probably be extended into higher ranges, although this has not been experimentally proven.

It has been found that a saturated ethanolic solution of 9-methyl-2:3:7-trihydroxy-6-fluorone is a suitable precipitant for bismuth in dilute nitric acid solution. This precipitate is dissolved in sulphuric acid, fumed, and the bismuth finally determined as the yellow iodo - bismuth complex, preferably with a Spekker or similar absorptiometer.

Whilst the major purpose of this investigation was to develop a method in which this reagent was used for the determination of bismuth in refined lead and antimonial lead, it soon became evident that with a little modification the method could be applied satisfactorily to tin metal, tin - lead solders and so on, thus greatly increasing its usefulness.

The method was next considered in comparison with those involving the use of thiourea as a colour-developing reagent,[1,2,3,4,5] some of these methods being set out in detail in booklets published by the Tin Research Institute.[6,7]

Experience here shows a definite saving of time when 9-methyl-2:3:7-trihydroxy-6-fluorone is used, and also that high bismuth figures are obtained with thiourea in the presence of tellurium.

In the case of antimonial lead it is first of all necessary to melt the sample under molten sodium hydroxide in an iron crucible. Antimony is then removed as sodium antimonate by the addition of sodium nitrate, and the bismuth is concentrated in the lead button. A similar procedure may be used for the removal of tin, although in the case of tin metal, or tin - lead alloy containing a high percentage of tin, the sample must first be alloyed with a suitable quantity of refined lead of known low bismuth content.

Results have shown that other impurities usually found in the above metals do not interfere, good recoveries being obtained in all cases.

Experimental

A saturated solution of 9-methyl-2:3:7-trihydroxy-6-fluorone in ethanol was found to precipitate bismuth equally well from a very dilute solution of either nitric or sulphuric acids, but, in determining bismuth in lead, the advantages of precipitating bismuth directly from the dissolving solution were so obvious that it was decided to work exclusively on the nitric acid solution.

Samples of 12 per cent. antimonial lead were melted under sodium hydroxide in an iron crucible, sodium nitrate being added in small amounts from time to time until the melt was quiescent, and the contents were then poured into a clean iron mould. When cool enough, the caustic slag was washed off in water, leaving a soft lead button containing the bismuth. Further samples were treated as above, and, after removal of the antimony, more sodium nitrate was added, thus removing lead in various amounts, but this did not affect the recovery of the bismuth from the resulting soft lead.

The fusion process could therefore be carried out very rapidly, without regard to undue loss of lead, but with a little experience a fairly sharp cut off between antimony and lead removal could be obtained.

Samples of bismuth-free tin were alloyed with their own weight of known bismuth lead and treated similarly, the findings being in keeping with those for antimonial lead.

With regard to the actual development of the yellow bismuth iodide colour, Robinson[8] used hypophosphorous acid to effect the rapid reduction of excess of iodine, and this procedure was adopted.

The effect of impurities was next considered, and 1 per cent. of copper, silver and zinc were added to refined lead. After solution of the sample had been effected, 0·05 per cent. of bismuth was added in nitric acid solution and the method was applied. The results obtained showed that these impurities present to this amount had no effect on the amount of bismuth recovered. If tellurium was present in amounts of about 1 per cent., however, some small quantity was carried through to the final stages, and on the addition of hypophosphorous acid was reduced to the elemental state. It was found, however, that after heating to 70° C and cooling to 20° C the tellurium could be easily retained on a fine filter-paper.

Method

Reagents—

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

9-Methyl-2:3:7-trihydroxy-6-fluorone reagent—A 0·05 per cent. solution in ethanol; heat and filter before use.

Standard bismuth solution—In nitric acid solution, with 1 ml = 0·0001 g of bismuth.

Procedure for refined lead—

Transfer 1 g of rolled sample to a 250-ml conical beaker, add 15 ml of water and 5 ml of nitric acid, sp.gr. 1·42. Heat gently until dissolved, boil gently to expel brown fumes, add 100 ml of water and, while swirling, add 20 per cent. sodium hydroxide solution until the first permanent turbidity. Add nitric acid dropwise until the solution just clears and heat to nearly boiling. Add 20 per cent. sodium hydroxide until the first slight turbidity, then 1 drop of nitric acid. The solution should now be clear.

Add 10 ml of saturated solution of 9-methyl-2:3:7-trihydroxy-6-fluorone, and bring just to boiling (do not boil vigorously), stand in a warm place for 1 minute or so, add another 10 ml of reagent, shake and filter, washing the filter-paper free of lead with hot water. Open out the paper and wash the precipitate back into the original beaker with a minimum of water, cleaning the filter-paper with 10 ml of hot 25 per cent. sulphuric acid. Evaporate the solution, fume strongly until it is colourless and then cool. Add 50 ml of water, 5 ml of hypophosphorous acid, cool, make up to 100 ml in calibrated flask, add 1·5 g of potassium iodide, heat to 70° C, cool to 20° C and measure the colour intensity on a Spekker absorptiometer, using 2-cm cells and Kodak No. 2 filters (430 mμ). Carry through a blank on reagents. Prepare a calibration curve by adding various amounts of bismuth to refined lead of low bismuth content, observing the above procedure.

Carry out a blank on the lead used.

Results—Typical results were as follows—

Bismuth added, per cent.	..	..	0·012	0·016	0·022	0·022	0·022
Bismuth found, per cent.	..	..	0·013	0·016	0·022	0·021	0·021

Procedure for antimonial lead—

Weigh 10 g of sample into an iron crucible (about 4 inches diameter) containing 100 g of sodium hydroxide and melt, raising the temperature to about 800° to 850° C; a Meker-type burner is quite suitable.

Add sodium nitrate cautiously, swirling occasionally, until violent action has ceased, and the melt is beginning to assume a dark yellow colour, and pour into a clean iron mould. When cool enough, extract the melt with water, recover the lead button (with care this will all be in one piece), and dry and weigh it.

Take a weight of the lead button equivalent to 1 g of the original antimonial lead, and follow the procedure for refined lead, using reagents only as a blank.

Results—Typical results were as follows—

Bismuth added, per cent.	..	..	0·106	0·106	0·106	0·106	0·106
Bismuth found, per cent.	..	..	0·102	0·104	0·102	0·103	0·103

Procedure for tin—

Weigh 10 g of tin, alloy it with 10 g of refined lead of known low bismuth content in an iron crucible and fuse with sodium hydroxide. Dry and weigh the lead button. Take a

weight of the button equivalent to 1 g of the original tin, and follow the procedure for refined lead, using as a blank 1 g of the refined lead used for alloying.

Results—Typical results were as follows—

Bismuth added, per cent.	..	..	0·106	0·106	0·106	0·106	0·106
Bismuth found, per cent.	..	..	0·103	0·101	0·102	0·101	0·101

Recovery of bismuth from lead containing impurities

In each case 1 per cent. of copper, tellurium, silver and zinc were added to refined lead, tellurium being added in this instance to prove the efficiency of the recovery in its presence. Normally, this metal would be removed in the sodium hydroxide - sodium nitrate treatment.

Results—Typical results were as follows—

Bismuth added, per cent.	..	..	0·05	0·05	0·05	0·05	0·05
Bismuth found, per cent.	..	..	0·0480	0·0475	0·0480	0·0475	0·0475

Notes on the method—

Since this reagent will precipitate antimony under the stated conditions, it is considered necessary to fuse the sample with sodium hydroxide if its presence is either known or suspected.

Small amounts of antimony, such as might be found in refined lead, or residual amounts from a fusion treatment, although precipitated by the reagent, will not develop a colour with potassium iodide sufficient to invalidate the final determination of bismuth, provided the stated conditions are adhered to.

Recoveries of added bismuth are quite good, being 95 per cent. or better in each case.

It is important to prepare a calibration curve by adding various amounts of bismuth to lead and following the procedure, but a curve constructed by measuring known quantities of bismuth in sulphuric acid solution from a burette and immediately developing the colour with potassium iodide showed recoveries of 90 per cent. or better in all cases.

Although the bismuth reported in the tin was added as a constituent of the refined lead, this was done to test the efficiency of the recovery, with a full knowledge of the amount added.

With 1 per cent. of tellurium present, it was found that on adjusting the acidity of the solution, before the precipitation of bismuth, a slight precipitate persisted. This was ignored, and the small amount of tellurium which thus escaped separation at this stage was filtered off after developing the bismuth iodide colour.

The method is quite rapid, a single determination on refined lead requiring about 1¼ hours, and a single determination on either tin or antimonial lead about 2 hours.

The amounts of bismuth used in the determination are in the range 0·01 to 0·11 per cent., but its scope could easily be extended by increasing or decreasing the amount of sample taken. However, it is not advisable to fuse much less than 10 g of sample with sodium hydroxide.

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Notes

DETERMINATION OF SODIUM AND POTASSIUM IN COMPLEX CYANIDE SOLUTIONS BY MEANS OF ANION-EXCHANGE RESINS

THE separation of sodium and potassium from complex cyanides by means of cation-exchange resins has been studied by Samuelson.[1,2] The solutions are in this case passed through a hydrogen-saturated cation-exchange resin of sulphonic acid type. The alkali metals are taken up quantitatively, whereas many cyanide complexes are so stable that they pass through the resin layer without decomposition. The alkali metals are determined in one of the following ways: (*i*) after passing through the cation-exchange resin in hydrogen form, the solution is titrated with alkali, methyl red being used as indicator, (*ii*) the alkali metals are eluted with hydrochloric acid and are weighed as chlorides after evaporation of the solutions, and (*iii*) the chlorides obtained by procedure (*ii*) are dissolved in water and passed through a hydrogen-saturated cation-exchange resin. The effluent is titrated with standard alkali.

Accurate values were obtained by these methods. The cyanide complexes used in the investigations were $K_3Fe(CN)_6$, $K_4Fe(CN)_6.3H_2O$, $Na_2Fe(CN)_5NO.2H_2O$, $K_3Co(CN)_6$, $K_3Cr(CN)_6$, $K_4Mo(CN)_8.2H_2O$ and $K_4W(CN)_8.2H_2O$. The separation was, however, not complete for complex zinc and nickel cyanides, probably owing to the fact that these complexes are not stable in acid solution.

In an investigation by the author on the determination of alkali metals in copper cyanide and zinc cyanide plating solutions, it was found very convenient to allow the solutions to percolate through a strongly basic anion-exchange resin in the hydroxyl form.[3] In the effluent only alkali hydroxide was present, which was titrated with 0·1 *N* hydrochloric acid against methyl orange. The maximum relative error was found to be less than 0·5 per cent. This method was also used in the investigations described in this note.

EXPERIMENTAL

The ion-exchange column was of ordinary construction and the anion-exchange resin used was of strongly basic type (Dowex 2). The height and diameter of the resin bed were 140 and 10 mm, respectively, and the particle size of the resin was 0·15 to 0·30 mm in air-dry condition. The anion-exchange resin was transformed into the hydroxyl form by treatment with 200 ml of 4 *N* ammonium hydroxide and subsequently washed with water until the effluent had a neutral reaction.

The chemicals used were of analytical-reagent grade. Solutions of these chemicals were passed through the ion-exchange column at a flow-rate of about 4 to 5 ml per minute. After

TABLE I

RECOVERY OF ALKALI METALS

Substance	Alkali metal added, g	Alkali metal found, g	Relative error, %
$K_3Fe(CN)_6$	0·0894	0·0894	±0·0
	0·0547	0·0549	+0·4
$K_4Fe(CN)_6.3H_2O$	0·0924	0·0926	+0·2
	0·0402	0·0401	−0·3
$Na_2Zn(CN)_4$*	0·1144	0·1139	−0·4
	0·0692	0·0689	−0·4
$Na_2Cu(CN)_3$*	0·1056	0·1059	+0·3
	0·0583	0·0583	±0·0
$K_2Ni(CN)_4.H_2O$	0·1054	0·1050	−0·4
	0·0552	0·0550	−0·4

* These salts were prepared by dissolving zinc cyanide and cuprous cyanide, respectively, in a solution of sodium cyanide, the amounts of sodium cyanide corresponding to the formulae of the complexes given here. These salts were not isolated in solid form.

the resin had been washed with 50 ml of distilled water, the combined effluent and wash water were titrated with 0·1 *N* hydrochloric acid, with methyl orange as indicator.

The results, which are presented in Table I, show that the accuracy is very high, a maximum relative error of ±0·4 per cent. being obtained. As is seen from the table, the method can also be used for nickel and zinc cyanide complexes, although the cation-exchange method described

in the introduction could not be used for these complexes. This depends upon the fact that the alkali metals pass through the anion-exchange layer, whereas nickel and zinc ions, if present, are precipitated as hydroxides and retained in the column. Because of this, only alkali metals could be detected in the effluent in these experiments.

CAPACITY OF THE RESIN BED—

In order to determine the capacity of the ion-exchange layer a 0·165 *N* potassium ferrocyanide solution was prepared. The solution was allowed to percolate through the resin bed at a flow-rate of 4 ml per minute. When the water in the column had been displaced, the effluent was collected in fractions of 10 ml, which were titrated with 0·100 *N* hydrochloric acid, phenolphthalein being used as indicator, until the consumption of hydrochloric acid was 0·15 ml, *i.e.*, the same as in the influent.

The results are presented in Fig. 1, in which C and C_0 denote the effluent and influent con-

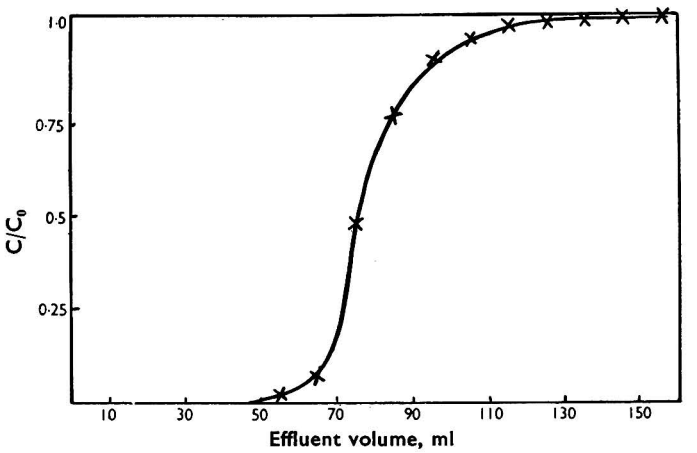

Fig. 1. Breakthrough curve for 0·165 *N* potassium ferrocyanide solution: influent concentration, C_0; effluent concentration, C; flow-rate, 4 ml per minute

centrations, respectively. As is seen from the figure, the breakthrough point is passed after 50 ml. The capacity of the resin bed is therefore—

$$\frac{50 \times 0{\cdot}165}{1{\cdot}000} \times 1{\cdot}000 = 8{\cdot}25 \text{ milli-equivalents},$$

which means that, under prevailing condition, the maximum uptake of the compounds used in this investigation is 0·91 g of $K_3Fe(CN)_6$, 0·87 g of $K_4Fe(CN)_6.3H_2O$, 0·90 g of $Na_2Zn(CN)_4$, 0·79 g of $Na_2Cu(CN)_3$ and 1·1 g of $K_2Ni(CN)_4.H_2O$. From the figure it is also seen that complete exchange is obtained for about 70 per cent. of the resin's capacity.

As a control, the presence of ferrocyanide ions in the effluent was tested for by addition of a few drops of a copper sulphate solution (5 g of copper sulphate, $CuSO_4.5H_2O$, in 100 ml of water) to the fractions of the effluent after the titration with hydrochloric acid. The brown precipitate of copper ferrocyanide was obtained in the sixth fraction, *i.e.*, at the breakthrough point according to Fig. 1.

REGENERATION OF THE RESIN—

In the paper mentioned above[3] it was stated that the anion-exchange resin was regenerated by passing 150 ml of *N* sodium hydroxide solution through the resin bed. It was, however, noticed that, after the passage of the complex cyanide solution, the colour of the resin darkened and that the original pale colour was not restored by the sodium hydroxide treatment. In the experiments described in this paper it was found that the capacity of the ion-exchange resin was gradually diminished and was not restored at the passage of sodium hydroxide. The regeneration was therefore performed in the following manner. The resin was treated with an excess of *N* sulphuric acid in a beaker (NOTE: there is a risk of hydrogen cyanide evolution). Later the resin

was poured into the column and treated with 200 ml of 4 *N* ammonium hydroxide, after which it was washed with distilled water until the effluent had a neutral reaction.

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September 8th, 1954

THE MICRO KJELDAHL DETERMINATION OF NITRO NITROGEN

WHITE and Long,[1] describing their sealed-tube micro Kjeldahl technique, show that low results are obtained for *p*-nitroacetanilide and state that "the method cannot be used for nitrogen linkages requiring reduction prior to the usual Kjeldahl digestion."

We have found that the reduction of nitro compounds can be conveniently effected by the addition of 50 mg of thiosalicylic acid[2] or glucose[3] to the digest; 25 mg were found to be insufficient. Typical results are shown in Table I.

TABLE I

EFFECT OF REDUCING AGENTS ON RECOVERY OF NITROGEN

Compound	Nitrogen calculated, %	Nitrogen found		
		Unmodified method, %	With thiosalicylic acid, %	With glucose, %
p-Nitroaniline	20·3	10·8	20·35	20·1
Picric acid	18·35	13·5	18·15	18·5
m-Dinitrobenzene	16·65	13·7	16·65	16·75
2-Amino-5-nitrothiazole	28·95	26·8	28·85	28·55
p-Nitroacetanilide	15·55	11·8	15·7	15·55
p-Nitrophenol	10·05	7·3	9·55	9·95

TABLE II

RESULTS OBTAINED WITHOUT REDUCING AGENTS

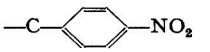
Compound	Nitrogen calculated, %	Nitrogen found, %
p-Nitrobenzoic acid	8·4	8·15
o-Nitrobenzoic acid	8·4	8·45
p-Nitrobenzaldehyde	9·25	9·1
p-Nitrophenylacetic acid	7·75	7·9
3-Benzyloxy-4-nitrotoluene	5·75	5·65
4-Benzoyloxy-4′-nitrodiphenyl	4·4	4·2
Di-*p*-nitrophenylmethanol	10·2	10·0
Di-*p*-nitrophenylmethyl acetate	8·85	8·95
Bisdi-*p*-nitrophenylmethyl ether	10·55	10·3
p-Nitro-N-methylephedrine hydrochloride	10·75	10·75
p-Nitrophenylalanine ethyl ester hydrochloride	10·2	10·3
N-Formyl-*p*-nitrophenylalanine	11·75	11·7
N-Phthaloyl-*p*-nitrophenylalanine ethyl ester	7·6	7·55
α-Benzoin oxime	6·15	6·25
1:5-Di-(4-hexanoyl-2-methylphenoxy)hexane dioxime	5·5	5·5
1:4-Di-(4-hexanoylphenyl)butane dioxime	6·4	6·35
Dimethylglyoxime	24·15	23·3*
2:6-Diamino-8-phenylpurine-7-oxide hydrochloride monohydrate	28·3	28·1
Phenazine di-N-oxide	13·2	13·2
Methyl β-anilino-α-nitroacrylate	12·6	12·5
Ethyl 2:2-dimethyl-5-nitro-1:3-dioxan-5-carboxylate	6·0	6·35

* Found, with thiosalicylic acid, 24·2 per cent.; with glucose, 24·0 per cent.

For compounds containing N–N linkages, glucose is slightly more efficient than thiosalicylic acid, but with neither reagent do the results approach the theoretical values.

The temperature of 470° C used by White and Long[1] has been found to be dangerously high,[4,5] and for this work the digestions, in which mercuric oxide and sulphuric acid were used, were carried out at 420° to 440° C for approximately 45 minutes.

During the course of this work, it was found that many nitro compounds gave correct results without reduction, and it appears that if the compound contains the following structure—



no reduction is necessary. Insufficient work has been done to determine whether *o*- or *m*-nitrophenyl compounds would behave in a similar manner. Furthermore, oximes and N-oxides do not normally require any reduction. (Dimethylglyoxime is an exception. This is not altogether surprising in view of its relatively high nitrogen content.) Zinneke,[6] using essentially open-tube digestions, found that oximes and nitroso compounds gave virtually quantitative yields of ammonia.

Only two aliphatic nitro compounds have been examined. These, rather unexpectedly, required no reduction.

Typical results obtained without reduction are shown in Table II.

Occasionally, slightly low results have been obtained, particularly with compounds containing more than 25 per cent. of nitrogen, the cause of which is not yet known. It is possible that these could be eliminated by a more critical control of the digestion conditions.

The author thanks Miss M. Priestley and Mr. J. D. Goatcher for experimental assistance.

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4. Grunbaum, B. W., Schaffer, F. L., and Kirk, P. L., *Anal. Chem.*, 1952, **24**, 1487.
5. Baker, P. R. W., *Analyst*, 1953, **78**, 500.
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CHEMICAL DIVISION
THE WELLCOME RESEARCH LABORATORIES
BECKENHAM, KENT

P. R. W. BAKER
December 10th, 1954

THE DETERMINATION OF SMALL AMOUNTS OF MAGNESIUM WITH ERIOCHROME BLACK

MUCH work has been carried out on the determination of calcium and magnesium with the disodium salt of ethylenediaminetetra-acetic acid (EDTA). This method, originally introduced by Schwarzenbach, has been applied to the determination of calcium and magnesium, as hardness in water,[1] in soils and plant material[2] and in limestone.[3] The recommended procedure for determining calcium and magnesium separately is to titrate with EDTA: (*a*) with Eriochrome black as indicator, which gives the total magnesium and calcium present, and (*b*) with murexide as indicator, when the calcium alone is determined. The magnesium is obtained by difference. In plant material the calcium content is usually in excess of the magnesium and this leads to a greater relative error in determining the magnesium.

Methods have also been published for the determination of magnesium in plant materials by titration with EDTA after the removal of calcium as oxalate[4] and, as hardness in water, absorptiometrically with Eriochrome black after removal of calcium as sulphate.[5] The latter determination was carried out in a methanol - water medium buffered to pH 10·2.

It was desired to determine the magnesium content of some fermented plant-water extracts with an accuracy of 5 per cent. Only limited quantities of material were available. The method had to be rapid, as the samples were part of a series. The method used was to determine the sum of the calcium and magnesium present by titration with EDTA, to determine the magnesium absorptiometrically with Eriochrome black and to apply a correction factor for the calcium present.

In the absorptiometric determination of magnesium with Eriochrome black,[5] the authors used a Beckmann spectrophotometer and recommended minimum slit-width. For the present work a Hilger Spekker photo-electric absorptiometer with Ilford colour filters was used.

ABSORPTION CURVES OF DYESTUFF AND MAGNESIUM COMPLEX—

The absorption curves for the dyestuff and magnesium complex at different pH values were determined by measuring the absorption at 40-mμ intervals, a Unicam grating spectrophotometer

being used. They were found to be similar to those given by Harvey, Komarmy and Wyatt.[5] Unlike these authors, very little formation of the calcium complex was noted at pH 10·1. This has also been found true in the use of Eriochrome black as indicator in titrations of calcium plus magnesium with EDTA. When magnesium was absent, it was necessary to add a small standard amount to obtain an end-point. The curves for the blank dyestuff and magnesium complex show maximum separation at 520 mμ.

It was found that the use of the filter with maximum transmission at this value gave the greatest difference in drum readings between the dye blank and magnesium complex solutions.

Method

Reagents—

Buffer solution—A saturated aqueous solution of borax, pH approximately 9·2.

Sodium hydroxide, N.

Dye solution—Weigh out 0·1000 g of Eriochrome black, dissolve in absolute methanol by warming, decant from any insoluble material into a 250-ml calibrated flask and dilute to the mark with absolute methanol.

Standard magnesium solution—Weigh out 1·0136 g of magnesium sulphate, $MgSO_4.7H_2O$, dissolve in distilled water and make to 1 litre. This solution contains 100 mg per litre of magnesium and should be checked by gravimetric analysis. More dilute standards are prepared from it by dilution.

Standard calcium solution—Weigh out 0·2500 g of calcium carbonate that has been dried at 105° C, dissolve in the minimum quantity of dilute hydrochloric acid, transfer to a 1-litre calibrated flask and dilute to the mark with distilled water. This solution contains 100 mg per litre of calcium and should be checked by a standard method. More dilute standard solutions are prepared from it by dilution with distilled water.

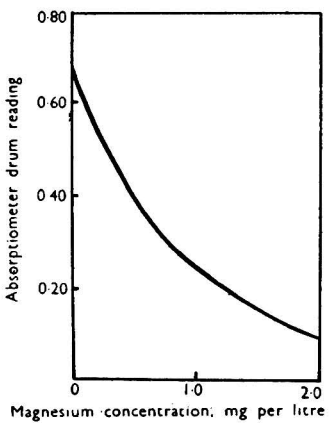

Fig. 1. Calibration curve for magnesium

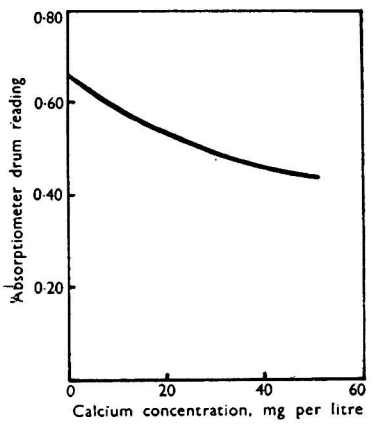

Fig. 2. Calibration curve for calcium used in calculating the correction factor

Apparatus—

Colour measurement—A Spekker photo-electric colorimeter was used, with 1-cm cells and Ilford No. 604 filters (max. transmission at 540 mμ). The drum of the instrument was set at 1·30 with distilled water in the colour cell. The drum readings give the density of the coloured solution.

pH measurement—A Cambridge pH meter was used to measure the pH of the test solutions.

Procedure—

With a pipette place a suitable aliquot containing 10 to 100 μg of magnesium in a 50-ml beaker; add water to bring the volume to about 20 ml. If the solution is acid, it should be made just alkaline to bromothymol blue paper by the dropwise addition of sodium hydroxide. Add 10 ml of borate buffer and 5 ml of dye solution. Adjust the solution to pH 9·5 $\pm$ 0·02 with sodium hydroxide, using a pH meter; wash the solution on the electrodes into the beaker; transfer

to a 50-ml calibrated flask and dilute to volume with distilled water. Measure the absorption using the Spekker absorptiometer.

A calibration curve is prepared by using standard magnesium solutions. A typical curve is shown in Fig. 1.

A calcium curve, which is a standard curve for calcium, is prepared from standard calcium solutions. An example is given in Fig. 2.

From these two curves a correction curve, Fig. 3, is plotted, which gives the correction factor to be subtracted from the uncorrected magnesium value in order to get the true magnesium value. The correction curve is constructed by plotting as ordinate and abscissa the calcium and magnesium values corresponding to a given absorptiometer reading, *e.g.*, from the the calibration and calcium curves given in Figs. 1 and 2, an absorptiometer reading of 0·575 corresponds to 0·141 mg of magnesium per litre and 15·0 mg of calcium per litre. This is plotted as the point X on the correction curve, Fig. 3.

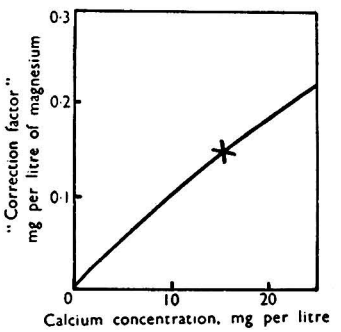

Fig. 3. Correction curve plotted from Figs. 1 and 2 as described in the text

In the absence of calcium the concentration of magnesium may be determined directly from the calibration curve.

In the presence of calcium the following method is used—

(*a*) The concentration of magnesium is determined from the calibration curve, the effect of calcium being neglected. This is the uncorrected value.

(*b*) The total calcium plus magnesium content is calculated from the results of the titration with EDTA. (Since it is necessary to work with concentrations of both calcium and magnesium, the values are most conveniently expressed in terms of either milligram-atoms per litre or milli-equivalents per litre.)

(*c*) The approximate calcium content of the sample is obtained from (*b*) − (*a*), and hence the calcium concentration in the dye solution.

(*d*) The concentration factor to be subtracted from the uncorrected value is read off from the correction curve. The uncorrected value minus the correction factor gives the true magnesium concentration.

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First submitted, *June 11th*, 1953
Amended, *December 29th*, 1954

SOME OBSERVATIONS ON THE DETERMINATION OF FRUCTOSE BY THE SELIWANOFF REACTION*

THE common method employed for determination of fructose in biological systems is based on the Seliwanoff reaction.[1] In order to overcome the deviations from Beer's laws that this reaction exhibits, it has been recommended that at least three working standards be employed. This

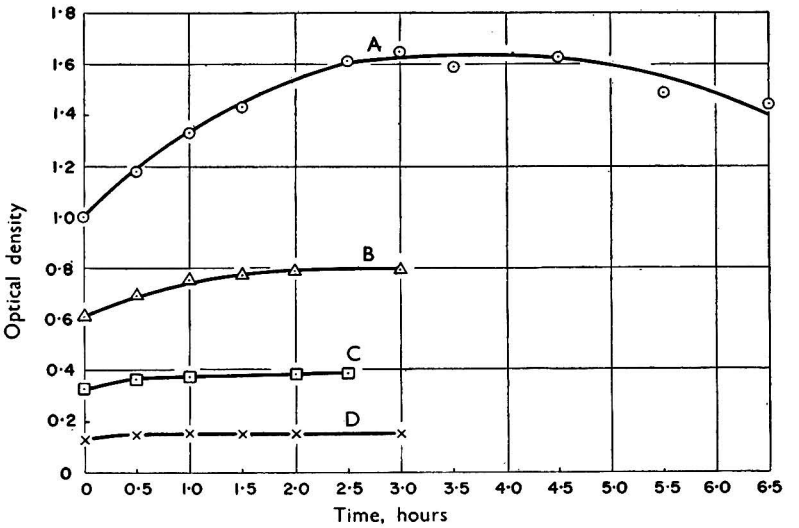

Fig. 1. Colour intensity as a function of time and fructose concentration: curve A, 200 μg per ml; curve B, 100 μg per ml; curve C, 50 μg per ml; and curve D, 20 μg per ml of fructose

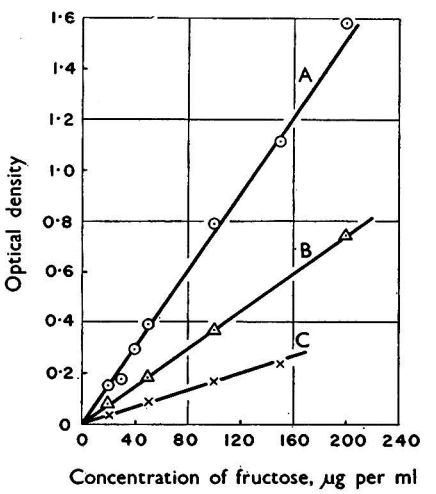

Fig. 2. Colour intensity as a function of concentration of fructose and of resorcinol: A, 0·1 per cent.; B, 0·5 per cent.; and C, 1·0 per cent. of resorcinol

we found to be awkward and, depending upon the number of samples to be analysed, somewhat inaccurate. However, we found that the necessity for the three standards could be eliminated by using a calibration curve that obeys Beer's law provided sufficient time is allowed for maximum colour development.

The stability of the colour with time was investigated by following the procedure of Roe.[1] All determinations were made with the Beckman model DU spectrophotometer at 514 mμ, the

* Communication from the Canada Department of Agriculture, Contribution No. 30.

absorption maximum of the coloured complex. The components were heated in a test tube for exactly 8 minutes and cooled, and the colour intensity was measured subsequently at various time intervals.

Fig. 1 indicates that the time required for the attainment of maximum depth of colour is dependent upon the concentration of fructose. At the high level of fructose (200 μg per ml) the colour was deepest approximately 3 hours after the cessation of the 8-minute heating. Furthermore, at this concentration the colour is stable for 1 hour and then fades. Even at a level of 50 μg per ml of fructose, approximately 1 hour is required for maximum colour development. Probably no serious error would be encountered at a level of 20 μg per ml, for here maximum colour intensity is attained very quickly. It should be noted that Berner and Sandlie[2] also found that at certain concentrations of fructose maximum depth of colour is attained after 1 to 2 days. Thus, provided that sufficient time is allowed for maximum colour development, a calibration curve can be obtained that obeys Beer's law up to 200 μg per ml quite accurately, as seen in Fig. 2. Steinitz[3] observed a deviation from the law above this level.

The values shown in Fig. 2 also indicate that the intensity of the colour is governed by the concentration of resorcinol employed. Concentrations of resorcinol above 0·1 per cent. w/v decreased the sensitivity of the reaction.

In summary, it has been found that the three working standards can be replaced by a calibration curve that shows Beer's law to be accurately followed provided sufficient time is allowed for maximum development of the colour. The use of such a calibration curve increases the speed of routine determinations. The method shows marked sensitivity to the concentration of resorcinol.

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Science Service Laboratory
Canada Department of Agriculture
University Sub Post Office
London, Ontario, Canada

W. Chefurka

December 6th, 1954

British Standards Institution

NEW SPECIFICATIONS*

B.S. 734:1955. Density Hydrometers for Use in Milk. Price 10s. 6d.
B.S. 736:1955. Centrifuge Tubes and Sedimentation Vessels for the Determination of Visible Dirt in Milk. Price 2s. 6d.
B.S. 1428: Part D5: 1955. Syringe Pattern Micro-Pipette. (Microchemical Apparatus: Group D: Volumetric Apparatus.) Price 2s. 6d.
B.S. 1428: Part D6: 1955. Micrometer-Operated Burette. (Microchemical Apparatus: Group D: Volumetric Apparatus.) Price 2s. 6d.
B.S. 2586:1955. Glass Electrodes. Price 3s.
B.S. 2587:1955. Open-Surface Milk Coolers. Price 3s. 6d.

AMENDMENT SLIP*

A printed slip bearing amendments to a British Standard has been issued by the Institution, as follows—
PD 2134—Amendment No. 1 (March, 1955) to B.S. 691:1953. Clinical Maximum Thermometers.

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

Book Reviews

THE DESIGN AND ANALYSIS OF EXPERIMENT. By M. H. QUENOUILLE, M.A., F.R.S.E. Pp. xiv + 356. London: Charles Griffin & Co. Ltd. 1953. Price 36s.

This book is intended for the experimenter who already has some grasp of the fundamental principles and calculations of statistics and needs help in the efficient planning of his experiments and the subsequent extraction from them of the maximum information. An introductory chapter on general principles could be read with great profit by any scientist, and certainly by any analyst. Subsequent sections discuss specific types of design, ranging from simple randomised blocks and factorial designs to incomplete blocks with "confounding." A whole section is then devoted to the important class of experiments in which time is a factor, either as one of the variables or because serial long-term tests are to be made. The final section deals with such complications as missing values; sequential experiments; and the transformation (here called "scaling") of observations.

The style is clear and the layout logical, the relative merits of the various designs being lucidly stated. Plenty of illustrative numerical examples are given, and although not many analysts would need to use the complicated designs described in the more advanced chapters, the first section of this book and certain parts of the last section can confidently be recommended to all who have the necessary elementary knowledge of statistics and have occasion to design experiments.

E. C. WOOD

STATISTICAL ANALYSIS IN CHEMISTRY AND THE CHEMICAL INDUSTRY. By C. A. BENNETT and N. L. FRANKLIN. Pp. xvi + 724. London: Chapman & Hall Ltd.; New York: John Wiley & Sons Inc. 1954. Price 58s.

The Committee on Applied Mathematical Statistics of the National Research Council of the United States decided in 1949 to sponsor a comprehensive book on statistics as applied to chemistry and the chemical industry. The present volume is the result. A knowledge of general mathematics such as a chemistry graduate should possess, but no special knowledge of statistics, is assumed; the ground is covered very completely—far more completely than most readers of *The Analyst* would require, for the first 470 pages are devoted to a full exposition of the mathematical basis of all the usual types of statistical test and computation. Experimental design is considered in the next 120 pages, and then follow 100 pages dealing with the analysis of counted data; control charts; tests for randomness. For the prospective user of statistical techniques who not only wishes to know what to do but also has sufficient interest in mathematical theory to enquire why it is done that way, this book provides all the answers authoritatively and—considering its size—at a reasonable price.

E. C. WOOD

MONOMERIC ACRYLIC ESTERS. By E. H. RIDDLE. Pp. viii + 221. New York: Reinhold Publishing Corp.; London: Chapman & Hall Ltd. 1954. Price 40s.

In this book an attempt is made to present information about the esters of acrylic and methacrylic acid in such a way as to be useful in the laboratory, on the pilot plant and in commerce.

There are chapters dealing with the physical properties of the esters, with their storage and handling, and with their polymerisation and copolymerisation. A useful description of the chemical reactions of the esters is included, and finally there is a short chapter of 18 pages on the analytical methods of examination of these monomeric substances.

Unfortunately the subject is not treated very critically, with the result that the book tends to be a catalogue of the American patent and technical literature in this field. There are 673 references, but the absence of an index must reduce the value of the book very considerably.

The chapter on analysis is very brief, being mainly concerned with the determination of unsaturation of the monomeric acrylic esters by the rather tricky pyridine sulphate dibromide method. Methods are given for the determination of ester and water content, as well as for the determination of the stabilisers hydroquinone and the monomethyl ether of hydroquinone.

The use of the term methyl polymethacrylate throughout the book instead of the more usual polymethyl methacrylate does draw attention to the need for more systematic methods of naming polymers.

J. HASLAM

Publications Received

A DICTIONARY OF DAIRYING. By J. G. DAVIS, D.Sc., Ph.D. (Lond.), F.R.I.C., M.I.Biol., F.R.San.I. Second Edition. Pp. xxviii + 1132. London: Leonard Hill Ltd. 1955. Price 65s.

DIE KOMPLEXOMETRISCHE TITRATION. By Prof. Dr. GEROLD SCHWARZENBACH. Pp. xii + 100. Stuttgart: Ferdinand Enke Verlag. 1955. Price DM19 (paper); DM21 (cloth boards).

X-RAY DIFFRACTION BY POLYCRYSTALLINE MATERIALS. Edited by H. S. PEISER, M.A., A.R.I.C., F.Inst.P., H. P. ROOKSBY, B.Sc., F.Inst.P., and A. J. C. WILSON, M.Sc., Ph.D., A.I.M., F.Inst.P. Pp. 725. London: The Institute of Physics. 1955. Price 63s.

MODERN GAS ANALYSIS. By PAUL W. MULLEN. Pp. x + 354. New York and London: Interscience Publishers Inc. 1955. Price $5.50; 40s.

METHODS OF BIOCHEMICAL ANALYSIS. Volume II. Edited by DAVID GLICK. Pp. vi + 470. New York and London: Interscience Publishers Inc. 1955. Price $9.50; 75s.

THE CHEMISTRY OF MICRO-ORGANISMS. By ARTHUR BRACKEN, B.Sc., Ph.D., F.R.I.C. Pp. vii + 343. London: Sir Isaac Pitman & Sons Ltd. 1955. Price 30s.

JOURNAL OF INORGANIC AND NUCLEAR CHEMISTRY. Edited by JOSEPH J. KATZ (Chicago), H. C. LONGUET-HIGGINS (Cambridge) and H. A. C. McKAY (Harwell). Volume I, Nos. 1/2, March, 1955. Pp. 164. London and New York: Pergamon Press Ltd. Subscription A (normal) 90s. per volume; subscription B (for individual subscriber's own use) 70s. per volume.
A new journal.

SEMIMICRO QUALITATIVE ANALYSIS. By EDWIN O. WIIG, Ph.D., WILLARD R. LINE, Ph.D., and JOHN F. FLAGG, Ph.D. Pp. viii + 238. New York: D. Van Nostrand Co. Inc. London: Macmillan & Co. Ltd. 1954. Price $3.25; 25s.

SYSTEMATIC HANDBOOK OF VOLUMETRIC ANALYSIS. By FRANCIS SUTTON, F.I.C., F.C.S., and JULIUS GRANT, M.Sc., Ph.D., F.R.I.C. Thirteenth Edition. Pp. xiv + 752. London: Butterworths Scientific Publications. 1955. Price 63s.

OUTLINES OF ENZYME CHEMISTRY. By J. B. Neilands and P. K. Stumpf. Pp. x + 315. New York: John Wiley & Sons Inc.; London: Chapman & Hall Ltd. 1955. Price 52s.

BRITISH POISONOUS PLANTS. Bulletin No. 161 of The Ministry of Agriculture and Fisheries. By A. A. FORSYTH, M.R.C.V.S., D.V.S.M. Pp. vi + 116. London: H.M. Stationery Office. 1954. Price 6s. 6d.

EINFÜHRUNG IN DIE ENERGETIK UND KINETIK BIOLOGISCHER VORGÄNGE. By Dr. phil. W. BLADERGROEN. Pp. x + 368. Basel: Wepf & Co. Verlag. 1955. Price Sw. Fr. 28.

ORGANIC REAGENTS FOR METALS. Volume I. By the Laboratory Staff of Hopkins & Williams Ltd. Edited by W. C. JOHNSON, M.B.E., F.R.I.C. Fifth Edition. Pp. viii + 199. Chadwell Heath, Essex: Hopkins & Williams Ltd. 1955. Price 15s.

Erratum

NOVEMBER (1954) ISSUE, p. 680, 2nd line of "REAGENTS." *For* "55 g" *read* "5·5 g."

ANALYST

THE ILFORD LABORATORIES of Thorium Limited, Rare Earth Chemical Manufacturers, have a vacancy for an Analyst with A.R.I.C. as a minimum qualification. The post would entail assisting the Head of the Analytical Section, both in process control and in a range of analysis requiring skill and experience. There is scope for development of new methods. The initial salary would be assessed on qualifications and experience, but would not be less than £700 per annum. Application forms may be obtained from the Research Manager, Thorium Limited, Uphall Road, Ilford, Essex.

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(3) Development and evaluation of new processing, plating and allied techniques.

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Applications in confidence, giving age, details of qualifications, experience and salary required to:—

THE PERSONNEL OFFICER
The de Havilland Aircraft Company Limited
Stag Lane, Edgware, Middlesex

APPLICATIONS invited from CHEMISTS who would like to work about 6 months each year in British West Indies and 3 months in England, remainder of year travelling and 2 months' leave. Applicants must be fully qualified (University degree or equivalent), preferably with both laboratory and factory experience of food manufacture and packing, including canning. Duties include taking charge of analytical and bacteriological laboratory with responsibility for technical control in factory of production and quality. Technical proficiency must be coupled with absolute integrity, initiative, self-reliance, tact and ability to enjoy really hard work for 9 months of the year. Write, in strict confidence, giving full particulars, past experience, age and other relevant personal data, to Box No. 530, c/o Smiths, 100 Fleet Street, London, E.C.4.

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EXPERIENCED CHEMIST (University graduate or member R.I.C.) wanted to take responsibility for all analytical and bacteriological work, technical supervision of production processes and quality of output, etc., in well-established modern factory producing range of high-grade products for export in bulk or canned. In addition to sound technical ability, such qualities as initiative, energy, tact in dealing with laboratory and factory staff, are essential. Good initial salary according to qualifications, excellent prospects. House provided conveniently near factory, attractive surroundings, healthy climate. Liberal home leave, first class passages. Apply, in strict confidence, giving full details past experience, age, married or single, when available, to Box No. 537, c/o Smiths, 100 Fleet Street, London, E.C.4.

ANALYST

THE DISTILLERS CO., LTD.

THE Research and Development Department of The Distillers Co., Ltd., Epsom, invite applications for the post of Analyst to undertake investigational work in connection with antibiotics fermentation research. Some two years' experience as an analyst is essential. Candidates should have an honours degree in Chemistry or Biochemical subjects.

Apply: Staff Manager, The Distillers Co., Ltd., 21 St. James's Square, London, S.W.1., ref. 20/55.

PARKE, DAVIS & COMPANY, LIMITED, invite applications for the following appointments in their Analytical Department:—

(*a*) A young lady graduate in bacteriology or biological subjects. Experience in germicidal and sterility testing on pharmaceutical products, although not essential, would be an advantage.

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Five-day week, staff restaurant, generous pension plan. Writing, giving particulars, to Personnel Manager, Staines Road, Hounslow, Middlesex.

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cont. next page

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CONTENTS

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