THE ANALYST

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THE ANALYST

EDITORIAL

A REPRINT OF *THE ANALYST* FROM 1876 TO 1951

FOR some years past the demand for back numbers of *The Analyst* has been continuous and insistent. The known locations of complete sets are but few; long runs appear but seldom in the second-hand market, and the stock of complete volumes for recent years remaining in the hands of our publishers is almost completely exhausted.

The reason for this lamentable position is not far to seek; the ever increasing appreciation of the importance of analytical chemistry in industry, public health, agriculture and in human activities in general has, during the last thirty years, led to the installation of many more chemical libraries in works and research laboratories, colleges, schools of chemistry and in private possession. To all of these a file of *The Analyst* is a necessity, and all are finding it increasingly difficult to fill their needs.

Plans that were in existence, many years ago, for reprinting the early volumes had perforce to be set aside on the outbreak of war; since 1945, and until recently, it has been impossible to obtain supplies of paper in excess of current requirements, or to plan ahead in face of ever rising costs.

It is therefore with no little pleasure that we are now able to announce a project for the reprinting of our back numbers up to 1951 and to know that it will be possible for the many gaps in chemical libraries to be filled.

On the last page of this issue there will be found a prospectus of the proposed reprinting, in which the 76 volumes to be dealt with have been divided into groups, some of which, for recent years, will also be sold as separate volumes. This grouping of the earlier volumes has been made in order to reduce the cost to subscribers to the lowest possible figure.

The reprinting must necessarily depend upon the response to our publisher's prospectus and order form, supplied with this issue; for it will be easily understood that a work of this magnitude cannot be undertaken unless a reasonable proportion of its cost can be guaranteed in advance.

The financial responsibility for this undertaking, which is far beyond the resources of the Society, will be borne by our publishers, W. Heffer & Sons, to whom all analytical chemists will be grateful for making *The Analyst* more readily available.

PROCEEDINGS OF THE SOCIETY OF PUBLIC ANALYSTS AND OTHER ANALYTICAL CHEMISTS

AN Ordinary Meeting of the Society was held at 7 p.m. on Wednesday, December 3rd, 1952, in the Meeting Room of the Chemical Society, Burlington House, London, W.1. The Chair was taken by the President, Dr. J. R. Nicholls, C.B.E., F.R.I.C.

A lecture on "Industrial Fluorosis" was given by H. H. Green, O.B.E., D.Sc.

NEW MEMBERS

Glynne Wentworth Beaumont, F.R.I.C.; Leslie Billington, A.R.I.C.; Elena Gagliardo, D.Chem. (Padua); Sheila Merrick, B.Sc. (Lond.).

DEATH

WE regret to record the death of

Leonard Owen Newton.

SCOTTISH SECTION

AN Ordinary Meeting of the Section was held on Thursday, December 11th, 1952, at 7.15 p.m. in the Biochemistry Department, The University, Glasgow. Mr. H. C. Moir, Chairman of the Section, presided.

A lecture entitled "Electrophoresis" was given by Dr. G. T. Mills, and was followed by a visit to the Electrophoresis Laboratory at Glasgow University.

Spectroscopic Properties of Vitamin A_2

Application to the Assay of Cod-Liver Oil

BY H. R. CAMA AND R. A. MORTON

(*Presented at the meeting of the Society on Wednesday, October 1st, 1952*)

Vitamin A_2, made by reducing retinene$_2$ with lithium aluminium hydride, shows a main ultra-violet maximum near 351 mμ ($E^{1\%}_{1cm}$ about 1400) and a secondary peak near 287 mμ ($E^{1\%}_{1cm}$ about 750). The blue solution (with antimony trichloride reagent) shows $\lambda_{max.}$ at 693 mμ, $E^{1\%}_{1cm}$ about 3900. The absorption intensities at different wavelengths and for different solvents have been measured and expressed as fractions of the maximum intensities.

The biological potency of vitamin A_2 is taken to be $1{\cdot}33 \times 10^6$ i.u. per g, that of vitamin A_1 being $3{\cdot}33 \times 10^6$ i.u. per g.

Fish-liver oils in general contain much more vitamin A_1 than A_2, depending on the species. In cod-liver oils vitamin A_2 may account for about one-seventh of the total vitamin A (molecule for molecule).

By determining $E^{1\%}_{1cm}$ at 693 mμ (A_2) and at 620 mμ (A_1) in the antimony trichloride colour test (applied to the unsaponifiable fraction), and the $E^{1\%}_{1cm}$ at 326 mμ, 351 mμ and 286 mμ in the ultra-violet, oils can be tested for both vitamins. The 693-mμ absorption measures vitamin A_2 directly and from it the vitamin-A_2 contributions to ultra-violet absorption at 351 mμ and 327 mμ can be calculated.

A conversion factor is given for calculating the probable vitamin-A_2 contribution to the potency. A cod-liver oil typical of those studied by spectrophotometric methods, corrected for all irrelevant absorption, gave an estimated vitamin-A potency about 6·5 per cent. lower than the estimate that included the possible vitamin-A_2 contribution.

VITAMIN A_2 is detected in fish-liver oils, by means of an absorption band near 693 mμ, in the blue solution produced by the interaction of the vitamin and the Carr - Price reagent (anhydrous antimony trichloride in chloroform). In low-potency oils where vitamin A_1 predominates over A_2, the 693-mμ band can only be seen when the unsaponifiable fraction is used, as the colour test is substantially inhibited when tests are made on the whole oil.

The ultra-violet absorption spectrum shown by most liver oils and unsaponifiable fractions is mainly due to vitamin A_1 ($\lambda_{max.}$ at 326 to 328 mμ), but the absorption curve will be distorted to a greater or lesser extent when vitamin A_2 ($\lambda_{max.}$ at 351 and at 286 mμ) is present. Fish-liver oils and extracts therefrom also exhibit irrelevant absorption from substances either unrelated to vitamins A or derived from them by oxidation. It is a matter of practical

TABLE I

INTENSITIES OF ABSORPTION EXPRESSED AS FRACTIONS OF $E_{max.}$ FOR VITAMIN-A_2 ALCOHOL ON DIFFERENT SOLVENTS

Wavelength, mμ	Solvent			
	*cyclo*Hexane	*iso*Propanol	Ethanol	Light petroleum
250	0·193	0·170	0·163	0·163
260	0·228	0·219	0·209	0·208
265	0·274	0·280	0·272	0·266
270	0·327	0·335	0·322	0·317
275	0·432	0·438	0·435	0·422
280	0·472	0·443	0·435	0·427
285	0·529	0·549	0·541	0·530
286	—	—	—	0·540
287	0·555	0·549	0·544	—
290	0·529	0·477	0·482	0·482
295	0·418	0·391	0·393	0·396
297	0·406	0·391	0·393	—
300	0·410	0·408	0·408	0·412
305	0·459	0·477	0·477	0·482
310	0·540	0·567	0·560	0·567
315	0·624	0·646	0·638	0·648
320	0·704	0·733	0·728	0·738
325	0·786	0·821	0·810	0·819
330	0·864	0·888	0·880	0·890
335	0·927	0·943	0·938	0·944
340	0·964	0·971	0·970	0·973
345	0·990	0·994	0·995	0·991
348	—	—	—	1·000
350	0·998	1·000	1·000	0·997
352	1·000	—	—	—
355	0·994	0·980	0·988	0·973
360	0·950	0·923	0·935	0·918
365	0·900	0·865	0·878	0·861
370	0·838	0·797	0·813	0·793
375	0·757	0·700	0·715	0·695
380	0·643	0·579	0·590	0·569
385	0·533	0·478	—	0·470
390	0·448	0·400	0·419	0·380
400	0·286	0·225	0·241	0·220
410	0·111	0·073	0·083	0·072
420	0·035	0·025	0·032	0·023

importance to the analyst to be able to determine the vitamin-A_2 content by studying spectrophotometrically the antimony trichloride colour and then translating the result into the equivalent observed ultra-violet absorption contribution of vitamin A_2. By subtracting the curve for the vitamin A_2 moiety from the observed curve (preferably measured on the unsaponifiable fraction) it is possible to assess the extent to which the residual absorption curve is distorted by adventitious interfering substances.

At present the analyst is handicapped by a lack of precise information to enable him to calculate the ultra-violet absorption of vitamin A_2 from the colour test measurements at 693 mμ.

Vitamin A_2 has not been isolated as a pure crystalline substance and the best preparations hitherto obtained[1,2] have been rather inadequately characterised. However, retinene$_2$, the aldehyde of vitamin A_2, has been obtained crystalline and apparently pure.[3] The well-known reducing agent lithium aluminium hydride ($LiAlH_4$) effects a fairly smooth conversion of retinene$_2$ to vitamin A_2 under appropriate experimental conditions. Although the process is not quantitative, the reduced material can be purified by chromatography and the vitamin-A_2 fraction can be studied both in respect of its ultra-violet absorption and by the antimony

trichloride colour test. With known values of $E^{1\%}_{1cm}$ at 693 mμ for the colour test and of $E^{1\%}_{1cm}$ at 351 mμ for the ultra-violet absorption test, it will be possible to calculate the ultra-violet absorption due to vitamin A_2 at 326 to 328 mμ (the maximum for vitamin A_1) from the colour test reading.

At this stage it is necessary to know the relative intensities of absorption at wavelengths over the range 250 to 400 mμ for vitamin A_2 in different solvents, and the most convenient specification is in terms of $E_{max.} = 1{\cdot}0$ (*i.e.*, at 350 to 352 mμ).

Experimental

Crystalline retinene$_2$ (m.p. 77° to 78° C, $E^{1\%}_{1cm}$ at 385 mμ = 1450 in *cyclo*hexane) was used (Cama *et al.*[3]). Lithium aluminium hydride (0·2 g) was finely ground and dissolved in 20 ml of anhydrous diethyl ether and 0·05 g of retinene$_2$ was dissolved in a further 50 ml of anhydrous ether. Both solutions were cooled to 0° C. The reagent solution was added slowly, with stirring, to the retinene$_2$ solution protected against light. When the solution

Table II

Spectroscopic properties of vitamin A_2

Workers	Solvent	$E_{max.}$ mμ	$E^{1\%}_{1cm}$	Inflection, mμ	E at smaller peak / E at main peak %
1	Ethanol	352	1460	None	0·55
		287	820	None	
2	"	352	1330	None	0·51
		288	678	None	
3	"	351	1410	277	0·54(5)
		286	698		
3	Light petroleum	348	1390	277	0·54
		286	750		
3	*cyclo*Hexane	351·5	1320	275	0·55(5)
		287	733		
3	*iso*Propanol	351	1370	277	0·55
		285	753		
3	Chloroform	356·5	1280	282	0·55
		291·5	704		
1	$SbCl_3$ colour test	693	4100		
2	"	693	3700		
3	"	693	3870		
		E at 620 (no peak)	1580		

1. Shantz.[1]
2. Farrar, Hamlet, Henbest and Jones.[2]
3. Cama and Morton; work described here.

had become colourless (4 to 5 minutes), the excess of lithium aluminium hydride was decomposed by adding ice-cold water dropwise to the mixture. If the reaction was allowed to continue for more than 5 minutes the mixture turned violet and the yield of vitamin A_2 fell progressively with time. The mixture was extracted with ether (redistilled over reduced iron) and the combined extracts were washed with water and dried with sodium sulphate. The solvent was removed under reduced pressure. The residue, which weighed 0·03 g and had an $E^{1\%}_{1cm}$ at 351 mμ of 970, was mainly vitamin A_2. It was chromatographed on alumina* weakened by stirring in water (10 per cent. w/w) under light petroleum. The light petroleum carried through the column a small amount of material showing $\lambda_{max.}$ at 350 and at 285 mμ—possibly an isomer of the normal vitamin A_2; this was followed by a small fraction showing maxima at 320 and 345 to 350 mμ. The main vitamin A_2 fraction was eluted with a mixture of light petroleum (95 volumes) and ether (5 volumes). By means of a two-fold increase in the proportion of diethyl ether, a little anhydrovitamin A_2 and some oxidised material was eluted. The main fraction was chromatographed again on a fresh column. The portion carried through by the mixture of light petroleum and diethyl ether (95 + 5) seemed to be quite homogeneous ($\lambda_{max.}$ at 350 and at 285 mμ, with a slight inflection at 275 mμ). The absorption spectrum of this material was measured in different solvents. The results are shown in Tables I and II.

* Supplied by P. Spence and Co., Ltd.

Discussion of results

The best preparations of vitamin A_2 recorded in the literature show the characteristics recorded in Table II. The absolute values of $E^{1\%}_{1cm}$ obtained in the present work are not particularly stressed because somewhat higher values are not unlikely to be found when vitamin A_2 can be crystallised.

The agreement is nevertheless good and the relative values at 693 and 351 mμ are sufficiently trustworthy to meet all practical needs. The absorption curves in the ultra-violet plotted on a scale relative to $E_{max.} = 1{\cdot}0$ agree well with the corresponding curves obtained by Salah[4] on Nile fishes; some of these fish yield liver oils showing vitamin A overwhelmingly preponderant in vitamin A_2. The small inflection near 275 mμ observed by us

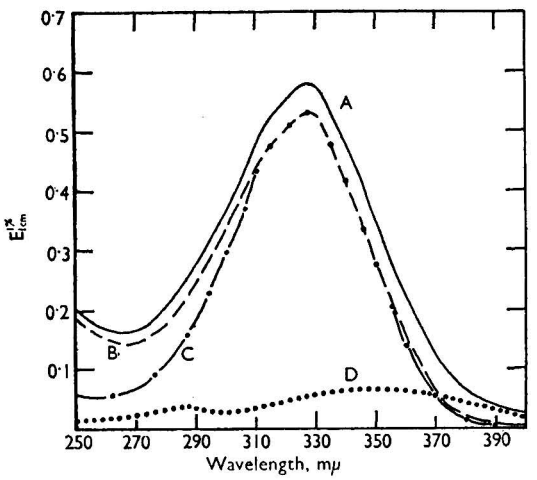

Fig. 1. Absorption spectrum of unsaponifiable fraction of cod-liver oil. Curve A, gross absorption; curve B, unsaponifiable (gross) — vitamin A_2; curve C, vitamin-A_1 contribution; curve D, vitamin-A_2 contribution

is regularly recorded by Salah, but it may be due to the presence of a small amount of an isomer of all-*trans* vitamin A_2. Incidentally, there is as yet no proof that natural vitamin A_2 or even synthetic vitamin A_2 is mainly all-*trans*.

Other properties of vitamin A_2 and its chemical constitution have been discussed in detail by Cama, Dalvi, Morton and Salah.[5]

The practical use of the information given in this paper is illustrated below.

Application of results to a sample of cod-liver oil—

A cod-liver oil tested on its unsaponifiable fraction by ultra-violet absorption gave $E^{1\%}_{1cm}$ at 327 mμ = 1·16 (uncorrected; solvent *cyclo*hexane); by the colour test, $E^{1\%}_{1cm}$ at 693 mμ = 0·384 and at 620 mμ = 2·80.

From the properties of vitamin A_2 (see Table II)—

$$E^{1\%}_{1cm} \text{ at } 351 \text{ m}\mu = \frac{0{\cdot}384 \times 1320}{3860} = 0{\cdot}13 \text{ (in } \textit{cyclo}\text{hexane).}$$

The contribution of vitamin A_2 to the observed ultra-violet absorption is obtained by multiplying all the figures in column 1 of Table I by the factor 0·13 (see Fig. 1). The resulting curve is subtracted from that obtained for the unsaponifiable fraction.

Now $E^{1\%}_{1cm}$ at 327 mμ (for vitamin A_2) = 0·13 × 0·8 (see Table I)
= 0·104,

and the intensity of absorption at 327 mμ corrected for vitamin A_2 = 1·16 − 0·104
= 1·056 (approximately 1·06).

In Fig. 1, the vitamin-A_1 curve is calculated on the basis of $E_{max.} = 0{\cdot}53$ for all-*trans* vitamin A_1 (0·5 per cent. solution) by multiplying by 0·53 all the figures for a *cyclo*hexane

solution of pure all-*trans* vitamin A_1 as observed by Cama, Collins and Morton.[6] The colour test for vitamin A_2 shows absorption at 620 mμ equal to that at 693 mμ divided by 2·45 (see Table II).

$$\text{The corrected colour test for vitamin } A_1 = 2{\cdot}80 - \frac{0{\cdot}384}{2{\cdot}45}$$
$$= 2{\cdot}64.$$
$$\text{The equivalent ultra-violet absorption} = \frac{2{\cdot}64}{2{\cdot}92}$$
$$= 0{\cdot}91.*$$

Shantz and Brinkman[7] have shown that the potency of vitamin A_2 is 40 per cent. of that of vitamin A_1 ($3{\cdot}33 \times 10^6$ i.u. per g), *i.e.*, $3{\cdot}33 \times 10^6 \times 0{\cdot}4$ or $1{\cdot}33 \times 10^6$ i.u. per g.

The conversion factor, therefore, for $E^{1\%}_{1cm}$ at 352 mμ (*cyclo*hexane) is 1100 and for the colour test at 693 mμ is 345.

The potency of vitamin A_2 in the oil is 136 ($0{\cdot}136 \times 1000$) i.u. per g or 132 ($0{\cdot}38 \times 345$) i.u. per g.

In the past it was found useful to multiply the gross $E^{1\%}_{1cm}$ value on "total unsaponifiable" by the factor 1600. It is now necessary to multiply the corrected $E^{1\%}_{1cm}$ value (on the unsaponifiable fraction) by the factor 1900; on applying these procedures to the above sample we have $1{\cdot}15 \times 1600 = 1856$ i.u. per g,
and $0{\cdot}99 \times 1900 = 1881$ i.u. per g.
The earlier empirical conversion factor was well chosen and is still useful.

A great many fish-liver oils contain a mixture of neovitamin A and all-*trans* vitamin A_1 in the ratio of 1 to 3, and a conversion factor that reflects the present state of knowledge concerning such a mixture is 1800; $E^{1\%}_{1cm}$ at 327 mμ (corrected for vitamin A_2) = 1·056.

Therefore potency = $1{\cdot}056 \times 1800 = 1900$ i.u. per g.

Three approaches lead to a figure of about 1870 and unless some entirely new problem arises it is probable that for most purposes sufficient accuracy has been reached.

We are indebted to the Research Committee of the Royal Liverpool United Hospital for financial assistance, which enabled one of us (H. R. C.) to participate in this work.

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3. Cama, H. R., Dalvi, P. D., Morton, R. A., Salah, M. K., Steinberg, G. R., and Stubbs, A. L., *Biochem. J.*, 1952, **52**, 535.
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July 17th, 1952

Discussion

Mr. S. A. Reed asked why a low value for vitamin A_1 was given on applying a conversion factor to the reading at 620 mμ.

Professor Morton replied that when the colour test was applied to cod-liver oils (unsaponified) $\lambda_{max.}$ was at 600 to 610 mμ, depending on the sample. When the colour test was carried out on the unsaponifiable fractions of the same oils, $\lambda_{max.}$ was at 617 to 620 mμ and $E_{max.}$ was much greater. The partial inhibition that occurred when oils were tested had not been fully explained; it certainly invalidated the test for quantitative purposes. The colour test given with unsaponifiable extracts was less variable in the amount of inhibition from sample to sample and showed a good correlation with the corrected ultra-violet absorption at 327 mμ. Nevertheless, taking the properties of all-*trans* vitamin-A alcohol as standard,

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* $\frac{E^{1\%}_{1cm} \text{ at } 328\ m\mu}{E^{1\%}_{1cm} \text{ at } 620\ m\mu} \times 1750 = 5070$ for all-*trans* vitamin-A alcohol. In this calculation the inhibition of the colour by other unsaponifiable constituents is assumed to compensate roughly for the more intense colour test shown by neo-vitamin-A alcohol, which is usually present. The approximation will be considered further in a future paper.

the colour test tended to give rather lower results than the ultra-violet absorption. It was not certain that the difference was due to residual colour test inhibition. Nevertheless, for a series of cod-liver oils, the colour test on the unsaponifiable fraction was a good measure of relative potencies.

DR. D. C. GARRATT asked whether the factors suggested by Professor Morton for conversion with the different techniques were sufficiently close to be used for all cod-liver oils or if they would be much in error for non-typical oils.

PROFESSOR MORTON said that it was difficult to generalise about non-typical cod-liver oils. Some low-potency oils exhibited absorption due to conjugated poly-ene acids, which exceeded in intensity that due to vitamin A. It was possible, however, to treat as normal the unsaponifiable fraction or vitamin fraction obtained by chromatography.

If an oil was untypical because it had deteriorated and contained an unusual amount of free vitamin A or anhydrovitamin A, recourse should be had to chromatography, because correction procedures were then of doubtful validity.

DR. E. C. WOOD, in supplementing the previous question, asked how much variation there was between cod-liver oils from different sources, *e.g.*, between those from Norway and Newfoundland.

PROFESSOR MORTON said that he had too little information about the origin of the oils he had tested to say whether there were consistent differences between the oils from different fishing grounds.

DR. J. E. PAGE enquired about the possibility of determining vitamins A_1 and A_2 in natural products by infra-red absorption spectroscopy. He said that the infra-red absorption spectrum of vitamin A_1 was significantly different from that of vitamin A_2 and that it should be possible to determine the two vitamins in simple mixtures.

PROFESSOR MORTON said in reply that without trial it was unwise to give a categorical answer, but it was unlikely that any standard infra-red absorption technique was as yet sufficiently quantitative to give E values for the accurate determination of vitamins A_1 and A_2 in oils or unsaponifiable extracts.

DR. R. E. STUCKEY said that certain cod-liver oils gave much irrelevant absorption on the unsaponifiable matter. He asked if Professor Morton would agree that a chromatographic procedure was necessary for these oils and what percentage of correction it would be reasonable to apply.

PROFESSOR MORTON said that it was often worth while to chromatograph anomalous oils (with or without saponification). Straight chromatography on weakened alumina and development with purified light petroleum often gave a vitamin-A ester fraction that after saponification proved to be spectroscopically more nearly normal. Corrections exceeding 20 per cent. of the gross value on the unsaponifiable matter were perhaps too great to be accepted uncritically.

MR. H. E. MONK asked if the different forms of vitamin A served any different biological function and whether richer oils, such as halibut-liver oil, contained amounts of neo-A and A_2 similar to those in cod-liver oil. He also asked if the green colour with vitamin A_2 was as fleeting as the blue with vitamin A_1 and whether there was any method of dealing with these transient colours.

PROFESSOR MORTON replied that there was at present no indication that vitamins A_1 and A_2 or their isomers had distinct physiological functions. Most fish-liver oils contained neovitamin A, but, although the all-*trans* form was usually predominant, the all-*trans* to neo ratio was a matter for measurement. In general, fish-liver oils contained 10 to 20 times as much A_1 as A_2, but for a few species of marine fishes the ratio of A_1 to A_2 could be as low as 5. The antimony trichloride colour test absorption was transient both for A_1 and A_2 and no method of prolonging the full intensity had been devised.

The Simultaneous Determination of Pentose and Hexose in Mixtures of Sugars

By W. R. FERNELL and H. K. KING

(*Presented at the meeting of the Society on Wednesday, October 1st, 1952*)

Pentose and hexose can be estimated when present together in mixtures by heating them with orcinol and acid either alone or with ferric chloride. The colours produced are measured at suitable wavelengths and the results calculated directly from the colorimeter readings by means of a nomogram.

During investigations into the chemical composition of bacterial cell material it became necessary to determine pentoses and hexoses when present together. An approximate answer, at least, was needed even when the exact nature of the sugars present was not known. Colorimetric methods based on the orcinol reaction proved suitable and required only small amounts of material.

Reaction of orcinol with acid decomposition products of sugars—

When orcinol is heated with sugars in the presence of sulphuric or hydrochloric acid the product has an absorption band in the blue end of the spectrum. Fig. 1 shows the absorption spectra obtained when the reaction is carried out under the conditions prescribed on p. 81. Pentoses and hexoses both give a peak at 425 mμ, but the absorption with the hexoses is the

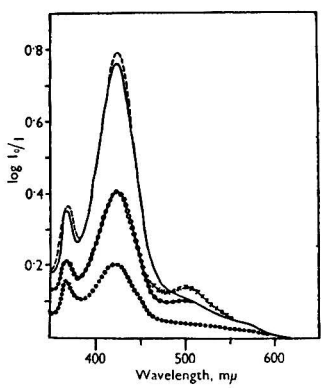

Fig. 1. Absorption spectra of material obtained on heating sugars with the orcinol-sulphuric acid reagent. Solutions containing 24 μg of the sugars per ml were treated with the reagent as described in the text. The absorption spectra of the solutions in a 1-cm cuvette were read on the Unicam spectrometer.

Xylose ————, ribose – – – – –, fructose •• •••, glucose o-o-o-o-o, galactose x-x-x-x-x

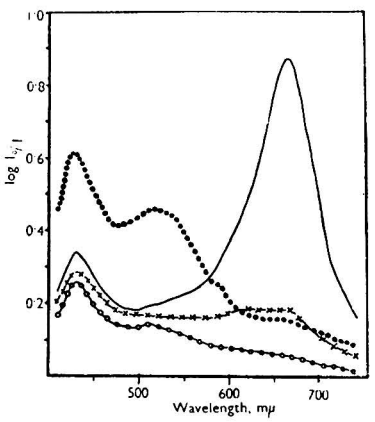

Fig. 2. Absorption spectra of material obtained on heating sugars with the orcinol-ferric chloride reagent. Solutions containing 24 μg of the two pentoses per ml or 72 μg of the hexoses per ml were treated with the orcinol-ferric chloride reagent as described in the text. The absorption spectra of the solutions in a 1-cm cuvette were read on the Unicam spectrometer.

Xylose ————, ribose ————, fructose ••••• ••, glucose o-o-o-o-o, galactose x-x-x-x-x

lesser. If the colour is read on an "EEL" colorimeter* incorporating the appropriate filter, the colour ratio of pentose to glucose is about 1·3 to 1, or 1·1 to 1 if molar concentrations are considered (see Table I). This is the basis of the Tillmans - Phillippi method for assaying total carbohydrate.[1,2] If, however, the reaction is carried out in the presence of ferric chloride,

* Made by Evans Electroselenium Ltd., Harlow, Essex.

pentoses give, in addition, a strong absorption band in the red, which is not given by hexoses. (See Fig. 2, which is essentially the same as that given by Brown.[3] Note that the hexoses were used at a concentration thrice that of the pentoses in Fig. 2, to permit accurate measurement of the small absorptions at the greater wavelengths.) The resulting green colour is used in the Bial test for pentoses, which has been adapted for quantitative use by Mejbaum[4] and Brown.[3] At the peak (660 mμ), xylose, ribose and arabinose (not shown in Fig. 2) give the same absorption, *viz.*, about 15 times that shown by fructose and 30 times that of glucose or galactose. Hence this reaction alone allows for estimation of pentose when an excess of hexose is not present. Brown[3] allowed for hexose by measuring the absorption at two wavelengths, at the pentose peak of 660 mμ, and also at 520 mμ where the difference between pentose and hexose was less marked. Although in principle his method could be used for determining both pentose and hexose present together, it has its disadvantages. The reagent blank at 520 mμ is considerable and variable. The large blank absorption at the blue end of the spectrum also limits the usefulness of measurements at what would otherwise be the most suitable wavelength, *viz.*, 425 mμ, when the absorption for both pentose and hexose is at a maximum and of the same order of magnitude. In the method recommended here the mixture of sugars is heated with orcinol and acid, alone or with ferric chloride, and the absorption is read at the most suitable wavelengths, *viz.*, 425 mμ and 660 mμ, respectively. The concentrations of both pentose and hexose can then be read from a nomogram.

TABLE I

COLORIMETER READINGS WITH VARIOUS SUGARS

Sugar	Readings with acid orcinol reagent and 621 (blue) filter	Readings with acid orcinol - ferric chloride reagent and 608 (red) filter
Glucose	19	2
Galactose	20	5
Fructose	6½	2
Sucrose	13	2
Maltose ($C_{12}H_{22}O_{11}.H_2O$) ..	17	2
Xylose	26	78
Ribose	26	78

METHOD

PREPARATION OF MATERIAL FOR ASSAY—

When only monoses or oligosaccharides are present, no preliminary preparation is required except for deproteinisation (*e.g.*, with trichloro-acetic acid) when necessary. Polysaccharides and combined sugars are hydrolysed with *N* hydrochloric acid at 100° C for 4 to 6 hours in a sealed tube.

REAGENT—

Orcinol—Traces of impurities are liable to yield derivatives similar to, but more deeply coloured than, those of orcinol itself. The blank does not provide full correction, and if the colour value much exceeds the normal range the linear relationship between carbohydrate present and colour produced no longer holds. The orcinol must therefore be freshly recrystallised from hot water with a little charcoal. This yields the monohydrate, which should be converted to the anhydrous material by drying in a vacuum-desiccator. If orcinol containing water is used for making the orcinol - sulphuric acid reagent a high blank may result.

ORCINOL - SULPHURIC ACID REACTION[2]—

Dissolve dry orcinol (0·2 g) in 100 ml of diluted sulphuric acid (2 + 1) *without heating*. The reagent is stable for 2 days at 0° C, but must be discarded if it shows any discoloration. This is particularly likely to happen if freshly-recrystallised orcinol is not used. Add 10 ml of the reagent to 1 ml of the test sample (containing 5 to 25 μg of carbohydrate) in a 6 × $\frac{5}{8}$-inch test tube. After mixing, cover the mouth of the tube with a glass bulb, heat the tube for 15 minutes at 100° C and cool rapidly to room temperature. Read the colour after 30 minutes, either in an "EEL" colorimeter with a 621 (dark blue) gelatin filter* or in a

* Transmission maximum at 450 mμ.

spectrophotometer at 425 mμ. The colour is stable for 6 hours. Make a blank determination and correct the absorption value found. If the blank value is abnormally high it is a sign of impurities in the reagent and the results should be rejected.

ORCINOL - FERRIC CHLORIDE REACTION[3]—

Dissolve 0·15 g of ferric chloride ($FeCl_3.6H_2O$) and 0·4 g of orcinol in 10 ml of cold water and make up to 200 ml with 30 per cent. w/v hydrochloric acid. Add 6 ml of this

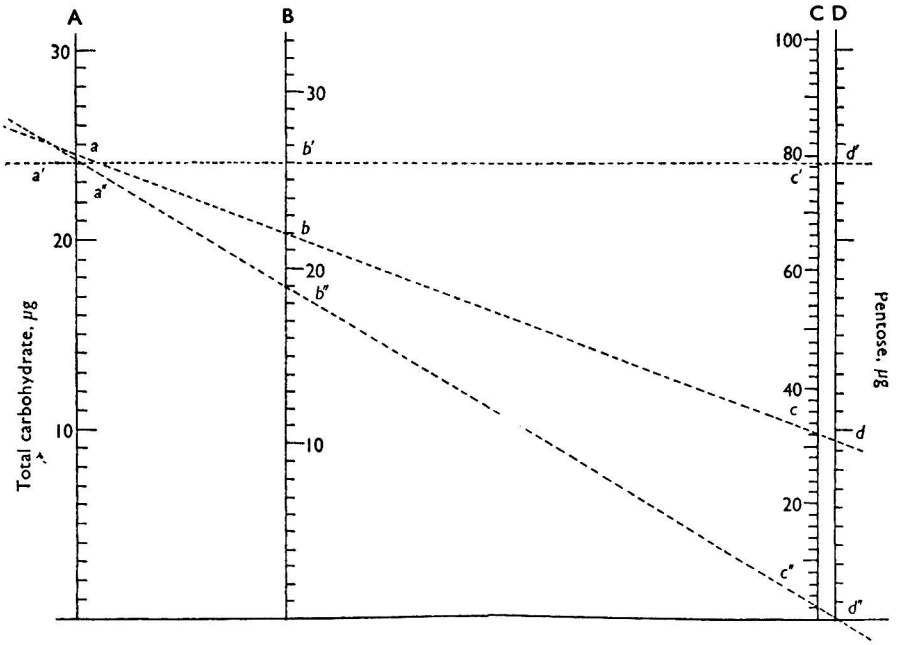

Fig. 3. Nomogram for calculating quantities of pentose and hexose from colorimeter readings. A, total carbohydrate (pentose plus hexose), μg per ml; B, colorimeter readings, orcinol-sulphuric acid method; C, colorimeter readings, orcinol-ferric chloride method; D, pentose, μg per ml

reagent to 2 ml of the sample (containing 5 to 25 μg of carbohydrate per ml) in a 6 × $\frac{5}{8}$-inch test tube. After mixing, cover the mouth of the tube with a glass bulb, heat the tube at 100° C for 20 minutes, and then cool rapidly to room temperature. Read the colour after 30 minutes either in an "EEL" colorimeter with a 608 (red) filter* or in a spectrophotometer at 660 mμ. The colour is stable for 2 hours. Make a blank determination and correct the zero setting of the colorimeter.

PRECAUTIONS—

Careful attention must be paid to the following points—

(1) The test solutions must not be diluted until shortly before the determination, or they may suffer appreciable decomposition.
(2) A large water-bath should be used, as time and temperature of heating are critical.
(3) The test tubes must be covered with loose glass bulbs or stoppers during heating so that the sulphuric acid is not diluted by absorption of steam or the hydrochloric acid by loss of hydrogen chloride.

CALCULATION OF RESULTS—

The amounts of hexose and pentose present can be calculated from the colorimeter readings by means of a nomogram (Fig. 3). The scales B and C correspond to the colorimeter readings for the acid-orcinol and the acid-orcinol - ferric chloride reactions, respectively. A line, *e.g.*, *bc*, is drawn connecting the two readings for the test sample. This line is extended

* Absorbs all wavelengths shorter than about 640 mμ.

at both ends to cut the other two scales at *a* and *d*. The total carbohydrate present (hexose plus pentose) is given at *a*, and *d* gives the pentose. The hexose is obtained by difference. The nomogram assumes that the hexose present is glucose. If a large proportion of fructose, (or sucrose) is present a substantial error will be introduced. If fructose is known to be the principle hexose present a special nomogram can be constructed.

Construction of the nomogram—Scales B and C are drawn to a ratio of 1 to 3, this being the ratio of colorimeter readings when the same amount of pentose is subjected to the acid-orcinol and acid-orcinol - ferric chloride reactions, respectively. Scales A and D are identical and give the concentrations of pentose (μg per ml) corresponding to the readings on either scale B or C. The distance between the four scales is such that AD/CD is the ratio of the

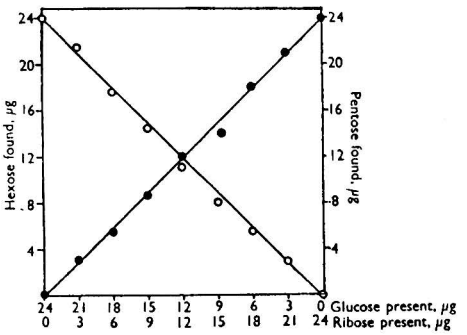

Fig. 4. Simultaneous estimation of pentose and hexose. Solutions were prepared containing glucose and ribose in various proportions. The total amount of sugar was always the same *viz.*, 24 μg per ml. The two sugars were estimated. Dots, pentose found; circles, hexose found. The two lines represent the theoretical readings

sensitivity for pentose to hexose in the acid-orcinol - ferric chloride method, and AD/BD in the acid-orcinol method. These ratios vary slightly with the filters and instruments used and each worker should construct his own nomogram. Although Fig. 3 applies to determinations carried out with an "EEL" colorimeter the same principles are used in constructing the nomogram for any other type of instrument, *e.g.*, a Beckmann spectrophotometer.

Results

Table I shows the colorimeter readings with solutions containing 24 μg per ml of various sugars and treated with the two reagents as described above. The disaccharides were not hydrolysed before estimation. Ribonucleic acid (yeast) recorded 40 per cent. of the theoretical amount of pentose before hydrolysis and slightly more after hydrolysis. This is in accordance with expectation, since the pyrimidine nucleotides resist hydrolysis.[5] Deoxyribosenucleic acid (thymus) gave no colour with either reagent.

In Fig. 3, *abcd* is the experimental line for a mixture containing 9 μg of ribose and 15 μg per ml; $a'b'c'd'$ for 24 μg per ml of ribose only, and $a''b''c''d''$ for 24 μg per ml of glucose only. Fig. 4 shows the concentrations of hexose and pentose found in a series of solutions containing various amounts of glucose and ribose.

We are grateful to Professor R. A. Morton, F.R.S., for his interest in this work, which was performed during the tenure by one of us (W. R. F.) of a grant from the Ministry of Education under the Further Education and Training Scheme.

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June 13th, 1952

The Control of Anticoagulant Therapy

By ROSEMARY BIGGS

(*Presented at the meeting of the Biological Methods Group on Friday, March 14th, 1952*)

The one-stage prothrombin test is the best available method for the control of anticoagulant therapy. This test does not necessarily measure prothrombin; its results are affected by the presence of factors V and VII. Patients treated with the dicoumarin derivative tromexan lack factor VII, and it is this deficiency that controls the results of the test in these patients. A method that probably gives a true measure of prothrombin is described; the results of this test confirm the belief that the amount of prothrombin is only slightly reduced by anticoagulant therapy with the dicoumarin group of drugs. This method is of no value in the control of anticoagulant therapy.

Dicoumarin and its derivative tromexan have an anticoagulant effect on the blood by interfering with the normal coagulation mechanism. To measure the anticoagulant effect of the dicoumarin group of drugs it is necessary to measure the extent of the clotting abnormality. In practice this problem is not difficult. It has been found that the so-called one-stage prothrombin test gives a measure of the abnormality and that if the anticoagulant is administered in doses sufficient to maintain the clotting time by this test within certain fairly definite limits, haemorrhage from overdosage is unlikely and it is probable that the anticoagulant has a therapeutic effect. This problem is technical and much has already been written on the subject, so this paper is restricted to a consideration of the reasons why the one-stage test is a reliable guide to therapy with dicoumarin.

In work on blood coagulation, theory has always been important. The process of clotting, although superficially simple, is complex. A chain of preliminary reactions precedes the appearance of fibrin. Clotting can be studied only by observing fibrin formation; hence information about the earlier stages of the process is derived from indirect evidence. Because of this difficulty it has become customary to observe some phenomenon associated with coagulation and then to deduce the existence of a coagulation "factor" to account for the observation. In this way a great many factors have been proposed. When proposed, most of these factors have little claim to reality. As they appear the factors are ignored, adversely criticised, or studied by opponents; and gradually, with the multiplication of experiments, a few factors emerge as being more probable than others. These more probable factors gradually become accepted because the hypothesis of their existence explains the observations of several groups of workers. Naturally these several groups of workers have themselves given names to the factors, so that even the most acceptable factors proposed in the past 50 years are known by many different names. It is against this mobile background of elusive hypothetical substances that an attempt is made to measure coagulation defects in terms of isolated substances.

Prothrombin, the substance long thought to be deficient in the plasma of patients treated with dicoumarin, is a widely recognised factor. It has survived more than 50 years of experiments and it now has only one name in common usage. Prothrombin is a substance occurring in plasma, and although not itself a coagulant of fibrinogen, it can, in suitable circumstances, be converted into a coagulant, thrombin. This conversion is greatly accelerated by tissue extracts called thromboplastins. According to the classical theory of blood coagulation, which was generally accepted during the first 40 years of this century, the reactions of blood could be written—

$$\text{Prothrombin} \xrightarrow[\text{Calcium}]{\text{Thromboplastin}} \text{Thrombin}$$

$$\text{Fibrinogen} \xrightarrow{\text{Thrombin}} \text{Fibrin}$$

The last of these two reactions, the thrombin - fibrinogen reaction, has been studied in detail, and it is now well established that when thrombin is added to fibrinogen, the clotting

time of the fibrinogen is inversely proportional to the thrombin concentration. Hence the clotting time of fibrinogen can be used as a measure of thrombin. If the classical theory of blood clotting is accepted and knowledge of the thrombin - fibrinogen reaction is applied, it becomes possible to devise methods for measuring prothrombin. These methods have proved useful, but they have given rise to endless practical and theoretical difficulties. They are known as the one-stage and the two-stage prothrombin tests.

THE ONE-STAGE "PROTHROMBIN" TEST—

The one-stage test consists in adding 0·1 ml of tissue extract to 0·1 ml of citrated plasma and then adding 0·1 ml of calcium chloride. The clotting time of the mixture is recorded. According to theory, the speed of thrombin formation in the mixture depends on the amount of prothrombin present, and the speed of clotting will depend on the speed of thrombin formation and the amount of thrombin formed. Thus, the clotting time is a measure of prothrombin. Even if this theory is accepted, there are some obvious disadvantages in the method. Thromboplastin preparations do not all behave in exactly the same way. Conditions such as the presence of heparin or reduction of fibrinogen below 100 mg per 100 g will influence the clotting time regardless of the prothrombin concentration. Moreover, endless difficulty has arisen over the expression of results. A common method is to test dilutions of plasma by the one-stage test and draw a dilution curve relating clotting time to so-called concentration of prothrombin. When this is done it is found that the shape of the curve depends greatly on the diluent used. In addition to this method of expressing the results, there is a second method in which the clotting times are expressed as a percentage. This method bears no relation to the previous method, but in written communications the two methods are often not differentiated and so a great deal of unnecessary confusion has arisen. The two-stage test is more complicated and will be discussed later.

The first difficulty that arose from these methods was that the results from the two tests on any one sample of plasma seldom agreed. This in itself suggests that there is something wrong with the theory.

When these tests were being developed a haemorrhagic disease of Canadian cattle was investigated and was found to be caused by the cattle eating spoiled sweet-clover hay. By a series of brilliant experiments (summarised by Link[1] in 1944) the haemorrhagic agent dicoumarin was isolated, and it was found that haemorrhage was due to reduction in prothrombin as measured by both one-stage and two-stage tests. Dicoumarin has been, and is, used widely as a therapeutic agent in various thrombosing diseases, and most workers believe that the plasma of patients treated with dicoumarin lacks prothrombin. This belief rested primarily on an acceptance of the classical theory of blood clotting, which is now known to be incomplete. There are at least two other factors that must be included in the theory, which should now probably be written[2]—

$$\text{Tissue extract} + \text{Factor VII} \xrightarrow{\text{Calcium}} \text{Thromboplastin}$$

$$\text{Prothrombin} + \text{Factor V} \xrightarrow[\text{Calcium}]{\text{Thromboplastin}} \text{Thrombin}$$

$$\text{Fibrinogen} \xrightarrow{\text{Thrombin}} \text{Fibrin}$$

Factors V and VII are essentially accelerators of blood clotting that affect the speed of thrombin formation, and a deficiency in either factor will influence the time for the one-stage prothrombin test. Factor V is a generally accepted factor but still has a number of alternative names, such as accelerator globulin,[3] prothrombin accelerator[4] and accelerin.[5] Factor VII[6] is not at present generally accepted, but it appears to be an essential factor and its existence will account for observations of various authors who postulate the following substances: co-thromboplastin,[7] serum prothrombin conversion accelerator[8] and convertin.[5] A fuller discussion of this problem has been given elsewhere.[2]

Fortunately there are simple tests for deficiencies of Factors V and VII. Plasma treated with aluminium hydroxide, barium sulphate or calcium phosphate lacks prothrombin and Factor VII but contains Factor V. Thus, if a plasma sample has a long clotting time by the one-stage method and if the clotting time is greatly shortened by the addition of

10 per cent. of plasma that has been treated with aluminium hydroxide, then Factor V is deficient in the plasma sample.

Normal serum contains Factor VII in large amounts, small amounts of Factor V and little prothrombin. If a plasma sample has a long clotting time by the one-stage test and this time is *not* shortened by adding 10 per cent. of plasma treated with aluminium hydroxide but *is* shortened by adding 10 per cent. of normal serum, then the plasma sample lacks Factor VII.

By these tests it is found that the plasma of patients treated with tromexan lacks Factor VII. The one-stage clotting time is not reduced by the presence of 10 per cent. of plasma treated with aluminium hydroxide, but is reduced, often to normal, by 10 per cent. of normal serum.

THE MEASUREMENT OF PROTHROMBIN—

This finding throws doubt on the usual conception that the plasma of patients treated with tromexan lacks prothrombin. To decide whether or not a sample lacks prothrombin it is necessary to have a reliable measure of prothrombin. If the one-stage test is not a reliable measure of prothrombin, the question arises whether the two-stage test is more so.

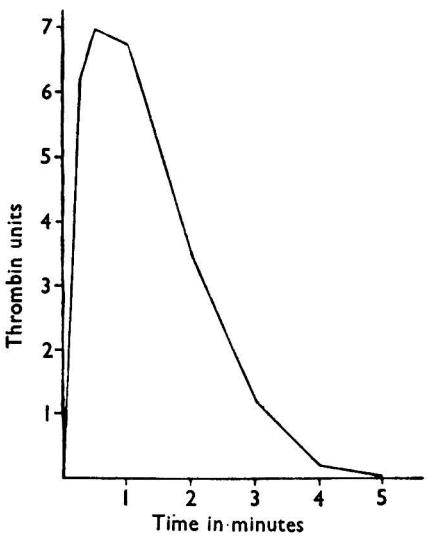

Fig. 1. The formation of thrombin and its neutralisation in normal plasma by the two-stage method

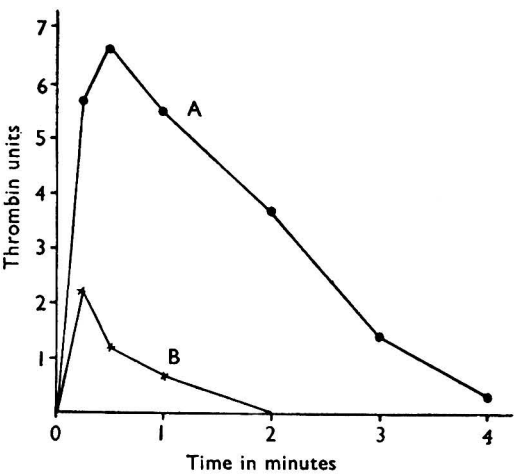

Fig. 2. The formation of thrombin in a normal and a prothrombin deficient patient's plasma tested by the two-stage method. Curve A, normal; curve B, prothrombin deficient

In the two-stage test, plasma and tissue extract are mixed and calcium chloride is added. At intervals, samples are removed from this incubation mixture, which is forming thrombin, and added to fibrinogen. The clotting times of the fibrinogen solutions give a measure of the amount of thrombin present at the time the sample was removed. In this way the progress of thrombin formation is recorded. From Fig. 1 it will be seen that thrombin formation proceeds rapidly until a maximum is reached and then the level of thrombin declines. The level of thrombin is not maintained, because thrombin in plasma is neutralised by an inhibitor, antithrombin.

The usual method of calculating a relative percentage of prothrombin by the two-stage method is to compare the maximum level of thrombin formed in the pathological plasma with that in a normal plasma and to express the result as a percentage. In the example shown in Fig. 2 the patient would be said to have about 33 per cent. of prothrombin. Another example shown in Fig. 3 will suggest that this method has disadvantages. In this illustration two examples of pathological plasma are compared. One curve is derived from the plasma of a patient who had uncomplicated prothrombin deficiency and the other is from the plasma of a patient treated with tromexan. The curve for normal plasma is not shown in the figure,

but from the levels of thrombin reached, the two plasma samples would be said to contain about the same amount of prothrombin. Yet it is clear that the plasma of the patient treated with tromexan had a much greater ability to form thrombin than that of the prothrombin-deficient patient. If it is taken that prothrombin is the precursor of thrombin, then the plasma of the patient treated with tromexan must have contained more prothrombin than the plasma of the other patient. It would seem that the duration of thrombin formation should be taken into account in assessing the amount of prothrombin present in the plasma.

The essential problem of this technique is that the level of thrombin is not maintained because thrombin is neutralised by antithrombin. Most workers have assumed that, for this reason, the test should be carried out on greatly diluted plasma because the effects of antithrombin are less obvious in diluted plasma. But although the effects of antithrombin are less obvious in diluted plasma, they are still present.[2,9] Moreover, some pathological

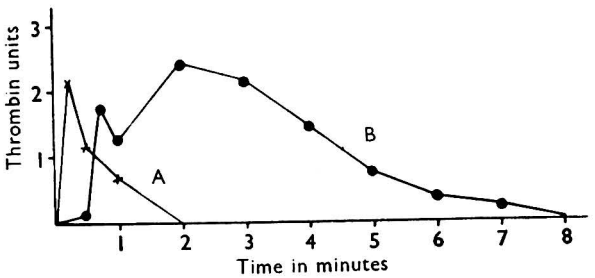

Fig. 3. The formation of thrombin in a prothrombin deficient patient's plasma (curve A) and in the plasma of a patient treated with tromexan (curve B)

plasma samples cannot be diluted because the clotting times become unduly long. If the problem of antithrombin in the two-stage method has to be taken into account, it is advisable to use undiluted plasma, because in undiluted plasma the effects of antithrombin can be studied in a few minutes, whereas in greatly diluted plasma the study would be very time-consuming.

From the curves shown in Fig. 3 it seems that a reasonable method of assessing the amount of prothrombin in these two plasma samples might be to compute the area beneath the curves as a measure of prothrombin. But it was difficult to know whether or not this procedure was likely to give reliable results. A method of testing the hypothesis was to study some theoretical system similar in general pattern to that of the thrombin generation system in plasma.

Suppose that a substance A is converted to B by a first-order reaction and that as B is formed it is converted to C, also by a first-order reaction; then the reaction is expressed in the curves of Fig. 4*a*. In the blood coagulation system A would be prothrombin, B thrombin, and C a neutralised thrombin - antithrombin association. In the two-stage test it is B that is measured, but the test must be designed to give a relative measure of A. The problem is how best to measure B. The factors that influence the shape of curve B are the amount of A present and the speeds of formation and destruction of B.

If the speeds of formation and destruction of B are constant but the amount of A varies, then the curves shown in Fig. 4*b* will result. A study of these curves shows that the level of B achieved and the areas enclosed by the curves would give a proportional measure of A.

If the amount of A is constant and the speed of destruction of B is constant but the speed of formation of B is varied, then the curves of Fig. 4*c* result. From these curves it is obvious that the levels of B achieved are now no guide to the amount of A present, but it is found that the areas enclosed by the curves are still proportional to the amount of A present.

If the amount of A is constant and the speed of formation of B is constant but the speed of destruction of B varies, then neither the levels of B achieved nor the areas enclosed by the curves can give a measure of A (Fig. 4*d*).

On applying these results to the coagulation system it appears that the measurement of the area enclosed by the curves will give a relative measure of prothrombin provided that the antithrombin activity is constant. It is important to know how closely the reactions of coagulation conform to the theoretical system and apparently the agreement is good.

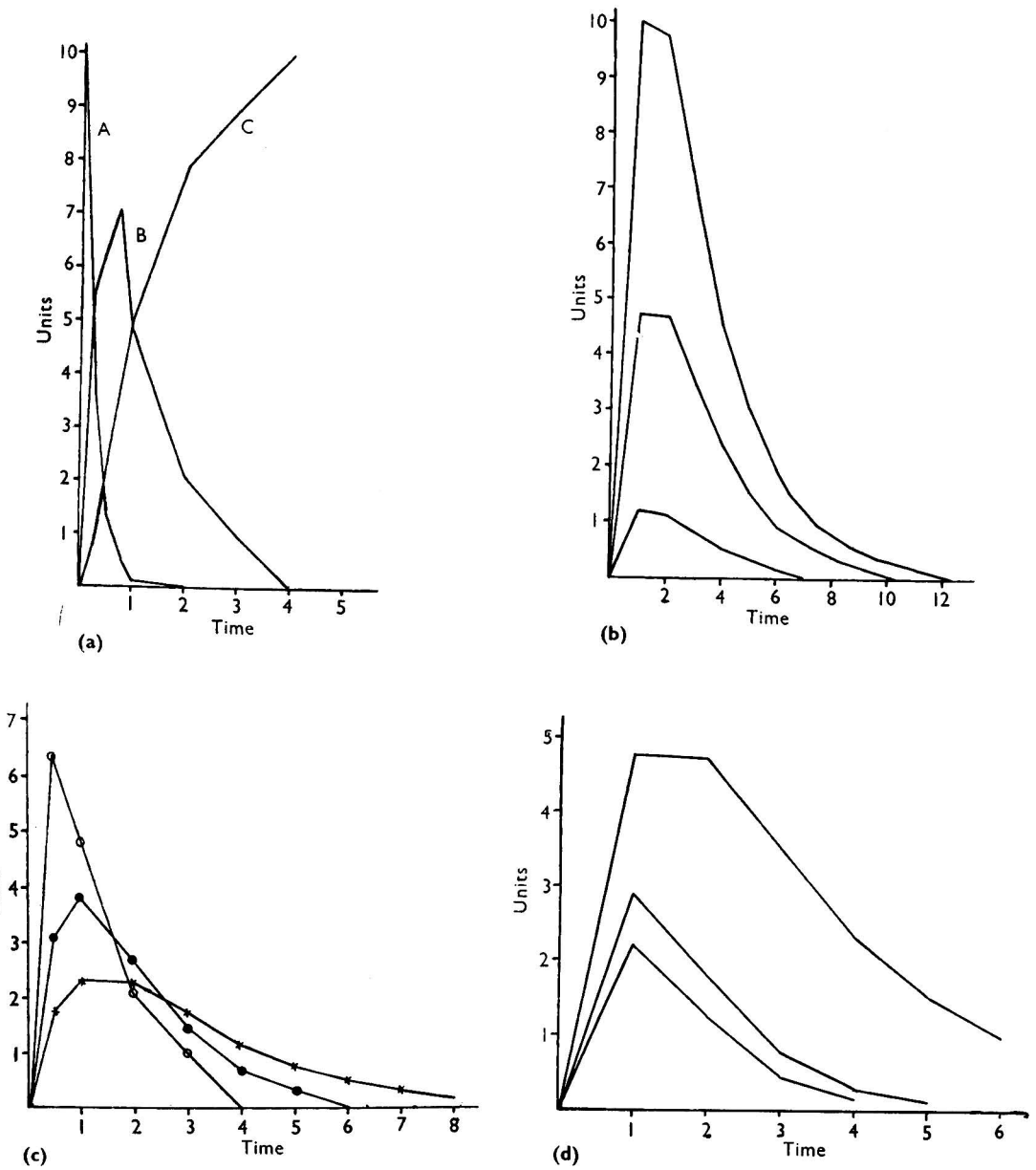

Fig. 4. Calculated curves for a theoretical reaction in which substance A is converted to B by a first-order reaction and simultaneously B is converted to C.

(*a*) Curves to show the disappearance of A and the formation of B and C.
(*b*) The concentrations of B at different times when formed from three different initial concentrations of A.
(*c*) The concentrations of B at different times assuming three different speeds of formation of B. The initial concentration of A and the speed of neutralisation of B are constant.
(*d*) Curves to show the effect on the concentration of B of varying the speed of conversion of B to C, the initial amount of A and the rate of formation of B being constant

In Fig. 5 the average curve for 15 normal subjects is compared with a theoretical curve. In Fig. 6 curves obtained by varying the amount of prothrombin are compared with theoretical curves. In Fig. 7 curves in which the speed of thrombin formation varies are compared with theoretical curves.

One more test can be applied to this method. The prothrombin content of normal and prothrombin-deficient plasma can be measured by the method and the prothrombin content of mixtures of the two can be measured. The theoretical prothrombin content of

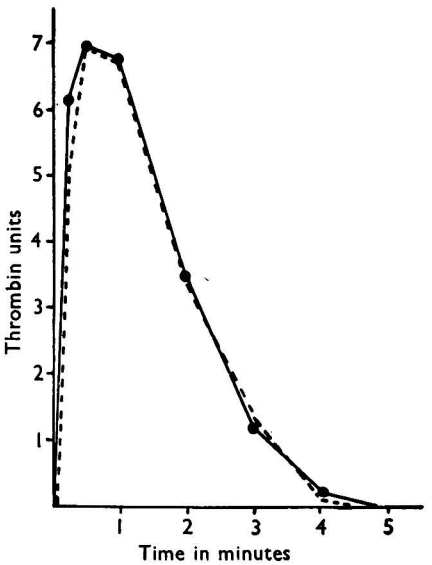

Fig. 5. The formation of thrombin in normal plasma tested by the two-stage method (continuous line) compared with a theoretical curve (broken line)

the mixtures can be calculated from a knowledge of the prothrombin in the normal and abnormal plasma samples. When this is done it is found that there is a reasonable agreement between the theoretical and observed areas (Fig. 8).

It is clear that the general pattern of the formation of thrombin agrees with the theoretical system. It is true that apparent agreements of this sort have no great significance and certainly do not mean that the reactions of blood clotting can be explained in terms of this simple pattern. But the deduction made from the agreement is empirical: it is suggested that in a system that follows this general pattern the measurement of area is likely to give a reasonable proportional measure of prothrombin. Provided that the experimental conditions are rigidly uniform, it is probable that this method of measuring prothrombin is more generally applicable than any other.

If this method of measuring prothrombin is applied to the plasma of patients undergoing treatment with tromexan, it is found that prothrombin is not greatly reduced, and in no instance has less than 50 per cent. of prothrombin been found.[10] There was no correlation between the results of the one-stage and two-stage tests. This finding is not surprising, as the one-stage test does not record a change in clotting time for the reduction of a clotting factor by 50 per cent. Thus the reduction in prothrombin would be unlikely to influence the one-stage test. Moreover, the one-stage clotting time is shortened nearly to normal by 10 per cent. of normal serum, and from this evidence alone the coagulation defect cannot be attributed to prothrombin deficiency, because serum diluted to one-tenth of its normal strength provides insignificant amounts of prothrombin.

From this work it appears that the main abnormality in the plasma of patients treated with tromexan is a deficiency of Factor VII. This deficiency is measured by the one-stage method, which, consequently, is useful. It may be thought that an attempt to define the

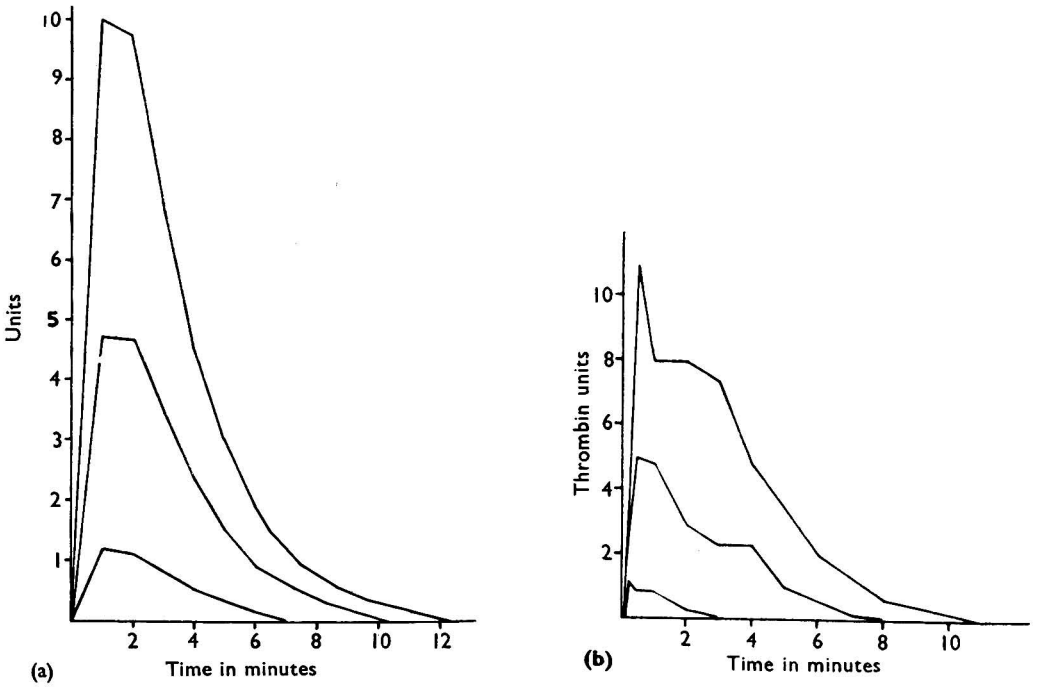

Fig. 6. Experimental observations in which the amount of prothrombin in plasma was varied compared with the theoretical curves

(*a*) Theoretical curves
(*b*) Experimental results. Different amounts of prothrombin prepared by adsorption were added to plasma of a patient with uncomplicated prothrombin deficiency and thrombin formation was followed by the two-stage method

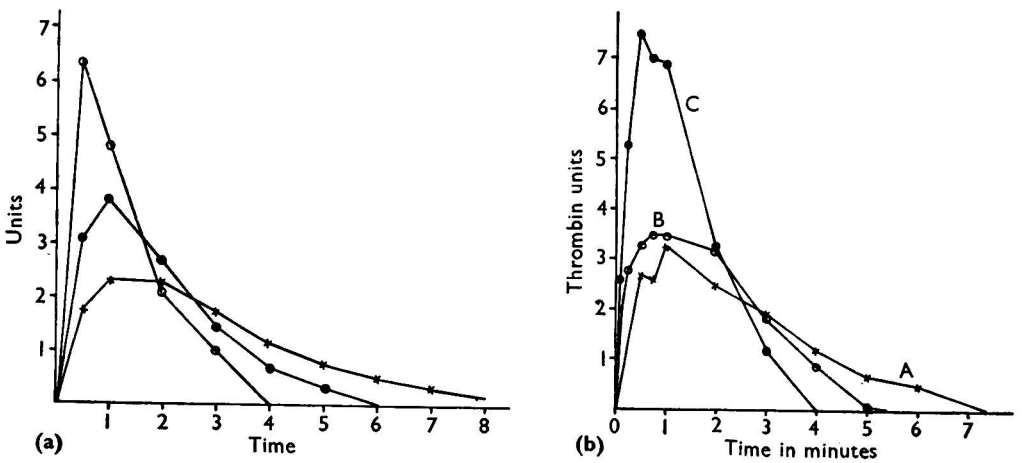

Fig. 7. Experimental observations in which the speed of thrombin formation was varied. Comparison with the theoretical curves

(*a*) Theoretical curves
(*b*) Experimental results. Curve A, thrombin formation in tromexan plasma; curve B, thrombin formation in tromexan plasma to which 10 per cent. of normal serum was added; curve C, thrombin formation in normal plasma

deficiency in the plasma of patients treated with tromexan is unnecessary because empirically the one-stage test has been found to be adequate. But although the one-stage test, carefully standardised, is valuable, much difficulty has arisen in practice from modifications of the technique and from deductions based on the theory that the test gives a measure of

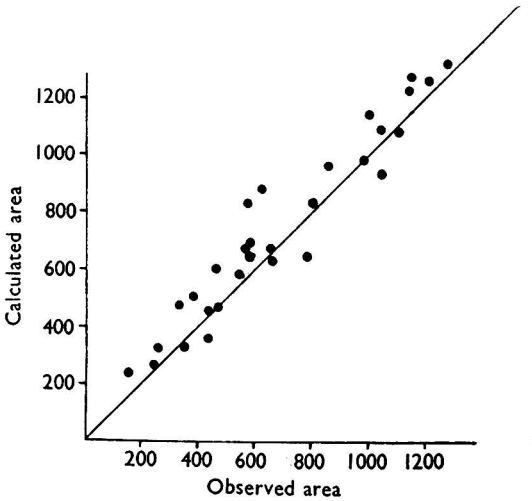

Fig. 8. The area enclosed by the two-stage curves was measured in normal plasma, abnormal plasma and in mixtures of the two in known proportions. The observed area in the mixtures is compared with the area calculated for the proportions of normal and abnormal plasma mixed

prothrombin. A clearer conception of the significance of the results of the test in particular coagulation defects may, in the end, help in the understanding of the normal coagulation mechanism.

The experimental work on which this paper was based was carried out in collaboration with Dr. A. S. Douglas and will be reported fully elsewhere.[11] All the experimental techniques used are described in detail by Biggs and Macfarlane.[2]

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June 6th, 1952

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Discussion

Mr. K. L. Smith suggested that the areas enclosed by the curves might be computed approximately as the product of the mean of all the points multiplied by the length of base.

Dr. H. O. J. Collier enquired about the use of the test in practice and asked how the results were related to medical findings.

DR. BIGGS said that a start had been made in the routine use of the test, but not enough work had yet been done to say how the results would compare with clinical findings. In reply to a question by Mr. A. L. Bacharach she said that Factor V was an additional factor rather than an intermediate product in the formation of thrombin.

DR. G. E. FOSTER enquired whether the findings were the same with animal as with human plasma.

DR. BIGGS replied that she had no experience with ox plasma, but she preferred to use human plasma, which was, for her, more important. As a source of thromboplastin she used human rather than rabbit brain.

DR. R. M. HARDISTY asked if Dr. Biggs' "Factor VII" was the same as "Factor VII" of Koller and the "proconvertin" of Owren. If so, what was the evidence for including it in the first stage of the coagulation process as combining with brain extract to produce thromboplastin.

DR. BIGGS, replying, referred to the work of R. F. Jacox (*J. Clin. Invest.*, 1949, **28**, 492) and of Owren (reference 5 of paper), and said that Factor VII was essentially an accelerator. She mentioned also a recent paper by F. D. Mann and M. H. Hurn (*Proc. Soc. Exp. Biol. N.Y.*, 1952, **79**, 19) dealing with species specificity of thromboplastin.

DR. E. BRASTED enquired whether the one-stage prothrombin test could be used as a measure of fibrinogen.

DR. BIGGS said that the one-stage prothrombin test could be used as a measure of fibrinogen deficiency, but it would give a very inaccurate and unreliable measure of fibrinogen; other and much better methods for measuring fibrinogen were in routine use.

DR. L. ELLIS asked whether it was possible to measure prothrombin in terms of an independent prothrombin rather than as a percentage of normal human prothrombin content.

DR. BIGGS said that prothrombin could not be isolated from plasma in a form freed from all other coagulation factors; an absolute measure of prothrombin was therefore impossible.

The Detection and Determination of Ultra-Violet Absorbers and Other Additives in Polymethyl Methacrylate and Methyl Methacrylate - Ethyl Acrylate Co-Polymers

BY J. HASLAM, S. GROSSMAN, D. C. M. SQUIRRELL AND S. F. LOVEDAY

Chemical and spectroscopic methods are described for the detection and determination of small amounts, 1 per cent. or less, of additives in polymethyl methacrylate and methyl methacrylate - ethyl acrylate co-polymers. These methods deal with the determination of lauryl mercaptan and total sulphur in such polymers, as well as the qualitative and quantitative detection and determination, both chemically and spectroscopically, of such ultra-violet absorbers as methyl salicylate, phenyl salicylate, 2:4-dihydroxybenzophenone, stilbene and resorcinol monobenzoate. Details are given of a direct spectroscopic method for the determination of small amounts of dibutyl phthalate, and observations are made on the behaviour of cresyl esters and hydroquinone and catechol monobenzoates in the appropriate tests.

WITHIN recent months it has become necessary to detect and determine small amounts of additives used in the preparation of polymethyl methacrylate and in co-polymers of methyl methacrylate with other monomers. It is proposed to describe here some of the methods that have been found most useful in connection with this work. The additives, which may be included either in the course of, or at the conclusion of, the polymerisation, are usually only present at small concentrations (less than 1 per cent.). Although there are many of these additives, it is proposed to describe the detection and determination only of those that have been encountered in day-to-day work in the plastics industry.

The particular substances dealt with are: dibutyl phthalate, lauryl mercaptan, methyl salicylate, phenyl salicylate, 2:4-dihydroxybenzophenone, stilbene and resorcinol monobenzoate. Lauryl mercaptan, it is understood, is an extremely useful substance because its

addition to a polymethyl methacrylate polymer slows down the depolymerisation that occurs on heating, and polymers incorporating this substance have, in general, a much higher softening point than those from which the mercaptan is absent. Phenyl salicylate, methyl salicylate, 2:4-dihydroxybenzophenone, stilbene and resorcinol monobenzoate can

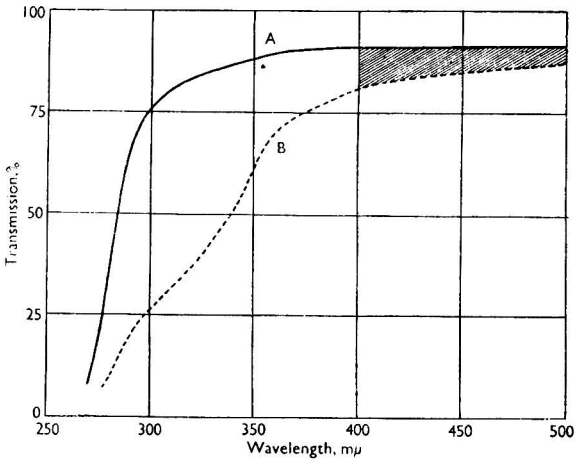

Fig. 1. Transmission curve of $\frac{1}{8}$-inch sheet polymethyl methacrylate. Curve A, before exposure; curve B, after exposure

be added to an acrylic polymer as ultra-violet absorbers. Their inclusion in the polymer sheet tends to reduce the "yellowing" that may occur on exposure to sunlight. This phenomenon may be more readily understood by reference to the ultra-violet exposure test diagrams shown in Figs. 1, 2 and 3, which show, as examples, the transmission curves of $\frac{1}{8}$-inch sheets of polymethyl methacrylate (*a*) unplasticised, (*b*) containing 1·0 per cent. of

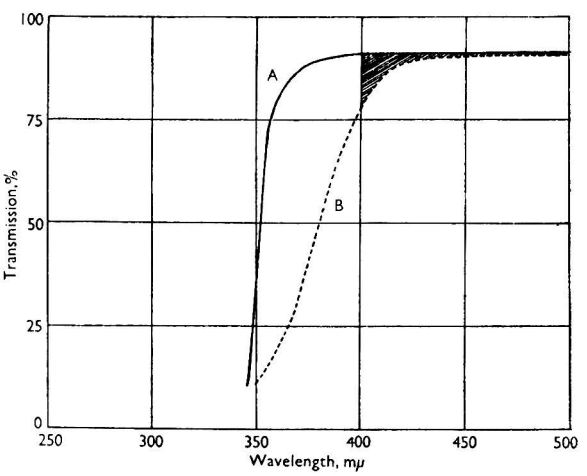

Fig. 2. Transmission curve of $\frac{1}{8}$-inch sheet of polymethyl methacrylate containing 1·0 per cent. of phenyl salicylate. Curve A, before exposure; curve B, after exposure

phenyl salicylate and (*c*) containing 0·44 per cent. of stilbene, before and after exposure to ultra-violet radiation.

The samples were exposed to ultra-violet radiation under fixed conditions for 50 hours, *i.e.*, the equivalent of 50 hours of mid-summer sunshine in Great Britain.

A comparison of the relative colour formation or "yellowing" of the samples after exposure, assessed in the visible region between the wavelengths 400 and 500 mμ and indicated by the shaded portions in the diagrams, is as follows—

Unplasticised	Phenyl salicylate, 1·0%	Stilbene, 0·44%
8	2·5	1

This, apart from showing the effect of the inclusion of an ultra-violet absorber in polymethyl methacrylate sheet, clearly demonstrates the difference in efficiency between the two absorbers used for the tests.

THE DETERMINATION OF MERCAPTANS AND TOTAL SULPHUR IN POLYMERS OF THE POLYMETHYL METHACRYLATE TYPE

The determination of mercaptans and total sulphur in polymethyl methacrylate-type polymers forms rather a different problem from that of the determination of dibutyl phthalate and ultra-violet absorbers, and will be dealt with separately.

Lauryl mercaptan in polymethyl methacrylate and methyl methacrylate co-polymers can be determined by a modification of the method originally developed by Kolthoff and

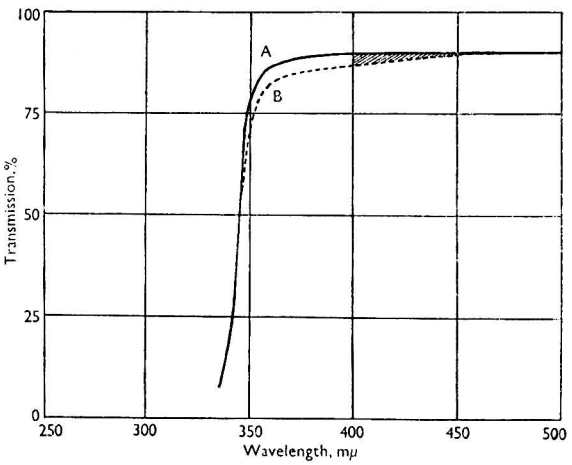

Fig. 3. Transmission curve of $\frac{1}{8}$-inch sheet polymethyl methacrylate containing 0·44 per cent. of stilbene. Curve A, before exposure; curve B, after exposure

Harris[1] for the amperometric titration of mercaptans. In the test the polymer is dissolved directly in acetone; ammonium hydroxide and ammonium nitrate are added and the mercaptan is titrated amperometrically with standard silver solution. Full details of the test are given below.

THE DETERMINATION OF MERCAPTANS

APPARATUS—

The apparatus is similar to that of Kolthoff and Harris,[1] namely, a rotating platinum-wire indicator electrode, and a reference half-cell containing mercury in contact with a potassium iodide, mercuric iodide and potassium chloride solution, connected through the test solution by means of a salt-bridge and short-circuited through a micro-ammeter.

REAGENTS—

Acetone—Analytical reagent quality.
Ammonium hydroxide—A 5 *M* solution.
Ammonium nitrate—A *M* solution.
Silver nitrate—A 0·005 *N* standard solution.

PROCEDURE—

Accurately weigh 2 g of the sample into a 250-ml beaker and stir magnetically with 100 ml of acetone until solution is complete (this usually takes about 1 hour). Add 5 ml of the ammonium hydroxide solution and then 5 ml of the ammonium nitrate solution and stir for a further 15 to 20 minutes, during which time any polymer precipitated by the addition of the aqueous solutions redissolves.

Transfer the beaker and its contents to the titration apparatus and titrate the mercaptan in the solution amperometrically with the 0·005 *N* silver nitrate solution by the general procedure used by Kolthoff and Harris.[1]

The accuracy of the test is adduced in Table I from results of experiments that involved the amperometric titration of 100-ml portions of (*a*) solutions of known amounts of lauryl mercaptan in acetone, (*b*) solutions of known amounts of lauryl mercaptan in a mixture of equal volumes of acetone and alcohol and (*c*) solutions containing, in addition to known amounts of lauryl mercaptan, 2 g of dissolved polymethyl methacrylate.

TABLE I

DETERMINATION OF LAURYL MERCAPTAN

Medium	Lauryl mercaptan added, mg	Lauryl mercaptan found, mg
Acetone	4·83	4·82
	9·66	9·67
Ethyl alcohol - acetone mixture (1 + 1)	4·83	4·96
	9·66	9·77
	(High results owing to small blank on alcohol)	
Acetone containing 2 per cent. of dissolved polymer	0·00	0·00
	4·83	4·82
	4·83	4·86

It may often be desirable to determine the total sulphur content of the polymer sample, for although a certain amount of a substance such as lauryl mercaptan may have been added initially in the preparation of the polymer, only a part of this may exist as lauryl mercaptan in the final product. Again such substances as thio-ethers may have been used and these would not respond to the amperometric test for mercaptans. For these reasons it is desirable to have available a method for determining the total sulphur content (0 to 0·3 per cent.) in these polymer preparations; the following method has been found suitable.

THE DETERMINATION OF TOTAL SULPHUR

APPARATUS—

A Mahler - Cook bomb was used for combustion of the sample in a rapid and quantitative way; this forms part of the Mahler - Cook New Quick-seal bomb calorimeter ordinarily used for the determination of the calorific value of coal.*

REAGENTS—

Hydrogen peroxide—A 5-volume solution.

Sulphuric acid—A 0·005 *N* solution.

Hydrochloric acid—A *N* solution.

Precipitating reagent—Solution A. Dissolve 0·2 g of peptone in 50 ml of 1 per cent. barium chloride ($BaCl_2.2H_2O$) solution. Buffer to a pH of 5·0 with 0·02 *N* hydrochloric acid solution; add 10 g of sodium chloride (analytical grade) and dilute to 100 ml. Heat in a water-bath for 10 minutes and add a few drops of chloroform.

Solution B. Dissolve 0·4 g of ground gum ghatti in 200 ml of distilled water by warming slightly. When solution is complete add 2·0 g of barium chloride ($BaCl_2.2H_2O$).

Store solutions A and B separately and prepare the final precipitating reagent just before use by diluting 10 ml of solution A to 100 ml with solution B.

* The Mahler - Cook New Quick-seal bomb is manufactured by Messrs. Chas. W. Cook & Sons Limited, Birmingham.

PROCEDURE—

Accurately weigh 0·5 g of the sample into a glass crucible through which passes the platinum resistance wire, connected to the firing terminals. Add 10 ml of distilled water to the bomb and place the crucible and sealing cap in position. Seal the bomb and introduce oxygen to give a pressure of 25 atmospheres. Immerse the bomb in water, fire it, and after 10 minutes release the gases by bubbling them through 50 ml of the 5-volume hydrogen peroxide solution, using a sintered-glass distribution plate.

Dismantle the bomb and wash it thoroughly with distilled water. Bulk the washings and scrubbing solution together in a 250-ml beaker, add 15 ml of *N* hydrochloric acid solution and boil down to a volume of approximately 25 ml.

Filter the cooled solution into a 50-ml calibrated flask and add 5 ml of the prepared precipitating reagent; dilute to 50 ml, shake well and set aside for 30 minutes. Measure the optical density of the resulting solution in a Spekker absorptiometer using a 1-cm cell and Spectrum red 608 filters.

Prepare a calibration curve by putting known aliquots of 0·005 *N* sulphuric acid solution (2 to 16 ml cover a suitable range) through the above precipitation procedure and plot a graph relating the number of millilitres of 0·005 *N* sulphuric acid solution per 50 ml of final solution to the absorptiometer indicator drum reading. From this graph calculate the sulphur content of the test sample.

The method was tested by determining sulphur in samples of polymethyl methacrylate containing known amounts of lauryl mercaptan or dioctyl thio-ether, which had been included in the polymerisation process. The results were as shown in Table II.

TABLE II

DETERMINATION OF SULPHUR IN PREPARED SAMPLES OF POLYMETHYL METHACRYLATE

Sulphur added as lauryl mercaptan, %	Sulphur found, %	Sulphur added as lauryl mercaptan and dioctyl thio-ether, %	Sulphur found, %
nil	nil	0·12	0·13
0·06	0·05		
0·06	0·05		
0·09	0·09		

THE QUALITATIVE DETECTION OF ULTRA-VIOLET ABSORBERS AND OTHER ADDITIVES

Our experience has been principally of the ultra-violet absorbers phenyl salicylate, methyl salicylate, 2:4-dihydroxybenzophenone, stilbene and, to a lesser extent, resorcinol monobenzoate.* It has been shown, for instance, that qualitative evidence of phenyl salicylate, methyl salicylate, 2:4-dihydroxybenzophenone and resorcinol monobenzoate can be obtained by applying comparatively simple tests to the polymer or co-polymer. With each, the sample is dissolved in acetone and the polymer precipitated with alcohol and water. The polymer is filtered off and the appropriate tests applied to the acetone - alcohol - water solution containing the ultra-violet absorber. The addition of alkali to this solution and the immediate production of a yellow colour is presumptive evidence of the presence of 2:4-dihydroxybenzophenone. The tests for phenyl salicylate, methyl salicylate and resorcinol monobenzoate are based on treating the solution with a known amount of alkali, neutralising the resulting solution and applying three tests: a 2:6-dibromoquinonechlorimide test for phenols, Millon's tests for phenols and the ferric chloride test for salicylates. Phenyl salicylate, methyl salicylate and resorcinol monobenzoate behave differently in these tests, the details of which are given below.

CHEMICAL EXAMINATION

REAGENTS FOR SOLUTION OF SAMPLE—

Acetone—Analytical reagent quality.
Absolute alcohol, 99·9 per cent.

* Preliminary tests under an ultra-violet lamp often give useful pointers, particularly if samples containing known additives are available for comparison.

PREPARATION OF SAMPLE SOLUTION—

Weigh 1 g of the finely divided sample into a 150-ml conical flask, add from a pipette 10 ml of acetone, stopper, and set aside overnight to ensure complete solution. Place the flask on a magnetic stirrer and allow the glass-enclosed metal stirrer to revolve for a few minutes to homogenise the solution before adding 45 ml of absolute alcohol dropwise from a burette to precipitate the polymer. Set the solution aside for 10 minutes and then add 48 ml of distilled water from a burette in the same manner. After setting aside for a further period of 15 minutes, filter the solution through a Whatman No. 1 filter-paper and retain the filtrate.

Notes—Certain polymethyl methacrylate samples, when treated in this way, yield highly viscous solutions in acetone, which on treatment with alcohol form insoluble gels. In such circumstances it is desirable to first dissolve the polymer in a mixture of 10 ml of acetone and 10 ml of alcohol, stirring constantly to maintain mobility. The polymer is precipitated finally by adding 35 ml of absolute alcohol and 48 ml of distilled water.

The ternary mixture of 10 ml of acetone, 45 ml of absolute alcohol and 48 ml of distilled water gives a final volume of 100 ml.

REAGENTS FOR QUALITATIVE TESTS—

Sodium hydroxide solution, N.

Nitric acid solution, N.

2:6-*Dibromoquinonechlorimide solution*—Dissolve 0·1 g of 2:6-dibromoquinonechlorimide in 25 ml of 95 per cent. ethyl alcohol just before the solution is required for use.

Ferric chloride solution—A 10 per cent. w/v solution of ferric chloride ($FeCl_3.6H_2O$) in distilled water.

Millon's reagent—Dissolve 2 ml of mercury in 20 ml of analytical reagent grade concentrated nitric acid. Allow to cool and add 35 ml of distilled water; then add 10 per cent. w/v sodium hydroxide solution dropwise until a faint permanent turbidity is formed and finally 5 ml of 20 per cent. v/v nitric acid solution to clear the solution.

Borate buffer solution—Dissolve 23·4 g of sodium borate ($Na_2B_4O_7.10H_2O$) and 3·27 g of sodium hydroxide pellets in distilled water and dilute to 1 litre.

QUALITATIVE TESTS—

1. (*a*) Add 2·0 ml of *N* sodium hydroxide solution to 50 ml of the prepared solution. The immediate formation of a yellow colour, which is destroyed by the addition of 2·2 ml of *N* nitric acid solution, is indicative of 2:4-dihydroxybenzophenone.

(*b*) If no yellow colour forms immediately, bring the alkaline solution to the boil and continue to boil for 15 minutes. The formation of a yellow-green colour is indicative of resorcinol monobenzoate. (*N.B.* The formation of a pale straw-yellow colour at this stage should be ignored.)

2. If test 1 (*a*) is negative, transfer a fresh 20-ml aliquot of the prepared sample solution to a 50-ml calibrated flask containing 10 ml of distilled water. Add 4·0 ml of *N* sodium hydroxide solution and stand the flask in a thermostat maintained at 25° C for 4 hours, after which time add 4·2 ml of *N* nitric acid solution. To this faintly acid solution apply the following tests—

(*a*) To a 10-ml aliquot contained in a 50-ml Nessler cylinder, add 5 ml of Millon's reagent. A pink colour, developing after 5 minutes and becoming deeper over a period of 30 minutes, is given by phenyl esters such as phenyl salicylate.

(*b*) To a 1-ml aliquot contained in a 6 × ½-inch test tube add 2 ml of the borate buffer solution. Mix well and add 5 drops of the 2:6-dibromoquinonechlorimide reagent. In this test, resorcinol monobenzoate yields an immediate violet colouration, and compounds such as phenyl salicylate give a blue colour that develops within 5 minutes.

(*c*) To the remainder of the slightly acid solution, add 2 drops of the ferric chloride reagent, and dilute to 50 ml with distilled water. The immediate formation of a violet-coloured ferric salicylate complex is indicative of a salicylate in the hydrolysis products.

From the results of these tests the probable presence of the undernoted ultra-violet absorbers is inferred—

(*i*)	Positive reaction in test 1 (*a*)	Probably 2:4-dihydroxybenzophenone
(*ii*)	Positive reaction in test 2 (*c*) .. .. " 2 (*a*) .. .. Blue colour in test 2 (*b*)	Probably phenyl salicylate.
(*iii*)	Positive reaction in test 2 (*c*) .. .. Negative " 2 (*a*) and 2 (*b*)	Probably methyl salicylate.
(*iv*)	Negative reaction in test 2 (*c*) .. .. Immediate violet positive reaction in test 2 (*b*) .. .. Positive reaction in test 1 (*b*)	Probably resorcinol monobenzoate.

Note on test for salicylates—Under these test conditions we have found that methyl salicylate gives a more intense colour than an equal weight of phenyl salicylate, owing, no doubt, to the more complete hydrolysis of methyl salicylate by aqueous alkali.

It is always desirable to supplement this qualitative chemical information by the corresponding qualitative spectroscopic information obtained by applying the ultra-violet absorption tests detailed below.

Spectroscopic examination

When the sample submitted for analysis is in the form of clear sheet, useful information can often be obtained by a straightforward examination of the light transmission of the sheet itself. Ultra-violet absorbers have a bathochromic effect, *i.e.*, with polymethyl methacrylate sheet, they cause a shift towards the red of the straight-line vertical portion of the transmission curve. For example, experience has shown that in the examination of the light transmission of $\frac{1}{8}$-inch sheet of polymethyl methacrylate, when the wavelength at 50 per cent. transmission is greater than 310 mμ the sample contains an ultra-violet absorber. This can be seen from the data given in Table III, taken from the transmission curves shown in Figs. 1, 2 and 3.

Table III

Bathochromic effect of stilbene and phenyl salicylate on $\frac{1}{8}$-inch polymer sheet

	Wavelength for 50 per cent. transmission, mμ
Unplasticised polymethyl methacrylate	284
Polymethyl methacrylate containing 0·44 per cent. of stilbene	345
Polymethyl methacrylate containing 1·0 per cent. of phenyl salicylate	351

A plasticiser such as dibutyl phthalate exhibits an effect somewhat similar to that of ultra-violet absorbers, but to a much smaller extent.

It is always desirable, however, to carry out a general qualitative examination for additives such as dibutyl phthalate and the ultra-violet absorbers phenyl salicylate, methyl salicylate, 2:4-dihydroxybenzophenone, stilbene and resorcinol monobenzoate in the following way.

Preparation of test solution—

Weigh 0·3 to 0·4 g of sample in the form of small chips or fine drillings into a 100-ml calibrated flask, add about 60 ml of chloroform B.P. and shake at once to swell the polymer. Shake mechanically until the solution clears; 30 minutes is usually adequate. Make up to 100 ml with chloroform and shake for a few minutes until homogeneous. This constitutes the test solution.

Procedure—

Transfer a portion of this test solution to a 1-cm cell, cover with a ground-glass lid and examine spectrophotometrically in the region 265 to 340 mμ against a paired cell containing

chloroform from the same batch as that used in the preparation of the solution. It may be necessary with an unknown sample to dilute the solution at this stage.

Under the conditions of the test, solutions of polymethyl methacrylate, without additives, are relatively transparent in the region examined. The probable presence of the additives under investigation, namely, dibutyl phthalate, methyl salicylate, phenyl salicylate, 2:4-dihydroxybenzophenone, stilbene and resorcinol monobenzoate, is denoted by the characteristic absorption maxima in polymer solution shown in Table IV.

TABLE IV

ABSORPTION DATA OF CERTAIN ADDITIVES

Additive	$\lambda_{max.}$, mμ	$\lambda_{min.}$, mμ
Resorcinol monobenzoate	274	—
Dibutyl phthalate	276	—
2:4-Dihydroxybenzophenone	289, 324	313
Stilbene	299, 310	306
Methyl salicylate	308	—
Phenyl salicylate	312	—

Note—As a further check, it is current practice in this laboratory to plot the absorption spectra of the sample on transparent paper and compare this with the spectra of samples of known composition accumulated over a period of time.

THE QUANTITATIVE DETERMINATION OF ULTRA-VIOLET ABSORBERS AND OTHER ADDITIVES

The qualitative chemical tests supplemented by the corresponding qualitative spectroscopic tests will have given sound evidence of the presence or absence of the various additives such as methyl salicylate, 2:4-dihydroxybenzophenone, dibutyl phthalate, and so on.

It will often be desirable to carry out quantitative determinations of the various substances of which qualitative evidence has been obtained.

The chemical and spectroscopic methods we have found most useful in this connection are indicated below.

CHEMICAL EXAMINATION

The sample solution is prepared exactly as described in the qualitative tests from an accurately weighed 1-g portion of the sample. After precipitation of the polymer the additives are contained in 100 ml of solvent.

REAGENTS FOR THE DETERMINATION OF METHYL SALICYLATE—

Sodium hydroxide solution, N.

Hydrochloric acid solution, N.

Ferric chloride solution—A 10 per cent. w/v solution of ferric chloride ($FeCl_3.6H_2O$) in distilled water. Filter the solution immediately before use.

PROCEDURE FOR THE DETERMINATION OF METHYL SALICYLATE—

Transfer 10 ml of the prepared sample solution by means of a pipette to a 50-ml calibrated flask containing 5 ml of distilled water. Add 2·0 ml of *N* sodium hydroxide solution and stand the flask in a thermostatically controlled water-bath at 25° C for 4 hours. Remove the flask from the bath and add 2·2 ml of *N* hydrochloric acid followed by 2 drops of the ferric chloride solution. Mix the solution well and dilute to the 50-ml mark and set aside for 15 minutes before filtering through a Whatman No. 1 filter-paper into a 4-cm cell.

Measure the optical density of the solution in this cell, against a blank solution of the reagents used, in a Spekker absorptiometer, using Ilford green 604 filters and Calorex heat absorbers.

Prepare a calibration curve covering the range from 0 to 1·0 mg of methyl salicylate per 50 ml of final coloured solution (or 0 to 1·0 per cent. working on 1 g of sample) by the following procedure.

Dissolve 0·1 g of methyl salicylate in 100 ml of acetone contained in a 1-litre flask. Add 450 ml of absolute alcohol and dilute the solution to 1 litre with distilled water; 1 ml of

this solution is equivalent to 0·1 mg of compound. Into ten 50-ml calibrated flasks place 1, 2, 3 . . . 10 ml of this solution and dilute to 10 ml with a mixture containing 10 parts by volume of acetone, 45 parts by volume of absolute alcohol and 48 parts by volume of water. Add 5 ml of water to each flask followed by 2 ml of *N* sodium hydroxide solution and proceed as in the method described. Draw a graph relating milligrams of compound per 50 ml of final coloured solution to the Spekker indicator drum reading. Calculate the methyl salicylate content of the original sample from this calibration graph, which, in our experience, is a straight line passing through the following points—

Methyl salicylate corresponding to 50 ml of coloured solution, mg	0·0,	0·2,	0·4,	0·6,	0·8,	1·0
Indicator drum reading	0·0,	0·074,	0·146,	0·220,	0·295,	0·380

DETERMINATION OF PHENYL SALICYLATE—

For samples containing up to 0·5 per cent. of methyl salicylate, we have found it sufficient to carry out one precipitation of the polymer, but for samples containing phenyl salicylate, a second precipitation should be carried out by the following procedure.

Procedure—Precipitate the polymer, as already described in the method for the determination of methyl salicylate, from 10 ml of acetone by the addition of 45 ml of absolute alcohol and 48 ml of distilled water. Set aside for 15 minutes and then decant the supernatant solution through a Whatman No. 1 filter-paper, leaving as much of the polymer as possible in the flask. Collect the filtrate in a 100-ml calibrated flask and make up to the mark with a mixture of acetone (10 parts by volume), absolute alcohol (45 parts) and water (48 parts) by washing through the filter-paper. Determine the salicylate content of this solution by the procedure described for methyl salicylate, using a calibration curve prepared with a standard solution of phenyl salicylate.

Transfer the filter-paper and funnel to the original flask and puncture the filter-paper. Wash the polymer on the paper through the hole into the flask with 10 ml of acetone and stir for about 2 hours, during which time the polymer will redissolve or become well penetrated by the solvent. After this digestion, re-precipitate the polymer by adding 45 ml of absolute alcohol and 48 ml of distilled water before determining the salicylate in this, now much more dilute, solution by the procedure already described. Calculate the total phenyl salicylate content of the sample from the sum of these two determinations.

The calibration graph for phenyl salicylate is, in our experience, a smooth curve passing through the following points—

Phenyl salicylate corresponding to 50 ml of final solution, mg	0·0,	0·2,	0·4,	0·6,	0·8,	1·0
Indicator drum reading	0·0,	0·060,	0·118,	0·178,	0·237,	0·296

The need for a second precipitation may be made clear by noting that a sample made to contain 1·0 per cent. of phenyl salicylate gave a figure of only of the order of 0·9 per cent. when the determination was carried out on the filtrate from a single precipitation only. By the double precipitation method described above the figure was 0·97 per cent.

DETERMINATION OF 2:4-DIHYDROXYBENZOPHENONE—

Procedure—To 50 ml of the prepared sample solution contained in a 50-ml calibrated flask add 2·0 ml of *N* sodium hydroxide solution; shake well and set aside for 10 minutes. Filter the solution until it is optically clear through a Whatman No. 1 filter-paper into a 4-cm cell and measure the optical density against a blank of the reagents used in a Spekker absorptiometer, using Spectrum violet 601 filters and Calorex heat absorbers.

Prepare a calibration curve as follows—

Dissolve 0·1 g of 2:4-dihydroxybenzophenone in 100 ml of acetone in a 1-litre calibrated flask and dilute the solution to 1 litre by the addition of 450 ml of absolute alcohol and the balance of distilled water. Run 0, 5, 10 and so on up to 30 ml of this solution into seven 50-ml calibrated flasks and dilute to the marks by adding a 10 to 45 to 48 mixture by volume of acetone, alcohol and water. Add 2·0 ml of *N* sodium hydroxide solution to each flask and after well mixing and setting aside for 10 minutes, measure the optical density exactly as described above.

From this calibration curve relating optical density to mg of 2:4-dihydroxybenzophenone per 50 ml of final solution and covering the range 0 to 3 mg (*i.e.*, 0 to 0·6 per cent. of 2:4-dihydroxybenzophenone for 1 g of sample), calculate the 2:4-dihydroxybenzophenone content of the original sample.

The calibration graph that we obtained by the above method is a smooth curve passing through the following points—

2:4-Dihydroxybenzophenone per 50 ml of final solution, mg ..	0·0,	0·5,	1·0,	1·5,	2·0,	2·5,	3·0
Indicator drum reading	0·0,	0·045,	0·083,	0·120,	0·150,	0·180,	0·208

Spectroscopic examination

Determination of methyl salicylate—

Preparation of test solution—Accurately weigh 0·25 to 0·35 g of finely divided sample into a 100-ml calibrated flask, add 60 ml of chloroform B.P. and shake at once to swell the polymer. Agitate mechanically until the solution clears; 30 minutes is usually adequate. Make up the solution to 100 ml with chloroform B.P. and shake for a few minutes until homogeneous. The resulting clear solution is used for the test.

Procedure—Transfer a portion of the test solution to a 1-cm cell, cover with a ground-glass lid and measure the density at $\lambda_{max.} = 308$ mμ against a paired cell containing chloroform from the same batch as that used in the preparation of the solution. Take triplicate measurements.

Clean the cell thoroughly with chloroform, dry in a stream of filtered air and repeat the measurements on a fresh portion of the sample solution.

From the mean value of the density readings obtained, calculate the corresponding $E^{1\%}_{1\,cm}$ value by the following relationship—

$$E^{1\%}_{1\,cm} = \frac{D}{c \times l}$$

where D = optical density,
c = concentration of sample in g per 100 ml of solution
and l = cell length in centimetres.

Read off the methyl salicylate content in the original sample from a calibration graph relating $E^{1\%}_{1\,cm}$ at 308 mμ to percentage of methyl salicylate in polymethyl methacrylate covering the range 0 to 1 per cent. prepared as described below.

Weigh 3·0 g of polymethyl methacrylate drillings, transfer into a 500-ml calibrated flask and dissolve in 300 ml of chloroform by mechanical shaking. Make up the volume to 500 ml. To 25-ml aliquots of this solution contained in eleven 100-ml calibrated flasks add 0, 1·0, 2·0, 3·0 . . . 10·0 ml of a standard solution of methyl salicylate in chloroform B.P. The concentration of this solution should be such that 1 ml is equivalent to 0·15 mg of methyl salicylate. Make up all the solutions to 100 ml with chloroform.

Measure the density of each of these solutions at 308 mμ and calculate the $E^{1\%}_{1\,cm}$ values exactly as described in the method above.

Plot a calibration curve relating $E^{1\%}_{1\,cm}$ at 308 mμ to percentage of methyl salicylate calculated as a percentage of the total polymethyl methacrylate plus methyl salicylate.

We have found that under these conditions the calibration graph is a smooth curve passing through the following points—

Methyl salicylate, per cent.	0·0,	0·20,	0·40,	0·60,	0·80,	1·00
$E^{1\%}_{1\,cm}$ at 308 mμ	0·02,	0·57,	1·12,	1·67,	2·22,	2·77

Determination of phenyl salicylate—

Procedure—Carry out the procedure for the determination of phenyl salicylate by following, in general, the lines adopted for the corresponding determination of methyl salicylate except that—

(*i*) Prepare a test solution containing 0·15 to 0·20 g of accurately weighed sample per 100 ml of final solution.

(*ii*) Measure the density of the test solution at 312 mμ and calculate the corresponding value of $E^{1\%}_{1\,cm}$.

(*iii*) Read off the phenyl salicylate content in the original sample from a prepared calibration graph relating $E^{1\%}_{1\,cm}$ at 312 mμ to percentage of phenyl salicylate in polymethyl methacrylate.

(*iv*) Prepare a calibration curve relating $E^{1\%}_{1\,cm}$ at 312 mμ to percentage of phenyl salicylate in polymethyl methacrylate to cover the range 0 to 2 per cent. Use a standard solution of phenyl salicylate such that 1 ml $\equiv$ 0·30 mg of phenyl salicylate.

The calibration graph for phenyl salicylate is, in our experience, a smooth curve passing through the following points—

Phenyl salicylate, per cent.	0·0,	0·40,	0·80,	1·20,	1·60,	2·00
$E^{1\%}_{1cm}$ at 312 mμ	0·02,	0·98,	1·93,	2·89,	3·85,	4·82

DETERMINATION OF 2:4-DIHYDROXYBENZOPHENONE—

Procedure—Carry out the procedure for the determination of 2:4-dihydroxybenzophenone by following, in general, the lines adopted for the determination of methyl salicylate, except that—

(*i*) Prepare a test solution containing 0·15 to 0·20 g of accurately weighed sample per 100 ml of final solution.
(*ii*) Measure the density of the test solution at 289 and 324 mμ and calculate the corresponding values of $E^{1\%}_{1cm}$ at these wavelengths.
(*iii*) Read off the 2:4-dihydroxybenzophenone content in the original sample from a prepared calibration graph relating $E^{1\%}_{1cm}$ at 289 mμ to percentage of 2:4-dihydroxybenzophenone in polymethyl methacrylate. Check this result in a similar way by using $E^{1\%}_{1cm}$ at 324 mμ.
(*iv*) Prepare calibration curves relating $E^{1\%}_{1cm}$ at 289 and 324 mμ to percentage of 2:4-dihydroxybenzophenone in polymethyl methacrylate to cover the range 0 to 0·5 per cent. Use a standard solution of 2:4-dihydroxybenzophenone such that 1 ml is equivalent to 0·075 mg of 2:4-dihydroxybenzophenone.

The calibration graph for 2:4-dihydroxybenzophenone is, in our experience, a smooth curve passing through the following points—

2:4-Dihydroxybenzophenone, per cent. . .	0·0,	0·10,	0·20,	0·30,	0·40,	0·50
$E^{1\%}_{1cm}$ at 289 mμ	0·02,	0·62,	1·25,	1·86,	2·48,	3·09
$E^{1\%}_{1cm}$ at 324 mμ	0·01,	0·43,	0·86,	1·28,	1·70,	2·12

DETERMINATION OF STILBENE—

Procedure—Carry out the procedure for the determination of stilbene by following, in general, the lines adopted for the determination of methyl salicylate, except that—

(*i*) Prepare a test solution containing 0·20 to 0·25 g of accurately weighed sample per 250 ml of final solution.
(*ii*) Measure the density of the test solution at 299 and 310 mμ and calculate the corresponding values of $E^{1\%}_{1cm}$ at these wavelengths.
(*iii*) Read off the stilbene content in the original sample from a prepared calibration graph relating $E^{1\%}_{1cm}$ at 299 mμ to percentage of stilbene in polymethyl methacrylate. Check this result in a similar way by using $E^{1\%}_{1cm}$ at 310 mμ.
(*iv*) Prepare calibration curves relating $E^{1\%}_{1cm}$ at 299 and 310 mμ to percentage of stilbene in polymethyl methacrylate to cover the range 0 to 0·5 per cent. Use a standard solution of stilbene (*sym*diphenylethylene) such that 1 ml is equivalent to 0·075 mg of stilbene. Measure standard synthetic solutions containing 0 to 0·25 per cent. and 0·25 to 0·50 per cent. in 1·0 and 0·5-cm cells, respectively.

The calibration graph for stilbene is, in our experience, a smooth curve passing through the following points—

Stilbene, per cent.	0·0,	0·10,	0·20,	0·30,	0·40,	0·50
$E^{1\%}_{1cm}$ at 299 mμ	0·02,	1·47,	2·92,	4·37,	5·82,	7·27
$E^{1\%}_{1cm}$ at 310 mμ	0·02,	1·45,	2·88,	4·31,	5·74,	7·17

DETERMINATION OF DIBUTYL PHTHALATE IN POLYMETHYL METHACRYLATE SHEET—

Particular attention has been directed in this laboratory to the determination of small amounts, *i.e.*, less than 1 per cent., of dibutyl phthalate.

The determination of dibutyl phthalate plasticiser, normally present to the extent of about 5 per cent. in plasticised polymethyl methacrylate, has been previously described by Haslam and Soppet.[2] The principle of the method involves solution of the sample in acetone, with subsequent precipitation of the polymer with light petroleum (b.p. 40° to 60° C), filtration and recovery of the dibutyl phthalate by evaporation of the light petroleum solution. With samples containing small amounts of plasticiser and particularly with methyl

methacrylate - ethyl acrylate interpolymer compositions, the result obtained for the small percentage of dibutyl phthalate present may not be as accurate as is desirable because of the presence in the recovered dibutyl phthalate (*i*) of low molecular weight polymer and (*ii*) of small amounts of other additives in the polymer. For these reasons a direct ultra-violet absorption method of determining dibutyl phthalate in polymethyl methacrylate sheet has been worked out, and details of this method are described below.

Preparation of test solution—Accurately weigh 0·40 to 0·50 g of finely divided sample into a 100-ml calibrated flask, add 60 ml of chloroform B.P. and shake at once to swell the polymer. Transfer the flask to a mechanical shaker and agitate until a clear solution is obtained; 30 minutes is usually adequate. Make up the solution to 100 ml with chloroform and shake for a few minutes until homogeneous. The resulting solution is used for the test.

Procedure—Transfer a portion of the test solution to a 2-cm cell, cover with a ground-glass lid and measure the density on a spectrophotometer at $\lambda_{max.} = 276$ mμ against a paired cell containing chloroform from the same batch as that used in the preparation of the solution. Measure the optical density in triplicate.

Clean the cell thoroughly with chloroform, dry in a stream of filtered air and repeat the above measurements on a fresh portion of the sample solution.

From the mean of the density readings, calculate the corresponding $E^{1\%}_{1\,cm}$ value by the following relationship—

$$E^{1\%}_{1\,cm} = \frac{D}{c \times l}$$

where D = optical density,
c = concentration of sample in g per 100 ml of solution
and l = cell length in centimetres.

Read off the dibutyl phthalate content in the original sample from a calibration graph relating $E^{1\%}_{1\,cm}$ at 276 mμ to percentage of dibutyl phthalate in polymethyl methacrylate covering the range 0 to 1 per cent. prepared as described below.

Weigh 5·0 g of drillings of polymethyl methacrylate sheet, transfer to a 500-ml calibrated flask and dissolve in 300 ml of chloroform B.P. by shaking mechanically. Make up the

TABLE V

ANALYSIS OF POLYMER SOLUTIONS CONTAINING ULTRA-VIOLET ABSORBERS AND DIBUTYL PHTHALATE

Compound added	By chemical tests		By spectroscopic tests	
	Added, %	Found, %	Added, %	Found, %
Methyl salicylate	0·58	0·63	0·39	0·38
"	1·00	1·02	1·08	1·10
Stilbene	—	—	0·29	0·29
"	—	—	0·25	0·24
Phenyl salicylate	0·49	0·53	0·55	0·53
"	0·89	0·83	0·99	0·99
2:4-Dihydroxybenzophenone	0·30	0·29	0·25	0·24
"	0·40	0·36	0·34	0·30
"	0·50	0·45	—	—
Resorcinol monobenzoate	0·50	Qualitatively detected	Qualitatively detected	
Dibutyl phthalate	0·50	Qualitatively detected	0·64	0·64
"	—	—	1·06	1·03

volume to 500 ml and shake until homogeneous. To 50-ml aliquots of this solution contained in eleven 100-ml calibrated flasks add 0, 1·0, 2·0, 3·0 . . . 10·0 ml of a standard solution of dibutyl phthalate in chloroform B.P. The concentration of this solution should be such that 1 ml is equivalent to 0·5 mg of dibutyl phthalate. Make up each solution to 100 ml with chloroform and shake well. Measure the optical density of each of these solutions in a 2-cm cell and calculate the $E^{1\%}_{1\,cm}$ value exactly as described above.

Plot a calibration graph relating $E^{1\%}_{1\,cm}$ at 276 mμ to percentage of dibutyl phthalate calculated as a percentage of the total polymethyl methacrylate plus dibutyl phthalate.

TABLE VI

ANALYSIS OF POLYMERS CONTAINING ULTRA-VIOLET ABSORBERS AND PLASTICISERS

Intended compositions of polymers	Chemical results	Spectroscopic results
Unplasticised polymethyl methacrylate containing no ultra-violet absorber	No evidence of salicylate, 2:4-dihydroxybenzophenone or resorcinol monobenzoate	No evidence of plasticiser or ultra-violet absorber
Polymethyl methacrylate containing 0·5 per cent. of dibutyl phthalate	Phthalate detected	0·50 per cent. dibutyl phthalate
Unplasticised polymethyl methacrylate containing 0·5 per cent. of methyl salicylate	0·50 per cent. methyl salicylate	0·49 per cent. methyl salicylate
Polymethyl methacrylate containing 0·5 per cent. of methyl salicylate and 0·5 per cent. of dibutyl phthalate	0·49 per cent. methyl salicylate	0·49 per cent. methyl salicylate
Unplasticised polymethyl methacrylate containing 0·5 per cent. of phenyl salicylate	0·47 per cent. phenyl salicylate	0·43 per cent. phenyl salicylate
Polymethyl methacrylate containing 0·5 per cent. of phenyl salicylate and 0·5 per cent. of dibutyl phthalate	0·46 per cent. phenyl salicylate	0·43 per cent. phenyl alicylate
Unplasticised polymethyl methacrylate containing 0·5 per cent. of 2:4-dihydroxybenzophenone	0·48 per cent. 2:4-dihydroxybenzophenone	0·49 per cent. 2:4-dihydroxybenzophenone
Unplasticised polymethyl methacrylate containing 0·5 per cent. of stilbene	No evidence of salicylate, 2:4-dihydroxybenzophenone or resorcinol monobenzoate	0·44 per cent. stilbene

TABLE VII

ANALYSIS OF COMMERCIAL AND UNKNOWN SAMPLES

Sample	Chemical	Spectroscopic
1	Evidence of salicylate but not phenyl or cresyl. 0·49 per cent. as methyl salicylate	Characteristic of methyl salicylate, 0·49 per cent.
2	No evidence of salicylates, 2:4-dihydroxybenzophenone or resorcinol monobenzoate	No evidence of dibutyl phthalate or ultra-violet absorbers
3	Evidence of phenyl salicylate 0·98 per cent.	Characteristic of phenyl salicylate, 0·98 per cent.
4*	Evidence of salicylate but not phenyl or cresyl. 0·50 per cent. as methyl salicylate	Characteristic of methyl salicylate, 0·50 per cent.
5	Evidence of 2:4-dihydroxybenzophenone 0·26 per cent.	Characteristic of 2:4-dihydroxybenzophenone, 0·30 per cent.
6	No evidence of salicylates, resorcinol monobenzoate or 2:4-dihydroxybenzophenone, but strong evidence of presence of a phenolic or cresylic constituent. Suspected surface contamination—later proved	Small amounts of unknown absorbing material detected. Later shown conducive to presence of phenolic or cresylic constituent
7*	Evidence of salicylate but not phenyl or cresyl. 0·52 per cent. as methyl salicylate	Characteristic of methyl salicylate, 0·50 per cent.
8, 9*, 10*, 11	No evidence of salicylates, 2:4-dihydroxybenzophenone or resorcinol monobenzoate	No evidence of dibutyl phthalate or ultra-violet absorber
12	Evidence of 2:4-dihydroxybenzophenone 0·12 per cent.	Characteristic of 2:4-dihydroxybenzophenone, 0·11 per cent.

* These samples are methyl methacrylate - ethyl acrylate co-polymers.

Under these conditions we have found that the calibration graph is a smooth curve passing through the following points—

Dibutyl phthalate, per cent.	0·0,	0·20,	0·40,	0·60,	0·80,	1·00
$E^{1\%}_{1\,cm}$ at 276 mμ	0·02,	0·11,	0·19,	0·28,	0·37,	0·46

RESULTS

The methods described for the determination of dibutyl phthalate and ultra-violet absorbers in polymethyl methacrylate-type polymers have been tested by—

(*i*) Analysis of synthetic solutions containing known weights of pure polymethyl methacrylate and ultra-violet absorbers or dibutyl phthalate dissolved in acetone for the chemical tests and chloroform B.P. for the spectroscopic tests. The results shown in Table V were obtained.

(*ii*) Analysis of polymers prepared with the object of the final compositions containing known amounts of ultra-violet absorber and plasticiser. The intended compositions of the polymers, together with the results obtained, are shown in Table VI.

(*iii*) Analysis of commercial and unknown samples, the results of which indicate the agreement between the chemical and physical figures, as shown in Table VII.

NOTES ON THE TESTS—

The methods described above have been applied with equal success to the analysis of methyl methacrylate - ethyl acrylate co-polymers.

It is always desirable to pay particular attention to the odour of the sample when drillings are being taken; for example, methyl salicylate is quite distinctive.

When cresyl esters are suspected it may be noted that they differ in their reactions to the Millon's and indophenol tests as shown in Table VIII.

TABLE VIII

REACTIONS OF CRESYL ESTERS

Homologue	Millon's test	2:6-Dibromoquinonechlorimide test
Phenol	Pink colour deepening in 5 to 10 minutes	Deep blue developing in 5 minutes
o-Cresol	Pale yellow-pink deepening to yellow-brown	Immediate pronounced blue changing to deep purple
p-Cresol	Immediate pink deepening to deep claret	No colour
m-Cresol	Claret colour deepening	Pronounced blue

Such substances as hydroquinone monobenzoate and catechol monobenzoate behave as shown in Table IX in the appropriate Millon's and indophenol tests, as compared with resorcinol monobenzoate.

TABLE IX

REACTIONS OF MONOBENZOATES OF HYDROQUINONE AND CATECHOL COMPARED WITH THOSE OF RESORCINOL MONOBENZOATE

	Millon's test	2:6-Dibromoquinonechlorimide test
Resorcinol monobenzoate ..	Immediate yellow precipitate	Immediate violet—deepening
Hydroquinone monobenzoate	Immediate yellow precipitate	Fairly rapid reduction to brown-black—darkening
Catechol monobenzoate ..	Immediate yellow precipitate	As for hydroquinone, but much more slowly

The absorption maxima of laboratory prepared specimens of these compounds in ethyl alcohol solution are as follows: catechol monobenzoate, $\lambda_{max.} = 273$ mμ; resorcinol monobenzoate, $\lambda_{max.} = 274$ mμ; hydroquinone monobenzoate, $\lambda_{max.} = 275$ mμ.

Although we have no experience of the application of the method, it seems certain that the method described by Nicholls[3] for the determination of small amounts of benzoic acid by conversion to salicylic acid with hydrogen peroxide and ferric chloride could be used for the determination of resorcinol monobenzoate after an appropriate extraction and hydrolysis.

All absorption spectra measurements were made with the Hilger Uvispek spectrophotometer.

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The Determination of Calcium in Plants and Soils

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The method is based on the turbidity formed on adding a precipitating reagent to a sodium acetate - acetic acid buffer solution containing the calcium. The precipitating reagent consists of a solution of ammonium oxalate and citric acid in water, ethanol and butan-1-ol. The reagent is stable for 8 hours, and the turbidity, which is determined by means of a photo-electric absorptiometer, is constant from 5 to 60 minutes after adding the reagent.

The method, which was devised for calcium in plant and soil extracts, determines from 0·05 to 0·50 mg of calcium with an accuracy of within ±5 per cent. Permissible concentration limits of certain interfering ions are given, and examination of the amounts of these ions in plant and soil extracts indicates that significant interference will seldom be encountered.

METHODS previously published for the determination of calcium on a semi-micro scale by a turbidimetric procedure have been speedy, but have low accuracy; in our experience a 20 per cent. error is not exceptional. The simplicity of the turbidimetric procedure is a great asset, and investigation showed that the accuracy could be considerably increased. The accuracy of the method now proposed is satisfactory (within ±5 per cent.), and the manipulative simplicity inherent in the turbidimetric procedure is retained.

METHOD

PREPARATION OF THE SAMPLE—

The object of the preliminary treatment of the sample is to produce a solution containing no excessive amounts of organic matter or interfering ions and one in which the calcium concentration is such that 0·05 to 0·50 mg of calcium is contained in easily handled aliquots. In addition, the calcium extractant should be easily removable so that it can be replaced by Morgan's reagent,[1] the composition of which is given below (p. 107); if Morgan's reagent is itself the extractant, the required aliquot should be not more than 5 ml.

Plant tissues—Ash the sample and dissolve it in dilute hydrochloric acid; transfer a convenient aliquot to the reaction flask, evaporate to dryness and dissolve the residue in exactly 5 ml of Morgan's reagent.

Plant tissues that have been extracted with Morgan's reagent are used without treatment other than removal of excessive amounts of organic matter by activated carbon (Hester[2]). The carbon must contain no extractable calcium; purify ordinary grades by washing with acid.

Soils—Use solutions prepared by extracting the soil with Morgan's reagent, described by Peech and English,[3] as indicated above.

If the extract is prepared with neutral *N* ammonium acetate (Piper[4]) or 0·5 *N* acetic acid (Williams[5] and Williams and Stewart[6]), evaporate to dryness and dissolve the residue in a convenient volume of dilute hydrochloric acid; take the aliquot from this solution. Alternatively, evaporate a convenient aliquot of the initial extract to dryness in the reaction flask and dissolve the residue in 5 ml of Morgan's reagent. With either technique, destroy excessive amounts of organic matter by adding small amounts of 20-volume hydrogen peroxide as the evaporation nears completion.

REAGENTS—

Morgan's reagent[1]—Dissolve 100 g of hydrated sodium acetate and 30 ml of glacial acetic acid in water and dilute to 1 litre.

Precipitating reagent—A mixture of 95 per cent. ethanol (rectified spirits), butan-1-ol (technical *n*-butyl alcohol*), 0·5 per cent. w/v ammonium oxalate solution and 25 per cent. w/v citric acid solution. Mix 375 ml of 95 per cent. ethanol with 125 ml of butan-1-ol. After 5 minutes, add 500 ml of 0·5 per cent. ammonium oxalate solution rapidly to the mixture and stir thoroughly. After a further 5 minutes, pour this mixture rapidly into 40 ml of 25 per cent. citric acid solution and mix. The reagent is ready for use 1 hour after preparation and is stable for a further 8 hours.

Calcium stock reagent—Prepare a solution containing 2000 parts per million of calcium by adding 4·994 g of calcium carbonate to approximately 100 ml of water in a covered beaker and then add approximately 5 ml of glacial acetic acid. Warm the mixture and add approximately 500 ml of Morgan's reagent. Heat and stir the mixture until the carbonate dissolves. Cool and dilute accurately to 1 litre with Morgan's reagent.

Calcium standard solution—Prepare a solution containing 100 parts of calcium per million by diluting accurately 50 ml of calcium stock reagent to 1 litre with Morgan's reagent.

PROCEDURE—

Place an aliquot containing from 0·05 to 0·50 mg of calcium in the 100-ml conical flask and treat it as described above for preparing the sample, so that ultimately the calcium is dissolved in 5 ml of Morgan's reagent. Add 25 ml of the precipitating reagent to the solution whilst shaking the flask and contents. Set the mixture aside for not less than 5 minutes and not more than 1 hour, and during that period determine the turbidity; a Hilger Spekker photo-electric absorptiometer with 4-cm cells and glass turbidity filters is suitable for the determination.

Construct the graph for converting the absorptiometer readings to concentrations, by diluting 0, 1, 2, 3, 4 and 5 ml of the calcium standard solution to 5 ml with Morgan's reagent, adding the precipitating reagent, and determining the intensity of the turbidity produced under the conditions described above.

NOTES ON THE METHOD—

The calibration readings vary owing to minor variations in the preparation of the precipitating reagent. For example, 0·30 mg of calcium may give an absorptiometer reading between 0·37 and 0·27, and sometimes the reading for 0·50 mg of calcium is unusually low and slightly off-scale, *i.e.*, less than 0. This effect, however, is related to the precipitating reagent, and is compensated for by making a calibration graph for each batch of precipitating reagent prepared.

The effect of temperature (from 10° to 30° C) on the results was found to be negligible.

Typical calibration readings are as follows—

Calcium, mg . .	. .	Nil	0·05	0·10	0·15	0·20	0·25	0·30	0·35	0·40	0·45	0·50
Spekker reading	. .	0·99	0·92	0·81	0·67	0·53	0·41	0·31	0·21	0·12	0·06	0·02

INTERFERENCE FROM OTHER IONS

The effect of certain ions on the method was investigated, and it was found that some of these influenced the results when present in amounts outside the tolerance limits given in Table I. From Table II it is seen that high calcium values result from excessive amounts of magnesium, iron, manganese or aluminium in the aliquot. Within the established limits

* Supplied by British Industrial Solvents, Carshalton, Surrey.

iron and manganese can be controlled by citric acid, which, in addition, contributes to the precision of the method.

TABLE I

ION TOLERANCE LIMITS

Permissible amounts (milligrams) of ions in the aliquot taken for analysis

Calcium	.. ..	0·05–0·50			
Magnesium	.. ..	0–0·75	Sodium	.. ..	0–5·0*
Iron	.. .. ..	0–2·0	Chloride	.. ..	0–5·0*
Manganese	.. ..	0–0·25	Nitrate	.. ..	0–5·0*
Aluminium	.. ..	0–2·0	Phosphate	.. ..	0–5·0*
Ammonium	.. ..	0–5·0*	Sulphate	.. ..	0–5·0*
Potassium	.. ..	0–5·0*			

* The effect of greater amounts was not investigated.

TABLE II

ION INTERFERENCE

Effect of magnesium, iron, manganese and aluminium on the determination of calcium

Calcium present, mg	Magnesium added, mg	Iron added, mg	Manganese added, mg	Aluminium added, mg	Calcium found, mg	Percentage error
0·300	0·50				0·300	0
0·300	0·75				0·310	3·3
0·300	1·00				0·320	6·7
0·300		1·00			0·300	0
0·300		1·50			0·310	3·3
0·300		2·00			0·305	1·7
0·300		3·00			0·330	10·0
0·300			0·10		0·300	0
0·300			0·25		0·310	3·3
0·300			0·50		0·330	10·0
0·300				2·00	0·310	3·3
0·300				3·00	0·325	8·3

The tolerance limits are not often exceeded in solutions as usually prepared for analysis. More calcium than magnesium is present in most plants or plant organs, other than seeds (Cooper, Paden and Garman[7]), and in most soils (Russell[8]); interference from magnesium is therefore unlikely.

Hunter[9] summarises the position with regard to interference from iron, manganese and aluminium in the determination of magnesium, and concludes that only in exceptional plant analyses will the tolerance limits be exceeded; as the tolerance limits of these elements in the magnesium determination are equal to or less than those in the calcium, and the amounts of calcium present are usually greater than the amounts of magnesium, the possibility of interference from these elements in the determination of calcium in plants is remote.

Hunter[9] points out that in soil analysis the concentration of interfering ions in an extract will depend not only on their content and form in the sample, but also on the method used to prepare the extract. He tabulates the calcium, iron, manganese and aluminium concentrations in extracts of twelve soils, in some of which the calcium concentrations were low and interference therefore liable to occur; the extracting reagents used were (*i*) neutral *N* ammonium acetate solution (Piper[4]), (*ii*) Morgan's reagent, pH 4·8 (used as described by Peech and English[3] but with the extraction period increased to 2 hours), and (*iii*) 0·5 *N* acetic acid, pH 2·5 (Williams[5]). In none of these extracts is the concentration of magnesium, iron or aluminium high enough to interfere with determinations of calcium by the proposed method, and in only one acetic acid extract is the concentration of manganese excessive. Hence it can be taken that significant interference seldom arises in soil analysis.

Accuracy

To determine the accuracy of the method, solutions containing known amounts of calcium were prepared and their calcium contents determined twenty times. The calcium contents of the hydrochloric acid extract of hay ash and a Morgan's reagent extract of a soil were also determined twenty times. The results of a statistical analysis of the values obtained are shown in Table III.

Table III

Accuracy of the method

Statistical results for replicate determinations

Calcium per determination, mg	Number of determinations	Standard deviation	Standard error of the mean	Percentage error* (mean of 20 observations)	Percentage error* (mean of 2 observations)	Type of solution
0·05	20	0†	0	0	0	Standard
0·10	20	0·00134	0·000300	0·86	2·71	"
0·20	20	0·00138	0·000309	0·44	1·40	"
0·30	20	0·00216	0·000483	0·46	1·46	"
0·40	20	0·00351	0·000785	0·56	1·78	"
0·45	20	0·00900	0·002012	1·28	4·05	"
0·50	20	0·0156	0·003488	2·00	6·31	"
294‡	20	0·896	0·200	0·19	0·62	Plant extract
536‡	20	6·85	1·53	0·82	2·59	Soil extract

* This error will not be exceeded 99 times out of 100.
† Results identical.
‡ Milligrams of calcium per 100 g of plant dry matter or dry soil.

It will be seen that the standard deviation and standard error of the mean for each set of standard determinations increase with the amount of calcium present, indicating that greater variations can be expected with larger amounts of calcium. In order to estimate on a percentage basis the degree of accuracy at different calcium concentrations, the product of each standard error and the appropriate t value is expressed as a percentage of the mean determined. In this way the percentage accuracy that will be obtained 99 times out of 100 was estimated and is recorded in Table III for the means of both 20 and 2 determinations. The values show that, for a mean of 20 determinations, 99 times out of 100 the result will be within 1 per cent. of the true value with 0·05 to 0·40 mg of calcium, and within 3 per cent. with 0·40 to 0·50 mg of calcium. The results for the plant and soil extracts also indicate a high measure of reproducibility.

The calcium contents of numerous samples of plant tissue (ash extracted by hydrochloric acid) and soil (extracted by 0·5 N acetic acid) were determined by the method described. A comparison of these results with those obtained by an established macro-volumetric method (Piper[4]) and flame photometry* (Mitchell[10]) showed good agreement. Results by the proposed method and Piper's method are compared in Tables IV and V.

Table IV

Determination of calcium in plants

Material	Calcium per 100 g of dry matter: By macro-volumetric determination, mg	By turbidimetric determination, mg	Percentage difference
Tomato leaves	5340	5366	+0·49
Hay	367	350	−4·63
Swede bulbs	1831	1873	+2·29
Swede leaves	341	325	−4·69
Oat straw	309	300	−2·91
Oat grain	109·2	110·1	+0·82

* These results were supplied by Dr. R. L. Mitchell, Department of Spectrochemistry, The Macaulay Institute for Soil Research.

TABLE V

DETERMINATION OF CALCIUM IN SOILS

0·5 *N* acetic acid extraction

Soil pH	Percentage loss on ignition	Calcium per 100 g of soil: By macro-volumetric determination, mg	Calcium per 100 g of soil: By turbidimetric determination, mg	Percentage difference
4·9	4	12·3	12·9	+4·88
4·6	8	70·1	68·1	−2·85
4·3	8	122·4	116·8	−4·58
4·9	7	131·2	130·6	−0·46
4·6	54	272·5	275·0	+0·92
4·8	21	56·3	58·8	+4·44
5·3	26	69·8	67·8	−2·87
7·0	9	640·0	639·0	−0·16
6·8	10	182·6	187·5	+2·68

The degree of accuracy of the method can therefore be considered satisfactory by the standards usually recognised in this type of work.

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THE MACAULAY INSTITUTE FOR SOIL RESEARCH
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Micro-Determination of Phosphorus in Biological Material

BY H. W. HARVEY

An absorptiometric method for determining microgram amounts of phosphorus in small samples of algal growths has been devised and tested. It is based on the conversion of the phosphorus to phosphomolybdic acid and reduction to molybdenum blue by stannous chloride. The range of the method is from 1 to 70 μg of phosphorus.

IN connection with another research it was found necessary to determine phosphorus in uni-cellular algae, which had been separated by centrifugation and contained a few micrograms of phosphorus. The method proposed is suitable for 1 to 70-μg amounts of phosphorus. It consists in decomposing the organic material with sulphuric acid and hydrogen peroxide, heating with 5 *N* acid to convert the pyrophosphate to orthophosphate, decomposing residual hydrogen peroxide with sulphite and converting the orthophosphate to phosphomolybdic acid. The phosphorus is then determined by the molybdenum-blue method after controlled reduction by stannous chloride.

METHOD

REAGENTS—

Sulphuric acid—A 50 per cent. v/v solution.
Hydrogen peroxide—A 100-volume solution.

Sodium sulphite—An aqueous solution containing 33 g of $Na_2SO_3.7H_2O$ per 100 ml.

Acid molybdate solution—Dissolve 10 g of ammonium molybdate in 100 ml of water and add to 130 ml of concentrated sulphuric acid diluted with 170 ml of water.

Hydrochloric acid—Dilute 5 ml of concentrated hydrochloric acid to 100 ml with water.

Iodine solution—A 0·033 *N* solution of iodine in dilute potassium iodide solution.

Stannous chloride (stock solution)—Dissolve 40 g of $SnCl_2.2H_2O$ (clean unoxidised crystals) in 50 ml of concentrated hydrochloric acid and 50 ml of water. (This solution is reasonably stable.)

Stannous chloride (dilute solution)—Add about 0·7 ml of stock solution to 20 ml of 5 per cent. hydrochloric acid. This provides 2 mg of stannous ions in about 0·75 ml, and this amount decolorises 1 ml of 0·033 *N* iodine solution. The stock solution is conveniently added from a blood pipette. In order to ascertain the volume of dilute solution containing 2 mg of tin, it is necessary to titrate it against 1 ml of iodine solution. (This solution only retains its titre for a few hours.[1])

APPARATUS—

Hard-glass test tubes or centrifuge tubes—These must be not less than 12 cm long. For the determination of less than 1 μg of phosphorus, transparent silica tubes are preferable.[2]

Hard-glass flasks—Capacity 120 to 200 ml, with beakers to cover them.

(In order to rid new tubes and flasks of phosphate, moisten with sulphuric acid and heat in an oven before washing with distilled water.)

Pipettes—Two graduated pipettes of capacity 1 ml, provided with rubber teats (blood pipettes), and one 2-ml pipette, preferably of the syringe type.[3]

Thin quill tube—Drawn out and cut off. Marked to deliver about 0·2 ml.

PROCEDURE—

Add 0·2 ml of 50 per cent. v/v sulphuric acid to the organic matter in a hard-glass centrifuge tube or test tube, heat until charring starts, cool, add 1 drop of hydrogen peroxide and heat over a micro burner until fumes of sulphuric acid just fill the lower part of the tube. If the liquid is not then colourless add another drop of hydrogen peroxide and heat again. The resulting liquid contains orthophosphoric acid with a small amount of pyrophosphoric acid.

Add 0·6 ml of water to reduce the acid concentration to 5 *N* and heat to boiling twice. This treatment converts almost all of the phosphate to the ortho state (see p. 112).

Transfer the contents of the tube to 100 ml of distilled water contained in a wide-necked flask protected from dust by an inverted beaker, add 0·75 ml of sulphite solution, and then 2 ml of acid molybdate. The molybdate solution is conveniently added from a syringe pipette.[3] Mix, and then, not less than 3 minutes after adding the acid molybdate, add a volume of the dilute stannous chloride solution containing 2 mg of tin from a blood pipette and mix at once.

Between 5 and 7 minutes after adding the stannous chloride solution measure the optical density of the blue solution in a cuvette or vessel of suitable length, through a red filter (Chance OR1 or Ilford Spectrum red).

The optical density per centimetre of light path through the liquid (E_{1cm}) bears an *almost* linear relation to the phosphate content.

One or two reagent blanks are included in the series in order to correct for traces of extraneous phosphate.

One or two tubes containing a known quantity of phosphate can be included in the series in order to ascertain the value of E_{1cm} per μg of phosphorus, or this value may be determined independently. It remains constant for the reagent[6] (after correcting for temperature), provided that the same amount of stannous ion is added and that the amounts of 50 per cent. sulphuric acid and of acid molybdate solution are reasonably constant (see p. 113).

EXPERIMENTAL

COMBUSTION—

During the wet combustion there is no loss of phosphoric acid vapour, since all the acid fumes condense in the lower half of the tube. When phosphate was heated in this manner for a long time there was no loss. Any hydrogen peroxide condensing in the upper part of the tube is decomposed when the contents are washed into the sulphite solution.

CONVERSION OF PYROPHOSPHORIC TO ORTHOPHOSPHORIC ACID—

When orthophosphate was heated with acid and then (without conversion) washed into water, about 2 per cent. remained as the pyro acid, but when pyrophosphate was heated with acid, diluted with 0·6 ml of water and boiled, about 7 per cent. remained as pyrophosphate. Hence in the recommended procedure any loss of phosphorus by incomplete conversion of pyro to ortho acid is not likely to exceed 2 per cent. of 7 per cent., or, say, 0·2 per cent.

ACCURACY OF DETERMINATION OF ORTHOPHOSPHATE—

When a solution containing phosphomolybdic acid was reduced by stannous chloride, the blue colour increased to a maximum and then slowly faded. As the concentration of phosphorus was increased, the maximum colour was reached in a shorter time and fading started sooner. In the recommended procedure, the intensity of the colour is measured after a fixed reaction time (5 to 7 minutes). In this manner a plateau value is reached when the concentration of phosphorus lies between 15 and 90 μg per 100 ml, but the intensity has not quite risen to its maximum with small (1 to 2-μg) concentrations.

In addition to the effect of time, the intensity of the blue colour depends on the concentration of tin and, when phosphate concentration is high, on the ratio of tin to phosphate.

In consequence, the intensity of the colour developed with 2·0 mg of tin, after a reaction time of 5 to 7 minutes, departs slightly from strict linearity in the range 1 to 80 μg of phosphorus. However, neither this departure from linearity nor an increase in reaction time to 10 minutes after adding the stannous chloride appreciably affect the results. This was shown by the following experiment.

A series of solutions containing different amounts of phosphate in distilled water was treated with sulphuric acid, sodium sulphite, molybdate reagent and stannous chloride as described in the procedure (p. 111). The observed optical densities and calculations are shown in Table I. In the last column the values found for additions of 20 μg of phosphorus to 100 ml of water are used as a basis for calculating the "phosphate found" at the other dilutions. The difference between quantity added and found includes volumetric and instrumental errors.

TABLE I

EFFECT OF VARIATIONS IN REACTION TIME AT 13·5° C

Phosphorus added to 100 ml of water, μg	Internal length of cuvette, cm	E observed*	E_{1cm}	E_{1cm}— (reagent blank)	E_{1cm}— (reagent blank) per μg of phosphorus	Phosphorus found, μg
Reaction time, 5 minutes—						
0	15·0	0·021	0·0014 (reagent blank)	—	—	—
1	"	0·128	0·00854	0·00714	0·00714	1·07
2	"	0·220	0·01467	0·01327	0·00663	2·0
4	"	0·417	0·0278	0·0264	0·00660	3·9
20	2·0	0·267	0·1335	0·1321	0·00662	Basis
20	"	0·268	0·1340	0·1326		
48	1·0	0·313	0·313	0·3116	0·00649	47·1
80	"	0·510	0·510	0·5086	0·00636	76·8
Reaction time, 10 minutes—						
0	15·0	0·024	0·0016 (reagent blank)	—	—	—
1	"	0·131	0·00873	0·00713	—	1·07
2	"	0·229	0·0146	0·0130	—	1·97
4	"	0·426	0·0284	0·0268	—	4·04

* The difference between the optical densities of the solution and distilled water.

The intensity of blue depends also on the concentration of stannous tin. The effect of varying the concentration of stannous ion on different concentrations of phosphate is shown in Table II. It can be seen that the error caused by small differences in the concentration of stannous chloride is insignificant.

TABLE II

EFFECT OF VARIATIONS IN THE CONCENTRATION OF STANNOUS CHLORIDE FOR 2 TO 8 μg OF PHOSPHORUS

Phosphorus in 100 ml of reaction mixture, μg	Internal length of cuvette, cm	Tin added, mg	Optical density
About 2	15·0	1·7	0·182
„ 2	15·0	2·0	0·187, 0·186
„ 2	15·0	2·3	0·188
„ 20	2·0	1·7	0·257
„ 20	2·0	2·0	0·259, 0·259
„ 20	2·0	2·3	0·260
„ 80	1·0	1·7	0·398
„ 80	1·0	2·0	0·403, 0·404
„ 80	1·0	2·3	0·408

The optical density is also dependent upon the ratio of acid to molybdate in the reaction mixture. Table III shows the effect of variation in the volume of 50 per cent. sulphuric acid added. The greater the volume added the less the optical density after 5 minutes.

TABLE III

EFFECT OF ACIDITY ON OPTICAL DENSITY

Amount of 50 per cent. v/v sulphuric acid added, ml	Optical density of reaction mixture containing 20 μg of phosphorus, 5 to 7 minutes after adding 2 mg of tin, corrected for blank
0·1	0·272
0·15	0·267
0·2	0·260
0·25	0·258
0·3	0·250

As the addition of 0·2 ml of 50 per cent. sulphuric acid can be made to within 0·01 ml, the error due to variations in the volume is small. In addition, there is some loss of acid by reduction of the sulphuric acid to sulphurous acid during the wet combustion of the sample. If a relatively large quantity of organic matter, *e.g.*, 3 mg of sugar, is decomposed, and all the oxygen were derived from the sulphuric acid, the loss of sulphuric acid from this cause would amount to 0·022 ml; it would, in fact, be less, for some oxygen is supplied by the hydrogen peroxide.

The combined error due to variations in the volume taken and loss of sulphuric acid during combustion does not seem likely to exceed about 1 per cent.

Variations in the quantity of acid molybdate added from a syringe pipette were insignificant.

The effect of temperature on colour development has been investigated[2,4]; it amounts to an increase of 0·96 per cent. per 1° C.

EFFECT OF INTERFERING SUBSTANCES—

The formation of molybdenum blue is known to be hindered by several substances.[5,6] But in the proposed procedure in which the inorganic residue from 1 to 3 mg of organic matter is contained in 100 ml, none of these is likely to be present at a concentration sufficient to cause significant error, with the possible exception of copper.

The body fluids of several species of marine animals containing haemocyanin are relatively rich in copper, which has an outstandingly potent effect on the formation of molybdenum blue.

The depression in optical density caused by additions of copper to a solution containing 20 μg of phosphorus is as follows—

Copper added (as sulphate), μg	10	5	2
Optical density. Expressed as a percentage of that given by 20 μg of phosphorus	87·5	93·5	97·0

CONTAMINATION—

Many thousand duplicate determinations of phosphate and of organic phosphorus in sea water made in this laboratory, and involving 1-μg amounts of phosphorus, have shown that, provided simple precautions are taken, the danger of contamination is slight.

Contamination of the laboratory air by the phosphorus pentoxide evolved on igniting a lucifer match is sufficient to vitiate a determination.

For samples containing more than 1 to 2 μg of phosphorus the error due to solution of phosphate from hot acid-washed Pyrex or Hysil glass is insignificant. With samples containing less than this amount, silica tubes are best (p. 111).

The flasks required for this work should be washed thoroughly with water immediately after use.

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THE LABORATORY
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The Determination of Beta- and Gamma-Picolines, 2:6-Lutidine and 2-Ethylpyridine in Mixtures by Infra-red Spectroscopy

BY E. A. COULSON AND J. L. HALES

β-Picoline and γ-picoline, which are present in coal tar base fractions, are convenient starting materials for the preparation of nicotinic and *iso*-nicotinic acids. A method is described by which crude or purified tar-base fractions containing these two bases, 2:6-lutidine and smaller amounts of 2-ethylpyridine, can be analysed quantitatively by infra-red absorption spectroscopy.

COMMERCIAL picoline fractions, boiling at 140° to 145° C, derived from coal-tar bases, consist substantially of β- and γ-picolines and 2:6-lutidine.[1] In addition, 2-ethylpyridine may be present as a minor constituent. Both of the first-mentioned bases are currently of considerable technical interest. β-Picoline is used in the preparation of nicotinic acid and γ-picoline is the most convenient starting material in the preparation of the new chemotherapeutic agent *iso*nicotinic acid hydrazide (Rimifon, Nydrazid, or Isoniazid), which is now being tried against tubercular infections. At present there is no source of supply for these bases other than coal-tar picoline. Consequently a method for their detection in crude and purified fractions is a necessity for control of manufacture.

Colour reactions suitable for the detection and estimation of the two picolines in base mixtures have been described[2] and also methods that depend on the ultra-violet absorption spectra.[3] An infra-red absorption spectroscopic method has been used in this laboratory for some years and has been found most convenient in practice.[4] This method and those referred to above must be regarded as complementary. Pyridine, α-picoline and 2-ethylpyridine interfere with the determination of β-picoline, but not with that of γ-picoline, by the colorimetric method; however this method can be used to determine γ-picoline in the presence of a large excess of certain other materials, such as alcohol.

The ultra-violet absorption method can be used to estimate β- and γ-picolines and 2:6-lutidine directly in aqueous solution, but pyridine, α-picoline and 2-ethylpyridine interfere and must be removed by distillation before the former three bases can be determined. An

advantage of the proposed method is that pyridine, α-picoline and 2-ethylpyridine do not interfere and can be estimated; the procedure cannot be used directly to determine the bases in aqueous or alcoholic solutions.

EXPERIMENTAL—

Highly purified samples of the three chief components of tar-base fractions boiling in the range 140° to 145° C were available in this laboratory, their purities being estimated by the freezing-point method with the following results—

β-picoline, 99·97 ± 0·02 mole per cent.
γ-picoline, 99·75 ± 0·13 mole per cent.
2:6-lutidine, 99·93 ± 0·04 mole per cent.

A specimen of 2-ethylpyridine was made synthetically[5] and purified by distillation through a 50-plate column. It boiled at 144·5° to 144·8° C at a pressure of 700 mm of mercury and was of a quality comparable with that of the other three bases. All the spectral measurements were made on a modified Hilger D209 double-beam spectrometer[6] with a rock-salt prism. Scattered radiation was estimated, a 10 per cent. solution of carbon tetrachloride in *cyclo*hexane being used as a shutter; this proved superior to a mica shutter, which

TABLE I

DETERMINATION OF β- AND γ-PICOLINES, 2:6-LUTIDINE AND 2-ETHYLPYRIDINE IN SYNTHETIC MIXTURES

	β-Picoline		γ-Picoline		2:6-Lutidine		2-Ethylpyridine	
	taken, %	found, %	taken, %	found, %	taken, %	found, %	taken, %	found, %
	25·4	26·3	25·3	25·6	24·4	25·2	24·9	25·9
Difference	+0·9		+0·3		+0·8		+0·7	
	29·9	31·4	29·8	30·6	34·6	34·6	5·8	6·2
Difference	+1·6		+0·8		0		+0·4	
	50·3	50·8	20·1	21·1	—	—	29·6	31·1
Difference	+0·5		+1·0				+1·5	
	4·6	4·0	4·6	4·6	—	—	90·8	91·1
Difference	−0·6		0				+0·3	
	89·5	90·2	5·3	5·6	5·2	4·9	—	—
Difference	+0·7		+0·3		−0·3			
	4·7	5·2	4·7	5·7	90·6	90·9	—	—
Difference	+0·5		+1·0		+0·3			
	—	—	91·3	93·6	—	—	8·7	9·1
Difference			+2·3				+0·4	
	9·4	9·7	90·6	90·6	—	—	—	—
Difference	+0·3		0					

NOTE—The cell thickness was 0·516 mm and concentrations were chosen to give, where possible, an optical density of about 0·7 at the relevant key frequencies; in some experiments the best compromise was taken.

has a comparatively low optical density near 800 cm^{-1}. For all measurements the cell thickness was 0·516 mm; scattered radiation contributes about 3 per cent. of the total energy in this region and a corresponding correction was applied to all optical density measurements.

Investigation of the spectra of these four compounds suggested that the intense absorption bands near 800 cm^{-1}, arising probably from the aromatic ring, would be suitable for analytical purposes. The key frequency chosen for β-picoline was 784 cm^{-1}, for γ-picoline 794 cm^{-1}, for 2:6-lutidine 770 cm^{-1}, and for 2-ethylpyridine 745 cm^{-1}. Spectral examination of a dilute solution (1 g of each component in 100 ml of *cyclo*hexane) showed that the separation of the bands was adequate, the high resolution of the rock-salt prism in this region being advantageous.

For pyridine and α-picoline, both of which may be found as impurities in commercial mixtures, the nearest intense band to the key frequencies is at 750 cm^{-1}. Hence little interference will result even from fairly large proportions of these components; they could be estimated jointly by the band at 750 cm^{-1}.

In the adopted procedure the calibration curves for optical density against concentration

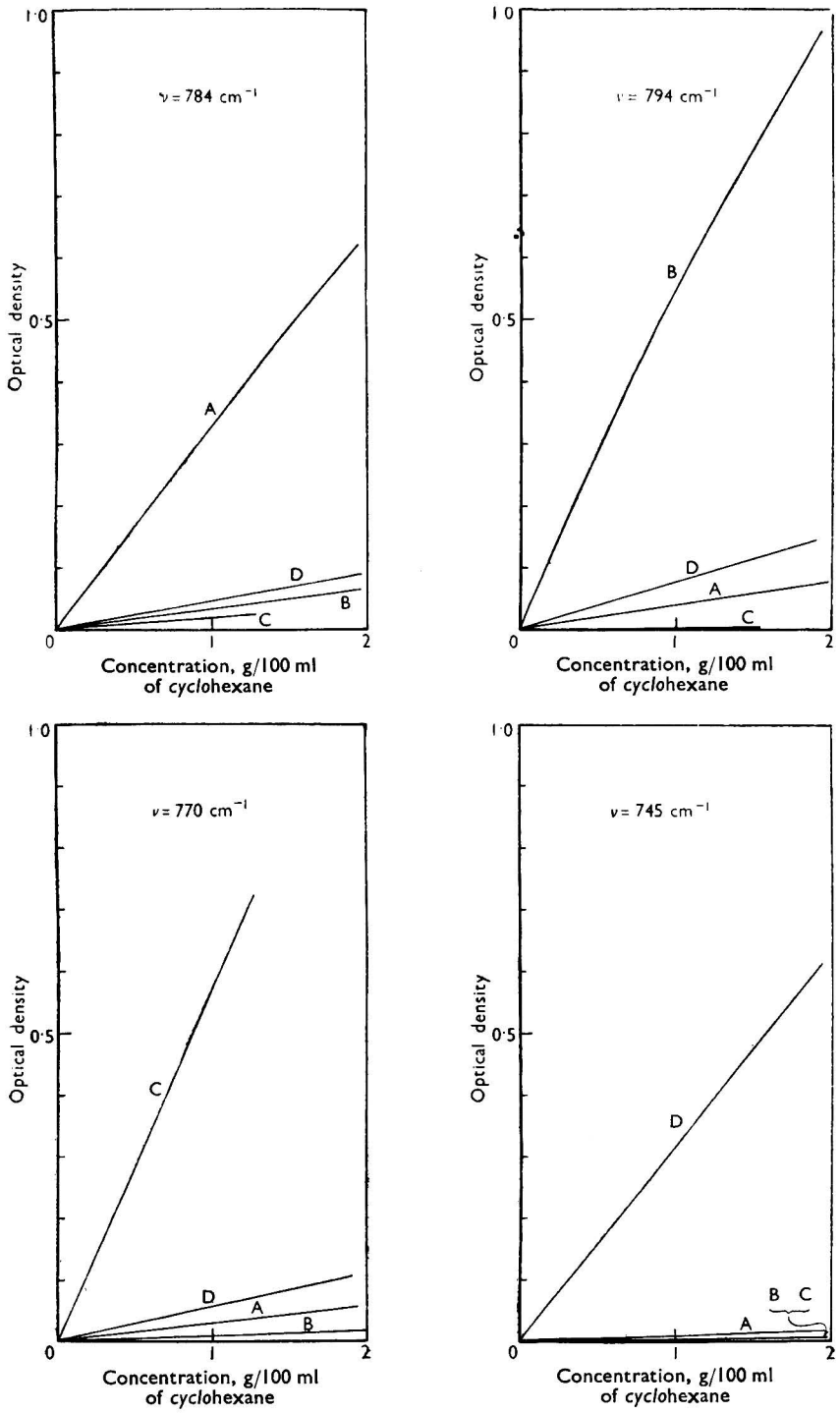

Fig. 1. Calibration curves. Curves A, β-picoline; curves B, γ-picoline; curves C, 2 : 6-lutidine; curves D, 2-ethylpyridine

were prepared (see Fig. 1). Standard solutions of the individual components were made up, and their optical densities measured at the four key frequencies.

A series of synthetic mixtures was prepared and analysed with the aid of the calibration curves, by the method of successive approximations[7]; the results are shown in Table I.

The work described has been carried out as part of the research programme of the Chemical Research Laboratory and this paper is published by permission of the Director.

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The Determination of Aluminium and Zinc after their Chromatographic Separation from Tin - Lead Alloys

BY J. R. BISHOP AND H. LIEBMANN

The determination of small quantities of aluminium and zinc in tin - lead solders is described. The method consists in separating first, by conventional means, the main constituents of the alloy from all trace-metal impurities, subjecting the solution containing these accompanying metals to chromatographic separation on cellulose columns and determining polarographically the aluminium and zinc so isolated.

It is shown that separations are good and that microgram amounts of the two metals in 2-g samples can be determined with reasonable accuracy.

IT has been generally accepted that serious faults in the behaviour of tin - lead solders can be attributed to metallic impurities.[1] Poor wetting by the molten alloy, as well as weakness of the soldered joints, have been ascribed to foreign materials. Lewis,[2] in a recent review, mentions zinc and aluminium as particular offenders in this respect.

Direct proof of the influence of trace constituents is lacking, and this is probably due to the analytical difficulties involved. In a number of instances spectrographic examination of solders has failed to show any difference between satisfactory and unsatisfactory samples, and the conclusion has been drawn that the interfering impurities must be present in amounts smaller than are detectable by this method. Owing to the difficulty of preparing analytical standards, no accurate published data indicating the sensitivity of the spectrographic determination of aluminium and zinc in tin - lead alloys are available. Nevertheless, estimates can be based on the work of Smith,[3] who gives figures for the spectrographic analysis of pure tin. According to his findings, 0·001 per cent. of aluminium and zinc are just detectable. Hence it appears that any investigation into the problem of impurities in solders would require analytical procedures capable of detecting and determining those trace impurities at concentrations smaller than 0·001 per cent.

Separations of trace elements from the main alloying constituents by orthodox methods present great difficulties, owing to co-precipitation and introduction of spurious metallic impurities by reagents. The development of quantitative methods of inorganic chromatography, mainly by workers at the Chemical Research Laboratory, Teddington,[4] seemed to permit of a new approach to the problem of solder impurities. By the use of these techniques, many of the operations necessary for separating one constituent from a complex system can be eliminated, and the separated element can be obtained in exceptional purity. In a

preliminary note from these laboratories,[5] it has been shown that chromatographic separation can be applied to tin - lead solders, and that small concentrations of zinc in these alloys can be determined relatively accurately. This work has now been extended to the determination of aluminium, and the limits for the detection and determination of zinc and aluminium in solders have been established.

Experimental

Although the conditions for the chromatographic separation of metallic ions had been elaborated in detail by other workers, three distinct problems had to be considered in the development of methods for the determination of aluminium and zinc in solders. It was necessary—

(1) to find a method of removing the major portions of tin and lead without removing the accompanying trace metals,
(2) to select solvents giving the most favourable chromatographic separations,
(3) to choose suitable methods for the determination of the metals in question.

These problems had to be attacked simultaneously, but it is convenient to discuss them separately.

Removal of tin and lead—

Tin, together with antimony and arsenic, can be removed from other metals by volatilisation as bromide. The remaining bromides can be converted into chlorides by successive treatment with perchloric acid and hydrochloric acid. The lead chloride is sparingly soluble and is removed easily by filtration. It remained to be shown whether zinc or aluminium could be subjected to these operations without loss by volatilisation or absorption. During the exploratory phases of the work, strip chromatograms were used to examine qualitatively whether any such losses occurred. There was no evidence of loss and it was later confirmed on a strictly quantitative basis that neither zinc nor aluminium was affected by the preliminary separations. Tin and lead are not removed completely, but they remain in amounts comparable with those of some of the accompanying impurities.

Chromatographic separation—

For quantitative work all chromatographic separations were carried out on cellulose columns, and the procedures described by Burstall[4] were adopted with little modification. The metal chlorides were applied to the column and eluted with a solution consisting of a mixture of 4 parts of a constant-boiling solution of hydrochloric acid and 96 parts of *n*-butanol. Under these conditions zinc first moves down the column, followed by iron and copper. Tin, lead, aluminium, nickel, manganese, cobalt and bismuth remain near the top.

If sufficient iron is present in the sample, it forms a visible zone that acts as a marker, and, in the determination of zinc, all of the eluate is collected until the iron band has come through. In practice sufficient ferric chloride solution was added beforehand to ensure the presence of this visible zone. Aluminium is isolated from other metals by eluting with a mixture consisting of 96 parts of methyl ethyl ketone and 4 parts of a constant-boiling solution of hydrochloric acid, which removes all the metals except aluminium and nickel; these were finally recovered by diluted hydrochloric acid (1 + 5). Zinc and aluminium can both be determined in a common sample by a combination of the two methods.

Determination of zinc and aluminium—

Polarographic methods were used for the determination of both metals. For zinc the conventional basal solution of ammonium chloride in ammonium hydroxide solution was used, with gelatin as maximum suppressor. The iron remaining from the marker solution was removed as hydroxide by centrifugation before recording the polarograms. Under these conditions 5 μg of zinc in the final solutions could be detected with certainty, but a measurement of the small wave obtained could lead to errors of the order of 50 per cent. As the whole method was based on sample weights of 2 g, $2{\cdot}5 \times 10^{-4}$ per cent. of zinc would represent the ultimate limit of detectability of zinc that might be achieved by the proposed procedure.

Aluminium was successfully determined by the polarographic method described by Willard and Dean,[6] in which use is made of the complex or compound formed by aluminium with a certain azo-dye. It gives a polarographic wave of a height proportional to the

aluminium concentration. Willard and Dean list nickel amongst the metals that interfere with this determination. It was found, however, that under the conditions finally adopted, nickel produces a separate additional wave, which can be distinguished from the one produced by aluminium. By modifying slightly the conditions laid down in the original paper, the final volume for polarography could be reduced to 2 ml; this made it possible to determine 1 μg with a precision of about 20 per cent. The precision increases rapidly as the concentration of aluminium increases.

The procedure finally adopted for the full analysis is given below.

Method

PROCEDURE—

Removal of tin and lead—Weigh 2 g of solder in the form of fine filings or drillings into a 250-ml flat-bottomed flask, and dissolve with gentle heating in 10 ml of a mixture of 9 ml of concentrated hydrobromic acid and 1 ml of bromine. Add 5 ml of 60 per cent. perchloric acid and boil until a clear colourless melt remains. Dissolve in 70 ml of a constant-boiling solution of hydrochloric acid, boil down to about 20 ml, add 20 ml of water, cool in ice, and filter off the crystalline lead chloride on a pulp pad previously washed with hydrochloric acid, and wash with ice-cold diluted hydrochloric acid (1 + 9). Evaporate the filtrate and washings in a 50 or 100-ml beaker until fumes of perchloric acid cease; cool, take up in about 10 ml of the constant-boiling solution of hydrochloric acid, add an equal volume of water, boil, cool in ice and filter and wash again, collecting the solutions in a 30-ml beaker.

This solution can be used for the aluminium determination; to determine zinc it is desirable to further separate lead chloride, by operations similar to the foregoing, but with even smaller volumes.

Determination of zinc—For the determination of zinc evaporate to dryness the solution in the 30-ml beaker, add 1 mg of iron, in the form of a ferric chloride solution previously freed from zinc by treatment with dithizone, and evaporate again to dryness. (These evaporations are best done under an infra-red lamp.) Take up the residue in the minimum amount of constant-boiling hydrochloric acid solution and add 0·5 ml of *n*-butanol.

Transfer this solution to a cellulose column* prepared according to the method given by Burstall.[4]

Wash the column with 100 ml of eluent prepared by mixing 80 ml of constant-boiling hydrochloric acid solution with 1920 ml of freshly distilled *n*-butanol. This removes residual zinc from the column. By means of a dropper, add the solution of metals to the top of the column. Elute the column, start collecting the eluate when the iron band is 2 inches from the bottom and continue to collect until the iron passes completely out of the column. This solution contains the zinc. Add 15 ml of absolute alcohol and 7 ml of water, evaporate to dryness, cool, and cover the bottom of the beaker with the minimum amount of perchloric acid. Destroy organic matter by heating the covered beaker to fuming, uncover and evaporate to dryness, and then repeat the perchloric acid treatment; take up in a few drops of constant-boiling hydrochloric acid solution and water, transfer to a 30-ml beaker and evaporate again to dryness. Take up the residue in the minimum amount of constant-boiling hydrochloric acid solution and transfer to a centrifuge tube graduated at 1 ml and 2 ml. Make just alkaline to methyl red by adding dilute ammonium hydroxide solution, add 0·33 ml of 0·2 per cent. gelatin solution and make the volume up to 1 ml. Dilute to 2 ml with a solution 1·5 *N* in respect of ammonia and 2·5 *N* regarding ammonium chloride. Centrifuge, decant into a dry micro beaker and record a polarogram between −1·1 and −1·6 volts, using a saturated calomel electrode as anode.

Determination of aluminium—For determining aluminium prepare the column with aqueous hydrochloric acid, so removing any contaminating aluminium at the start. For this purpose mix about 5 g of cellulose powder with 90 ml of diluted hydrochloric acid (1 + 5) and pour this slurry into a silicone-treated glass tube. Allow to settle and then to drain; wash successively with 200 ml of diluted hydrochloric acid (1 + 5), 200 ml of water, 100 ml of freshly distilled methyl ethyl ketone and 100 ml of an eluent prepared by mixing 80 ml of constant-boiling hydrochloric acid solution with 1920 ml of freshly distilled methyl ethyl ketone.

Evaporate to dryness the solution obtained after the lead chloride removals (above),

* The column is made from about 5 g of cellulose powder and is 5 inches long and ½-inch in diameter.

add about 1 ml of constant-boiling hydrochloric acid solution and heat to clarify the solution. Add 25 ml of the ketone and transfer to the column with small amounts of the ketone eluent. Allow to drain.

To remove all interfering metals other than nickel, which remains at the top of the column with the aluminium, elute with 200 ml of ketone eluent. Allow the column to drain completely and reject the eluate. Recover the aluminium and nickel from the column by passing 200 ml of diluted hydrochloric acid (1 + 5). Evaporate to dryness, add 0·5 ml of perchloric acid, cover the beaker and heat to fuming, uncover and evaporate to dryness. Take up the residue in a few drops of constant-boiling hydrochloric acid solution, remove most of this by heating and dissolve the residue in about 10 ml of water. Add a drop of methyl red, carefully make the solution alkaline with 10 per cent. sodium hydroxide solution and add 10 drops in excess. Bring to the boil and filter through a paper filter, which has been previously well washed first with hydrochloric acid and then with water, into a 30-ml beaker. Acidify the filtrate by adding perchloric acid dropwise, evaporate to dryness and take up the residue in 5 drops of water. Make just alkaline with 1 per cent. sodium hydroxide solution and just re-acidify with N perchloric acid. Now add x ml of N perchloric acid, x ml of 2 N sodium acetate solution and y ml of a 0·01 per cent. solution of Solochrome Violet, and adjust volume to z ml, where x, y and z are the volumes specified in Table I.

TABLE I

RECOMMENDED VOLUMES OF REAGENTS FOR ALUMINIUM DETERMINATION

Amount of aluminium expected, μg	x	y	z
0 to 5	0·2	0·5	2·0
5 to 50	1·0	4·0	10·0
50 to 500	10·0	40·0	100·0

Immerse the flask containing the solution for 5 minutes in a water-bath at 55° to 70° C, allow to cool, de-aerate, and record a polarogram between 0 and −0·8 volt against a standard calomel electrode.

RESULTS

As the amounts of metal to be determined are small, it is necessary to observe all the precautions usual in trace-metal analysis. Special attention must be paid to the selection of suitable batches of reagent. Fortunately the reagents required are few, and those used in large quantities, hydrochloric acid, *n*-butanol and methyl ethyl ketone, can be purified easily by distillation. With careful working the blank readings can be kept below or near to the limit of detection.

In order to test the chromatographic and polarographic procedures independently of the method of preliminary separation, solutions containing zinc and aluminium together with other metals were separated on cellulose columns and the metals under investigation were determined polarographically. The recoveries are shown in Tables II and III.

TABLE II

RECOVERY OF ZINC FROM SOLUTIONS CONTAINING 250 μg OF EACH OF ALUMINIUM, NICKEL, MANGANESE, LEAD, COBALT, BISMUTH AND COPPER

Zinc added, μg	Zinc recovered, μg	Recovery, %
600	607	101
600	590	98
500	514	103
500	504	101
500	472	94
500	482	96
100	102	102
50	50	100
25	29	116
12·5	13·5	104
5	5	100
5	4	80

TABLE III

RECOVERY OF ALUMINIUM FROM SOLUTIONS CONTAINING 250 μg OF EACH OF ZINC, NICKEL, MANGANESE, LEAD, COBALT, BISMUTH AND COPPER

Aluminium added, μg	Aluminium recovered, μg	Recovery, %
200*	192	96
150†	153	102
150†	169	113
100*	91	91
20	21	105
15	18	120
10	8·5	85
5	4·5	90
5	4·0	80
5	4·0	80
5	5·0	100

* Solutions containing 500 μg of each of Bi, Pb, Mn, Cu, Fe, Zn and 300 μg of Co.
† Solutions containing 300 μg of Ni in addition to other metals quoted.

TABLE IV

RECOVERY OF ADDED ZINC FROM A TIN - LEAD ALLOY CONTAINING 40 PER CENT. OF TIN

Zinc added, μg	Zinc recovered, μg	Recovery, %
100	101	101
100	90	90
50	56	112
50	55	110
50	52	104
50	51	102
50	47	94
50	52	104
50	50	100
50	51	102
25	23	92
25	28	112
12·5	12·5	100
12·5	16·0	128
5	7	140
5	4	80

TABLE V

RECOVERY OF ADDED ALUMINIUM FROM A TIN - LEAD ALLOY CONTAINING 40 PER CENT. OF TIN

Aluminium added, μg	Aluminium recovered, μg	Recovery, %
40	43·5	109
40	42·5	106
40	40·2	100
40	39·0	97
15	14·8	99
10	10·2	102
5	6·25	125
2	1·9	95
2	1·7	85
1	0·9	90

In further experiments known amounts of zinc and aluminium were added to 2-g samples of a tin - lead alloy containing 40 per cent. of tin. Tables IV and V show the results of these recovery experiments.

The figures reported in the foregoing tables confirm the original assumption that chromatographic separation might form a useful step in the analysis of tin - lead alloys. The main

advantage of the method lies in the fact that the separated trace metals are obtained in a pure form which permits sensitive methods to be used for their final determination. It appears, in fact, that the limits of the analysis are at present determined by the sensitivity of these final methods rather than by the processes of separation.

We are grateful to Mr. J. F. Elton, who carried out part of the experimental work at the later stages of this investigation, and we should like to record our thanks to the Directors of The Metal Box Co., Limited, for permission to publish this paper.

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An Apparatus for the Micro-Analysis of Gas Samples

By D. G. MADLEY and R. F. STRICKLAND-CONSTABLE

The design and operation of an apparatus for the rapid analysis of gas samples of the order of 20 cu. mm is described. The apparatus is simple and can easily be built in the laboratory, but is capable of fairly accurate results. Examples of typical test analyses on known mixtures of gases are given to indicate the accuracy attainable. Carbon dioxide, carbon monoxide, nitrous oxide, hydrogen and nitrogen or methane are amongst the gaseous constituents that can be estimated.

Methods for the analysis of small gas samples are required (*i*) for dealing with low pressure reactions, or other systems in which there is not enough gas available to apply the usual macro methods or (*ii*) when a number of gas samples are to be taken from a system at pressures approaching atmospheric, without altering the conditions in that system.

In general, macro methods can be used when more than 1 ml of sample is available,[1] but there is a need for an apparatus for dealing with much smaller samples. Many ingenious methods have been devised in the past, some of which are described by Farkas and Melville.[2] Except where binary mixtures of gases are concerned, the methods are often elaborate and are generally slow. The apparatus described below is simple in design, construction and operation, and capable of giving fairly accurate results rapidly. It has so far been used for the analysis of mixtures of nitrous oxide, carbon dioxide, carbon monoxide, oxygen, hydrogen, and nitrogen or methane; but certain other gases could also be included. For instance, nitrous oxide could be replaced by a condensable hydrocarbon, and carbon dioxide might be replaced by sulphur dioxide.

Some of the methods described in this paper are based on earlier work of Langmuir,[3] Sihvonen[4] and Meyer.[5] Earlier forms of the apparatus proposed have been described by Strickland-Constable,[6,7,8,9] in which brief descriptions are given of its application to particular gas mixtures. The apparatus has now been further modified by introducing a new form of reagent container, and by the use of "Hopcalite" for the determination of carbon monoxide.*

In its present form, designed for the analysis of a 20-cu. mm sample, the apparatus is shown in Fig. 1. The analysis bulb, P, is connected to a McLeod gauge, Y. The bulb, P, is fitted with two side-arms, each having at its end a special reagent container, R and S.

* Several reagents have been used for the oxidation of carbon monoxide to carbon dioxide at room temperature in the absence of gaseous oxygen. Hopcalite as supplied by Siebe Gorman Ltd. was found most suitable for the purpose.

T is a side-arm containing phosphorus pentoxide, F is a platinum filament, and Q an appendix around which a liquid air or other cooling-bath can be placed. The Wheatstone bridge is only required if mixtures containing methane are to be analysed, otherwise a variable resistance and ammeter are sufficient. The reagent containers are essentially taps having a special plug, with a pocket of about 0·5 ml instead of the usual hole (see Fig. 1, inset *a*). About 0·5 g of "Sofnolite" or "Carbosorb" is placed in S and a similar amount of "Hopcalite" in R. The platinum filament is made of 3·5 cm of 0·1-mm diameter wire. It was found that larger

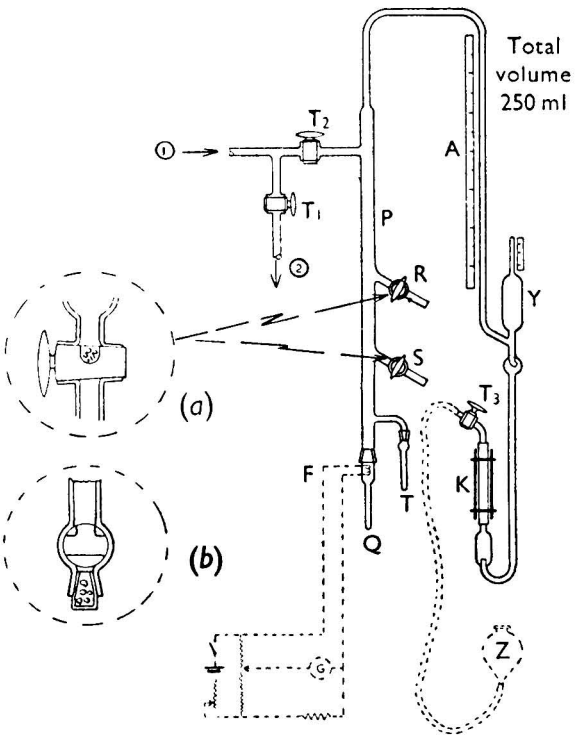

Fig. 1. Apparatus for analysis of 20 cu. mm of gas. The insets (*a*) and (*b*) show details of the reagent containers

filaments caused errors. The McLeod gauge should be of the type shown, in which the mercury is brought to a fixed mark on the compression stem, and the pressure read off the 700-mm scale, A. To measure a pressure, the mercury is brought approximately to the mark on the compression stem by raising the mercury reservoir, Z, tap T_3 is closed and a final adjustment is made by squeezing the length of rubber tubing in the specially made clamp, K. By this final adjustment a movement of about 1 mm of the mercury in the compression stem can be effected. In order to avoid parallax errors, the marks on the stem should be engraved on the glass. This can easily be done by rotating the capillary tube in a lathe and using a sharp glass-knife as the engraver; the whole McLeod gauge can in fact be made in the laboratory without recourse to a professional glassblower. The marks on the stem need not be accurately positioned, but they must be sufficiently close together to enable one mark to be calibrated with reference to the next, by using each mark for the measurement of one pressure. Three or four marks are usually sufficient.* The following dimensions are recommended for the McLeod gauge: capillary compression stem, 5 cm of 0·5-mm internal diameter tube; 50-ml bulb; connecting tubes not less than 9 mm

* It is sometimes convenient to blow small bulbs in the capillary between the marks. In this way a McLeod gauge with a wide range of pressure can be made.

in internal diameter. The particular form of mercury supply to the gauge, which allows of great rapidity of operation, is also important. The success of the whole method depends on the speed and accuracy of pressure measurements with this type of McLeod gauge.

ANALYSIS OF MIXTURE OF CARBON DIOXIDE, NITROUS OXIDE, CARBON MONOXIDE AND NITROGEN—

The operation of the analyser will be illustrated by considering the analysis of a sample containing carbon dioxide, nitrous oxide, carbon monoxide and nitrogen.

Procedure—Pass about 20 cu. mm of the gas into the analyser. The resulting pressure will be about 0·05 mm, and should be measured on the McLeod gauge. Remove the carbon dioxide by turning S so that the Sofnolite is placed in contact with the gas. After about 2 minutes, all the carbon dioxide will have been removed, and a second reading of the McLeod gauge gives the amount. Place liquid air around appendix Q and condense all the nitrous oxide in about 2 minutes (if a condensable hydrocarbon were present instead of nitrous oxide this would be determined by the same procedure). After reading the McLeod gauge again, bring the Hopcalite into contact with the gas for 2 minutes by turning R. The carbon monoxide is thereby oxidised to carbon dioxide, which is removed by Sofnolite or condensed in the liquid air. All the McLeod gauge readings should be duplicated to confirm the complete removal of each constituent.

The principle of this low-pressure analyser depends on the rates of diffusion being so high that rapid contact is established with any reagent present. It is quite unnecessary to circulate the gases through the reagents as in analysis at normal pressures. For this to be true, however, the liquid-air appendix, connecting tubes and reagent containers must not be too small. If the reagent containers are too big, it is necessary to apply time-consuming and uncertain volume corrections. With the dimensions given, no volume corrections are necessary, and yet the rates of diffusion are conveniently rapid. A proposed alternative form of reagent container that would facilitate the changing of reagents is shown in Fig. 1, inset *b*. This consists of a modified three-way tap, one connection of which is a ground-glass socket into which a cone containing the reagent fits. This design allows the barrel of the tap to be evacuated so that it does not contribute to the dead space of the reagent container.

The figures in Table I are shown as an example of test analyses of known mixtures. The time taken for this determination was 12 minutes.

TABLE I

ANALYSIS OF A MIXTURE OF CARBON DIOXIDE, NITROUS OXIDE, CARBON MONOXIDE AND NITROGEN

	Carbon dioxide	Nitrous oxide	Carbon monoxide	Nitrogen
Gas found, per cent. ..	24·5	25·1	24·5	25·9
Gas added, per cent. ..	25·0	25·0	25·0	25·0

We have carried out over 2000 complete gas analyses of mixtures similar to that shown in Table I with this form of apparatus, and have found that provided the usual precautions are taken when dealing with gases at these rather low pressures and the reagents are changed at frequent intervals, the accuracy of the results was always of the order shown in Table I.

ANALYSIS OF A MIXTURE OF CARBON DIOXIDE, CARBON MONOXIDE AND OXYGEN—

The analysis of a mixture of carbon dioxide, carbon monoxide and oxygen is carried out as follows.

Procedure—Introduce the sample and measure the pressure, which should be about 0·05 mm. Remove the carbon dioxide with Sofnolite as in the procedure above. If there is not enough oxygen present to oxidise all the carbon monoxide to carbon dioxide, introduce more oxygen and measure the amount added. Burn the carbon monoxide to carbon dioxide on the platinum filament. The temperature should be just below that at which visible reddening of the wire occurs. All the carbon monoxide is burned to carbon dioxide in about 2 minutes, and the amount originally present is given by the pressure drop. This can be checked by estimating the amount of carbon dioxide formed.

A typical analysis is shown in Table II. The time taken for this determination was 9 minutes.

TABLE II

ANALYSIS OF A MIXTURE OF CARBON DIOXIDE, CARBON MONOXIDE AND OXYGEN

	Carbon dioxide	Carbon monoxide	Oxygen
Gas found, per cent.	25·6	7·1	67·3
Gas added, per cent...	25·9	6·2	67·9

If required, oxygen can be determined on a separate sample by combustion with an excess of carbon monoxide. The use of Hopcalite for the estimation of carbon monoxide is then not possible, since this material adsorbs carbon dioxide and oxygen to some extent.

Smaller samples may be analysed, although with an apparatus of the dimensions given, the accuracy falls off at lower pressures. The analysis of a small sample of a simple mixture is shown in Table III.

TABLE III

ANALYSIS OF A SAMPLE OF VOLUME 1·7 cu. mm AT N.T.P.

	Carbon monoxide	Carbon dioxide	Oxygen
Gas found, per cent.	11·4	36·0	52·6
Gas added, per cent...	12·7	33·4	53·9

MIXTURES CONTAINING HYDROGEN IN ADDITION TO CARBON MONOXIDE—

Hydrogen can be estimated by burning on the platinum filament at the same temperature as for the carbon monoxide determination. All of the hydrogen is oxidised in about 4 minutes, the water formed being absorbed at once in the phosphorus pentoxide in T, and, if carbon monoxide is also present, the amount of each can be found by the usual method of noting the pressure change during oxidation and the further pressure change when the carbon dioxide formed is absorbed in Sofnolite. A result for a mixture of carbon monoxide, carbon dioxide, hydrogen and oxygen is shown in Table IV. The time taken for this determination was 12 minutes.

TABLE IV

ANALYSIS OF A SAMPLE, OF VOLUME 30-cu. mm AT N.T.P., CONTAINING HYDROGEN

	Carbon monoxide	Carbon dioxide	Hydrogen	Oxygen
Gas found, per cent. ..	20·1	6·7	38·5	34·7
Gas added, per cent. ..	18·5	6·9	40·0	34·6

When carbon monoxide and hydrogen are present together, the accuracy falls off somewhat when the initial pressure is less than 0·1 mm.

MIXTURES CONTAINING METHANE—

When methane is present as well as carbon monoxide and hydrogen, it can be determined as an unburnt residue. As in analysis at normal pressures, the combustion of the carbon monoxide and hydrogen must be carried out at a carefully controlled temperature; this temperature should be the lowest at which the carbon monoxide and the hydrogen will burn, which, as already stated, can be roughly gauged as that at which the platinum filament is just beginning to be faintly visibly red in total darkness. For accurate results, the filament should form one arm of a Wheatstone bridge, so that the temperature can be exactly controlled. This method[9] was used throughout a research in which several hundred analyses of mixtures containing methane were carried out. Such a Wheatstone bridge is shown in Fig. 1, but its use is unnecessary when carbon monoxide and hydrogen are to be determined in the absence of methane.

So far it has proved impossible to determine the methane itself by combustion on the platinum filament.

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June 3rd, 1952

Notes

THE COLORIMETRIC DETERMINATION OF CARDIAC GLYCOSIDES

CARDIAC glycosides react with alkaline dinitrobenzene in two stages, with the formation of red and blue colours. The second, blue, stage has already been described[1] and has been applied to the quantitative determination of cardiac glycosides. The blue colour is labile, and the determination must either be done rapidly at a low temperature[2] or the rate of decomposition[3] must be known if the results are to be reproducible.

When the cardiac glycoside and *m*-dinitrobenzene react in the presence of dilute sodium hydroxide or an alkaline buffer at a pH between 11 and 13, a red coloration ($\lambda_{max.}$ 560 mμ) is formed. The blue colouration is produced when the red is treated with more concentrated alkali.

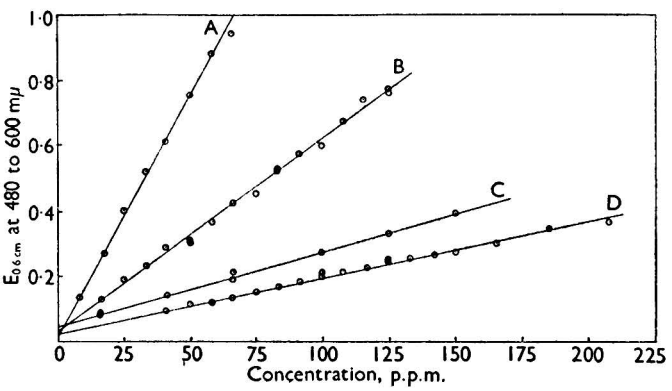

Fig. 1. The absorption of cardiac glycosides. Curve A, dianhydrogitoxigenin; curve B, deacetylanhydro-oleandrin; curve C, convallatoxin; curve D, digitoxin.

We have found that this red colouration can be used for the determination of dilute solutions of cardiac glycosides and aglycones. The colour is distinct from that given by the simple ketones, like acetone and dehydro*iso*androsterone ($\lambda_{max.}$ 520),[4] with dinitrobenzene and concentrated sodium hydroxide solution and the blue colouration appears to be dependent on the butenolide grouping in the cardiac glycoside or aglycone. No colouration is given by iso- and dihydro-derivatives. No blank correction for absorption at 560 mμ by the reagents is necessary.

The red colour is more stable than the blue for all the compounds studied. This stability permits the cardiac glycosides to be determined at room temperature. Optimum conditions for the development of a colour of maximum intensity, stable for at least 1 minute, were determined for each compound studied. The differences in optimum conditions are doubtless related to the variation in the rate of opening of the lactone ring and the formation of iso-compounds, which applies also to the blue colouration (Canbäck[3]). Higher concentrations of alkali produce a more intense colour, but they also accelerate the rate of fading.

THE CHOICE OF WAVELENGTH—

The absorption characteristics of the red solution were determined by means of a Beckman D.U. spectrophotometer. An absorption maximum of 560 mμ was obtained for all the glycosides and aglycones investigated.

THE CONCENTRATIONS OF SODIUM HYDROXIDE AND *m*-DINITROBENZENE—

The optimum concentration of each of these reagents was determined for each cardiac glycoside by comparing the intensity and stability of the colour obtained when different concentrations of one reagent were used with a fixed concentration of the other.

When suitable concentrations of *m*-dinitrobenzene and sodium hydroxide are used there is a linear relationship between concentration and absorption. With a Beckman D.U. spectrophotometer at a wavelength of 560 mμ the calibration curves passed through the point of origin. The results shown in Fig. 1 were obtained by means of an "EEL" direct reading colorimeter with a green filter (No. 404).

METHOD

REAGENTS—

m-*Dinitrobenzene*—Purified according to Callow and Callow's[5] method and made up to 2 per cent. w/v in absolute ethanol. The solution is stable when stored in a brown bottle.

Sodium hydroxide solution—A carbonate-free 0·10 *N* aqueous solution.

PROCEDURE—

The procedure for the estimation of convallatoxin is typical.

Treat an approximately 0·1 per cent. solution of the glycoside in absolute ethanol with 0·3 ml of *m*-dinitrobenzene solution and make up to 1·2 ml with absolute ethanol. Add 0·10 ml of sodium hydroxide solution. Determine the absorption at 560 mμ after 10 minutes by means of a Beckman D.U. spectrophotometer, an "EEL" direct reading colorimeter with green No. 404 filter, or other suitable instrument. Optimum conditions for other typical glycosides are shown in Table I.

TABLE I

CONDITIONS FOR COLOUR DEVELOPMENT WITH GLYCOSIDES AND AGLYCONES

Cardiac glycoside or aglycone	Volume of dinitrobenzene solution, ml	Volume of 0·1 *N* NaOH, ml	Development time, minutes
Deacetylanhydro-oleandrin	0·3	0·1	5
Dianhydrogitoxigenin	0·3	0·1	10
Digitoxin	0·3	0·2	Maximum absorption

We are indebted to Roche Products Ltd. for samples of cardiac glycosides.

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Amended, *September* 25*th*, 1952

Ministry of Food

STATUTORY INSTRUMENT*

1952—No. 2203. The Food Standards (Suet) Order, 1952. Price 2d.

This Order, which came into force on December 28th, 1952, and should be read with the Food Standards (General Provisions) Order, 1944 (S.R. & O., 1944, No. 42; Analyst, 1944, 69, 49), as amended (S.R. & O., 1944, No. 654; Analyst, 1944, 69, 247), prescribes a standard for block suet and revises the standard for shredded suet previously contained in the Food Standards (Shredded Suet) Order, 1944 (S.R. & O., 1944, No. 45; Analyst, 1944, 69, 49), which is now revoked, as follows—

STANDARD FOR BLOCK SUET AND SHREDDED SUET

1. Block suet shall consist of rendered beef suet, shall be free from fibrous tissue and shall contain not less than 99 per centum by weight of beef fat.

2. Shredded suet shall consist of rendered beef suet with farinaceous material. It shall be free from fibrous tissue, shall be shredded, flaked or otherwise comminuted and shall contain not less than 83 per centum by weight of beef fat.

CIRCULAR MF 15/52*

Sulphur Dioxide in Imported Dehydrated Vegetables

This circular (price 2d.), dated November 27th, 1952, amends the circular dealing with Regulation 60 CAA of the Defence (General) Regulations, 1939 (Circular FSL/9/45; in Scotland FSL/9S/45; Analyst, 1945, 70, 134),† by raising the permitted amount of sulphur dioxide in potatoes from 500 to 550 p.p.m., and by extending the table in paragraph 7 of that circular, so as to include certain other imported dehydrated vegetables, as follows—

	Parts per million by weight
Dehydrated Cabbage	3000
" Potatoes (including mashed Potato Powder)	550
" Runner Beans	2000
" Turnips	2000
" Spinach	2000
" Swedes	2000
" Peas	2000
" Mixed Vegetables	2000
" Parsnips	2000
" Carrots	2000

British Standards Institution

NEW SPECIFICATION‡

B.S. 756:1952. Dean and Stark Apparatus. Price 4s.

DRAFT SPECIFICATIONS

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

Draft Specification prepared by Sub-Committee DS/13/1—Hair.
CO(DS) 7782—Draft B.S. for Curled Hair and Curled Hair/Fibre Mixtures, Loose or Layered.

Draft Specification prepared by Technical Committee PVC/3—Oils, Varnishes, Putty, etc.
CO(PVC) 7785—Draft B.S. for White Spirit (Revision of B.S. 245).

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

† This circular, which appeared originally as FSL/2/45 and FSL/2S/45 (*Analyst*, 1945, **70**, 134) was subsequently re-numbered FSL/9/45 and FSL/9S/45.

‡ Obtainable from the British Standards Institution, Sales Department, 24, Victoria Street, London, S.W.1.

Book Reviews

THE MICROBIOLOGICAL ASSAY OF THE VITAMIN-B COMPLEX AND AMINO-ACIDS. By E. C. BARTON-WRIGHT, D.Sc., F.R.I.C. Pp. x + 179. London: Sir Isaac Pitman & Sons Ltd. 1952. Price 18s.

Modern microbiological techniques for assaying nutritional factors have developed during the past 13 years. Progress has been continuous, and to-day methods are available for the determination of the vitamins of the B group and of most of the amino-acids. Barton-Wright has been a worker in this field since the early days and his wide experience of microbiological methods qualifies him to write a practical manual for the guidance of newcomers to the field. That such a manual is needed there can be no doubt; the specificity, sensitivity and wide applicability of these methods are leading to their recognition as valuable analytical tools in the chemical laboratory.

Barton-Wright has adopted an arrangement of the text of his book that might well be followed by the authors of other practical treatises. Each assay is the subject of a separate chapter, within which will be found full working instructions for the method; despite the similarity of many of the basic operations of microbiological assays, the book is not marred by an irritating series of cross references.

In the preparation of a book such as this, which is so eminently practical that it will spend more time on the bench than on the bookshelf, more than usual care should be taken to avoid the occurrence in the text of errors and discrepancies. The need for such care becomes intensified when the book, like the one under review, relates to a relatively new sphere of analysis and is likely to be used as a working manual by analysts with no previous practical experience of the techniques that it embraces. It is to be regretted that this degree of care has not been taken during the preparation of Barton-Wright's book, with the result that a number of errors appear in the text. These are mainly omissions from the lists of ingredients of basal media, such as the omission of biotin on page 47; the omission of glucose, sodium acetate and sodium chloride on page 48; the omission of tryptophan on page 58; and the omission of salt on page 74. These omissions occur only in the compositions of media expressed on a percentage basis and are not repeated in the instructions for the preparation of the media. They are not likely, therefore, to lead a worker astray, but their occurrence is nevertheless a disturbing feature in a book that is pre-eminently a working manual. A more serious error is the omission of biotin from the list of ingredients quoted in the instructions for preparing the basal medium for the assay of methionine, cystine and tyrosine. Another omission that has escaped the proof-reading is the absence of instructions on page 42 to filter the medium after adjusting its pH to 4·5.

In the chapter on pantothenic acid, the author states that the various methods in use for the extraction of this vitamin must now be discarded in favour of an enzymatic method relying upon hydrolysis by a mixture of an alkaline phosphatase and an enzyme separated from chicken liver. The text then describes how the latter enzyme is prepared, but leaves the reader to refer to the original reference for the method and preparation of the alkaline phosphatase, although without this the chicken-liver enzyme is useless.

The references appended to more than 20 figures in the book, which have been reproduced from original papers, give only the names of the journals from which the figures have been abstracted; in no instance is mention made of the volume number, the page number or even the year of publication.

Despite the imperfections to which the reviewer has drawn attention, the book is of undoubted value to the worker in the field of microbiological assays and can save the time and trouble involved in making a search of original papers. The working instructions are given in detail and can be commended for their clarity. The value and reliability of the book would be enhanced by the correction of the omissions already mentioned, and it is to be hoped that this will be attended to in the next edition. The call for another edition will also provide opportunity to remedy two weaknesses of the present book, namely, the absence of methods for assaying vitamin B_{12}, and particularly the absence of information and guidance upon the application of statistical computations to the results of microbiological assays. To-day, more than ever before, it is necessary for the analyst to be able to quote the fiducial limits of a result he obtains by biological analysis.

A. J. AMOS

ASSOCIATED MEASUREMENTS. By M. H. QUENOUILLE, M.A. Pp. x + 242. London: Butterworths Scientific Publications. 1952. Price 35s.

Many different kinds of research, and not least analytical research, involve an examination of the extent to which two or more series of measurements are associated. Animals may be given graduated doses of a drug and their response measured in terms of some chemical or physical quantity; two analytical methods may be compared by applying them both to a series of samples; analyses of a large number of fruits or vegetables may be compared with observations of such factors as rainfall and temperature in an attempt to detect causal relationships. The usual textbooks of statistics give some discussion of the methods to be used in such cases, but Mr. Quenouille's book is the first to deal comprehensively with the matter.

And comprehensive it certainly is. The first section of 46 pages deals with graphical methods, in which one simply plots one measured quantity against the other and examines the result. Many interesting tests are described, some quite easy to apply, to provide an objective estimate of whether there is significant association, and the *graphical* fitting of the estimated "best" straight line or curve to a series of points—that is to say, by the intelligent eye rather than by an algebraic process—is well dealt with. Then follow 48 pages on numerical methods, including the analysis of covariance, the calculation of regression and correlation coefficients, multiple regression equations and curvilinear association. The next 45 pages are devoted to a commendable exposition of rapid methods for cutting down the arithmetical labour of these statistical operations, and there is finally a section of 59 pages dealing with special problems, including time series analysis and multivariate analysis. The book ends with a bibliography and an appendix of tables, many of which will not be found in other textbooks.

There is a wealth of numerical illustrations, and although few of these have been taken from the field of analytical chemistry, most problems coming the analyst's way will be found to have their analogues here. The book does not constitute light reading; but most of it requires concentration and a refusal to be frightened by algebra rather than a deep knowledge of mathematics, and the graphical section in particular could be read with pleasure and profit by any chemist. The book is well bound and published, it does not appear to contain any serious misprints and is by no means expensive as prices are to-day.

E. C. WOOD

A HANDBOOK OF SHELLAC ANALYSIS. By M. RANGASWAMI, B.A., A.I.I.Sc., and H. K. SEN, M.A., D.I.C., D.Sc., F.N.I. Second Edition. Revised by G. N. BHATTACHARYA, D.Sc., and P. K. BOSE, D.Sc., F.N.I. Pp. viii + 144. Bihar, India: Indian Lac Research Institute. 1952. Price 4Rs. 8ans.

Ten years have elapsed since the first edition of this book was well received and appreciated as a worthy attempt of the Lac Research Institute to bring some order into what was then, and still is, a rather chaotic chapter in organic chemical analysis.

The section of the book in which specifications are recorded seems to be much larger than before and has more participants; in all, 15 specifications are sponsored by the Government of India, the Indian Standards Institution, the Indian Stores Department, the U.S. Government, the American Society for Testing Materials, the U.S. Shellac Importers Association, the U.S. Bleached Shellac Manufacturers Association and the British Standards Institution, and, in the offing but not yet realised, there is an International Standard. Most of the above organisations and numerous others have also invented lac tests covering one analytical aspect or another. It is to be hoped that one day someone will have courage enough to say, after the manner of the famous war-time poster, "Is your specification really necessary?" and to do something about it if the answer is not satisfactory. It might then be found possible to transfer some of the many varied specifications and tests to the museum case, whereupon life for lac merchants and users would become simpler altogether.

In the meantime the authors have brought together nearly everything relating to tests, either in use or suggested, both chemical and physical in character. The arrangement is one chapter, one test; a method that facilitates comparison of the different methods and must surely lead eventually to the obvious goal, namely, the elimination of more or less private purchasing specifications and tests for lac.

As to the contents of these chapters on tests, which are very well done, only three comments will be offered, *viz.*, (*a*) for the determination of water no great confidence seems to be felt in the Karl Fischer method; (*b*) for the determination of acid and saponification values in non-aqueous solutions surely the glass-electrode technique has a place; and (*c*) in the paragraphs dealing with the determination of thiocyanogen number (miscellaneous tests) and its use as a basis for rosin

estimation, there is some uncertainty of expression about the relationship between the iodine value and the thiocyanogen number for rosin.

The authors are to be congratulated on the production of a practical and most serviceable handbook. L. A. JORDAN

THE CHEMISTRY OF SYNTHETIC DYES. Volume I. By K. VENKATARAMAN. Pp. xvi + 704. New York: Academic Press Inc. 1952. Price 116s.; $14.50.

There has been no general book on the subject of synthetic dyes since the days of "Cain and Thorpe." During this period progress in this important field has not been static and the time was fully ripe for a new work on this topic. The reports on the German dye industry have made generally available information that may have been known, at any rate in part, to the experts; that source has been freely drawn upon, but not so as to underestimate the contributions of other countries.

The opening history of the development of the dye industry is brief but effective; the outline of the primary raw materials includes a list of 217 cyclic compounds present in coal-tar—to which can now be added tetrahydrothiophen and 2:3:4:6-tetramethylpyridine. Dyes cannot be produced without an adequate supply of the necessary intermediates, the development of which may often involve, as Rowe stressed in 1938, greater difficulties than those of the dyes themselves. The allocation of 160 pages to a discussion on intermediates of a general character is therefore not excessive; those of a highly specialised kind are included later with the particular dyes for which they are needed.

Diazotisation is a process of special importance since dyes of the azo-class outnumber those of any other, so this subject merits general discussion, together with the resulting diazonium compounds. The classification of dyes on the basis of chemical constitution is given briefly, followed by that according to dyeing properties; with the latter are listed the commoner commercial class names. Methods of dyeing the various textile fibres naturally follow together with fastness tests and non-textile uses of dyes. Colour, its measurement and relation to chemical constitution are dealt with on general lines at some length.

Then comes the main subject and, as emphasis is mainly on the chemical side, a particular dye is usually referred to by a single name; multiplicity of names is acknowledged but we must await the forthcoming edition of the Colour Index for their unravelling. Four pages each suffice for the nitroso- and the nitro-dyes; the remaining 296 pages are devoted to the azo-dyes in their various aspects. The first chapter here is of a general nature and reviews the coupling reaction, the constitution, colour and dyeing properties of these dyes; in addition, their classification leads directly into the subsequent chapters, *e.g.*, on mono- and bis-azo-dyes, mordant azo-dyes, metal-dye complexes, tris- and poly-azo-dyes, urea and cyanuric derivatives, direct cotton dyes after-treated on the fibre, pyrazolones, thiazoles, stilbene dyes, dyes for cellulose acetate and the azoics. The extent of the discussion naturally varies much from chapter to chapter.

There are some minor flaws. Among the intermediates, there is an unnecessary tendency to digress into other fields of technology; Orema dyes are on the market to-day, although they are stated (p. 293) to have been withdrawn; the table on p. 660 purports to indicate those compounds which are only marketed as "Fast Salts," though in fact it does not; the expression (p. 357) "a pH range of −10 to +17" looks odd; there is the occasional misprint, *e.g.*, (p. 29) "prehinitene." Still, this is a useful, timely book, well documented and nicely produced, although the absence of an index to this single volume is a drawback. B. A. ELLIS

Publications Received

A TEXTBOOK OF PHARMACOGNOSY. By GEORGE EDWARD TREASE, B.Pharm., Ph.C., F.R.I.C., F.L.S. Sixth Edition. Pp. viii + 821. London: Baillière, Tindall and Cox. 1952. Price 37s. 6d.

STATISTICAL METHODS FOR CHEMICAL EXPERIMENTATION. By W. L. GORE. Pp. xii + 210. New York and London: Interscience Publishers Inc. 1952. Price 25s.; $3.50.

MALEIC ANHYDRIDE DERIVATIVES. REACTIONS OF THE DOUBLE BOND. By LAWRENCE H. FLETT and WILLIAM HOWLETT GARDNER. Pp. x + 269. New York: John Wiley & Sons Inc. London: Chapman & Hall Ltd. 1952. Price 52s.; $6.50.

INTRODUCTION TO THE STUDY OF PHYSICAL CHEMISTRY. By LOUIS P. HAMMETT. Pp. xii + 427. New York and London: McGraw-Hill Book Co. Inc. 1952. Price 51s.; $6.00.

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Assistant Male or Female required for Analytical Laboratory at Loughton, Essex (Central Line). Applicants should preferably be of at least Inter. B.Sc. standard. Previous analytical experience an advantage but not essential. Salary according to age and qualifications. Details to works Manager, Ozalid Co. Ltd., Lenthall Road, Loughton, Essex.

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CONTENTS

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