A CRITICAL INVESTIGATION INTO THE METHODS OF DETERMINING SULPHUR IN PLANT MATERIAL

A thesis submitted in part-fulfilment of the requirements for the degree of Master of Science

by

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1961.

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ACKNOWLEDGEMENTS.

The author wishes to express his gratitude and appreciation to the following:

Dr. U.J.A. Steyn, Ph.D., for his able direction of this thesis and constructive assistance at all times.

Professor W.F. Barker, Ph.D., for his assistance in procuring certain essential chemicals.

Professor L.D.C. Bok, Dr. Rer. Nat., for his generous assistance in the latter part of this research work.

The Staff of Rhodes University Library, for obtaining journals and microfilms of articles unobtainable in the Chemistry Department Library.

The Technical Staff of Rhodes University Chemistry Department and, particularly, Messrs. F. van der Water and G.W. Randell, for their assistance at all times.

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The author is indebted to the Council for Scientific and Industrial Research for a Research Grant (1960 - 1961) without which this work could not have been undertaken.

INTRODUCTION

Although sulphur is one of the more abundant elements present in plants, its importance as a plant nutrient has been underestimated until comparatively recently. Scientific literature over the past few years, however, shows that interest in the determination of sulphur in natural waters, soil and plant materials has been renewed.

Perhaps the main reason for the non-recognition of the importance of sulphur as a plant nutrient is the fact that sulphur-deficiency seldom occurs since sufficient sulphur is usually added to the soil in rainfall (particularly near industrial towns) to supply all the sulphur requirements of plants. In addition, sulphur is frequently present in fertilizers added to soils to counteract deficiency in other elements, especially phosphorus. Superphosphates, for example contain up to 60 % gypsum. Sulphur is also added to the soil in some instances in order to reduce the soil pH, the elemental sulphur being fairly readily oxidised in the soil to sulphuric acid. The organic fraction in the soil also contains sulphur.

Sulphur occurs in plants in a number of organic compounds, one of the more important being cystine. It also occurs in many plant proteins and in the respiratory pigment glutathione, which appears to act as an oxidation-reduction enzyme in sugar oxidation. Mustard-oil glycosides, organic storage compounds, often impart the distinctive odours and flavours to certain plants e.g. garlic, onions and mustard.

Symptoms of sulphur-deficiency often resemble those of nitrogen-deficiency. Cells are smaller and their walls thicker, there

is a relatively high proportion of fibers and lignified tissue, carbohydrates accumulate and nitrate nitrogen increases, organic sulphur becomes water-soluble and the cells lose some of their protoplasm. There may be interference with coll-division and fruiting. The entire plant is usually light green to yellowish green in colour. The chlorosis is most pronounced in young leaves, which become yellow.

Positive effects of adequate sulphur supplies include chlorophyll development (although chlorophyll does not contain sulphur), an increased root or absorbing system, longer stems and thicker leaves. In leguminous crops, there is generally an increase in the number of root-nodules, this being generally attributed to the action of sulphates on the legume bacteria. It might be mentioned here that sulphates, like nitrates, are easily leached from soils and severe losses of this nutrient occur during erosion and during drainage in wet climates.

There is thus sufficient evidence of the need for a rapid, accurate and reproducible method of determining sulphur, suitable for application to the routine laboratory analysis of plant and soil material. It would appear that investigations should be carried out on the possibilities of adapting existing methods to the analysis of plant and soil materials.

Over the years a number of different methods to replace the tedious and inaccurate gravimetric procedure have been investigated. Particular attention has been given to the volumetric procedures. However, most of these methods have disadvantages, being either too long or lacking in sensitivity with low sulphate concentrations. Thus

sulphate may be determined using benzidine hydrochloride (1) but the determination involves a filtration step and a blank correction for low sulphate-ion concentrations. Another volumetric procedure (2) uses barium chromate to precipitate the sulphate, the residual chromate being determined titrimetrically with thiosulphate. However, the precipitate must be removed by filtration before the titration step is possible and a calibration curve must be drawn to obtain accurate results. For the direct titration of sulphate with barium chloride solution, the internal indicator, disodium tetrahydroxyquinone, may be used (1) but, owing to the relatively slow formation of the barium sulphate precipitate at low sulphate concentrations, the technique is limited to solutions with high sulphate-ion concentrations. Other volumetric procedures include a photometric titration using lead nitrate and alcohol (3), reaction with barium iodate followed by iodometric titration (4), and treatment with stannous chloride followed by determination of the hydrogen sulphide iodometrically (5).

A polarographic method (6) involves the reduction of the sulphur to hydrogen sulphide, followed by the precipitation of cadmium sulphide which is then dissolved in hydrochloric acid and the cadmium determined polarographically. There are several methods of determining sulphur after reduction to hydrogen sulphide. Inter alia, there is the method involving the reduction by hydriodic acid and final colorimetric determination of the hydrogen sulphide by the methylene-blue reaction (7). This is probably the most sensitive of all the methods and investigations carried out on this method are recorded in Part II.

Another recent colorimetric method is the determination of the coloured acid-chloranilate ion liberated from barium chloranilate by the sulphate ion (8).

The rapid development of the complexometric titration methods using ethylenediaminetetra-acetic acid (E.D.T.A.) has led to indirect methods for the determination of sulphur by either determining the excess barium remaining after a known excess of barium chloride solution has been added to precipitate the sulphate (9), or dissolving the barium sulphate precipitate in ammoniacal standard E.D.T.A. solution and back-titrating the excess E.D.T.A. with standard magnesium solution (10, 11).

It would obviously have been impossible to investigate all the methods of determining sulphate and the author has confined himself to these last three methods, which seemed to offer the greatest possibilities in the field of plant analysis. The methods have been thoroughly tested and statistically compared as far as was possible.

Apart from the criteria of accuracy, speed and reproducibility, the time required for the laboratory worker to become thoroughly conversant with the techniques and economic considerations must also be taken into account. It is unfortunate that the methods of determining sulphur all appear to suffer in one or more of these respects.

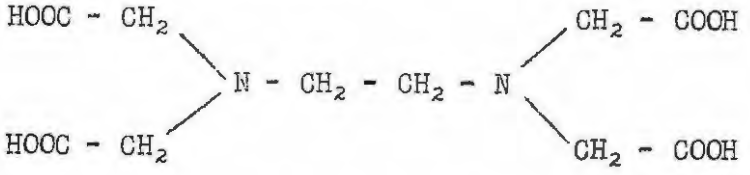
P A R T I

THE COMPLEXOMETRIC DETERMINATION OF SULPHUR.

1. REVIEW OF PUBLICATIONS ON THE COMPLEXOMETRIC METHODS.

1.1 The Properties of Ethylenediaminetetra-acetic acid and its salts.

Gerold Schwarzenbach started work on the metal complexes of aminopolycarboxylic acids or complexones in 1944 and since that date has published a number of papers on the subject of complexones. A large number of complexones have been developed, such as nitrilo-triacetic acid (NITA), ethylenediaminetetra-acetic acid (EDTA), diethylenetriaminepenta-acetic acid (DTPA) and diaminocyclohexane-tetra-acetic acid (DCYTA), to name only four. However, as it has proved most generally suitable for complexometric work, we shall consider only EDTA or more strictly its disodium salt, disodium-ethylenediamine-tetra-acetate or Versene (also generally referred to as EDTA). The acid has the following structure:-



This acid forms 1:1 complexes with a wide range of metals, the structures of which involve a number of five-membered rings. The acid has four replaceable hydrogen atoms and the resulting carboxyl groups together with the nitrogen atoms provide six atoms available for co-ordination to a metal cation. In general, the stability of the complex increases with an increase in the valency of the metal and most univalent metals are unable to form stable complexes. Use can occasionally be made of this to prevent complex-formation by reducing a metal

to a lower valency state. The stability also tends to decrease as the pH is lowered; thus, at low pH, only tri- and tetravalent metals will form complexes.

The disodium salt dihydrate is generally used in the laboratory as it is very much more soluble in water than the acid. It is easily obtainable as a pure material suitable for use as a primary standard. The two molecules of water tend to come off above 70°C and the salt should not be dried above this temperature.

In 1948, Schwarzenbach and Biedermann (12) titrated calcium and magnesium with EDTA and determined the end-point by measurement of pH changes. Later, Biedermann and Schwarzenbach (13) solved the problem of accurate end-point detection by the use of the dye Eriochrome-black T which, like EDTA, has the property of forming slightly ionized compounds with the alkaline earth metals. On the basis of these researches, Schwarzenbach and Ackermann (14, 15) developed a method for the determination of hardness in water. Schwarzenbach noted that calcium formed a weak unstable complex with the dye which did not permit solutions containing only calcium to be titrated with EDTA alone. However, by the inclusion of a small amount of magnesium in the titrating solution or the alkaline buffer, the end-point could be sharpened (15). More recent researches have confirmed the use of EDTA for determining hardness in water (16, 17, 18, 19).

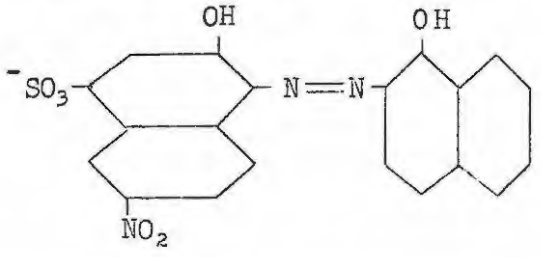
In 1949, the method for calcium and magnesium determination was applied to plant materials (20) but appeared to be unsuitable. Since then, the initial difficulties have been overcome and the method can now be used successfully for the determination of calcium and

magnesium in plant material.

In 1950, Munger, Nippler and Ingols (9) suggested that, since the EDTA method could be successfully applied to the determination of barium ions in solution, its use might possibly be extended to the determination of low sulphate-ion concentrations by titrating the excess barium ions after a known excess of barium chloride had been added to the sulphate solution.

1.2 The Choice and Stability of Indicators.

Munger, Nippler and Ingols (9) used a solution of Eriochrome Black T (Solochrome Black, Eriochromeschwartz T, F-241) in ethanol. This is also the indicator used by Schwarzenbach (13) in his early researches. It has the structure



It is an acid-base indicator with two sensitive regions of colour change. The equilibrium is as follows:-

$$H_2In^- \underset{\text{Wine-red (below pH 6)}}{\overset{\text{pK 6·3}}{\rightleftharpoons}} HIn^{--} \underset{\text{Blue}}{\overset{\text{pK 11·5}}{\rightleftharpoons}} In^{---} \text{ Orange (above pH 13)}$$

Belcher, Gibbons and West (10) stated that the ethanolic solution had to be discarded after three to four days. Goetz, Loomis and Diehl (21) recommended an aqueous solution of the dye mixed with hydroxylamine hydrochloride. Betz and Noll(17, 22) made up their

indicator solution in aqueous isopropyl alcohol with the addition of sodium carbonate, and claimed that it was stable after eight months. Diskant (23) carried out extensive investigations on the stability of the indicator and found a solution of the dye in diethanolamine or triethanolamine to be stable. A solution of the dye with sodium borate in methanol was used by van Thiel and Tucker (24).

Flaschka and Schöniger (25) have recommended a powdered mixture of the solid dye with sodium chloride and this was confirmed by Manns, Reschovsky and Certa (26) who found both the aqueous and alcoholic solutions of the dye unstable. Sporek (27) used ammonium chloride in place of sodium chloride.

Eriochrome Black T has also been used, screened with other dyes in order to obtain a clearer end-point. Belcher and his co-workers, for example, used dimethyl yellow (28). McCallum (29) used a mixed indicator of Phthalein purple, Methyl Red and Diamine Green B (Dianil Green) in water. This indicator was proposed by Pribil in 1954.

Other indicators which have been used are Naphthol phthalein (30), and Calcein (31).

1.3 Variations in the Method of Determination.

Variations in the method of Munger, Nippler and Ingols (9) may be divided into two groups; those in which the barium sulphate precipitate is dissolved and the barium ions determined, and the methods in which the excess barium ions after precipitation are determined.

Where Munger and his co-workers added magnesium ions separately, Manns and his co-workers (26) incorporated magnesium and

calcium ions in the standard EDTA solution. Similarly, Ueno and Yamaguchi (32) included zinc and magnesium ions in the titrating solution. Tettweiler and Pilz (33) added a little zinc complexonate before titrating with titriplex (versenate) while Langford (34) (33) precipitated the sulphate by the addition of a solution containing both barium and magnesium ions.

Many research workers preferred to remove the precipitate by filtration prior to titration with EDTA but, with low sulphate concentrations, they found that the presence of the precipitate had little effect on the clarity of the end-point.

Bakacs-Polgar (35) suggested shaking the solution, after precipitation, with carbon tetrachloride. The barium sulphate was transferred to the CCl_4 layer and the colour of the indicator could be clearly seen in the aqueous layer.

Casini (30) used tetrasodium EDTA and Naphtholphthalein as indicator at a pH 4-6, while McCallum (29), using a mixture of phthalein purple, methyl red and dianil green as indicator, carried out the titration in 50% ethanolic solution and back-titrated with EDTA, no magnesium being required.

Belcher and his co-workers (10) filtered off the $BaSO_4$ precipitate (after ageing it overnight) on a paper pulp pad. The precipitate was dissolved by boiling with ammoniacal EDTA solution and the excess EDTA was determined with standard magnesium chloride solution. Jackson (36) suggested the use of very small filter paper circles in place of the filter pulp pad. Later, Belcher and his co-workers (11) filtered off the precipitate using sintered glass crucibles.

Mukai and Goto (37) followed the method of Belcher (10) but back-titrated the excess EDTA with standard barium chloride solution using metal phthalein indicator. Rumler and his co-workers (38) suggested the replacement of ammonium hydroxide (for dissolving the $BaSO_4$ precipitate) by sodium hydroxide, ethanolamine or triethanolamine, to speed up the analysis. Sporek (27) precipitated the sulphate ions as lead sulphate in 50% isopropyl alcoholic solution and, after dissolution of the precipitate, back-titrated the excess EDTA with standard zinc solution.

1.4 Removal of Interfering Ions.

Whereas anion exchange proved to be of considerable value in the removal of anion interference, and particularly phosphate interference, in the determination of cations in plant material, the method is not applicable in the determination of sulphate since part or all of the sulphate ions are removed together with phosphate and other interfering ions.

Collier (39) extracted phosphate ions as the phosphomolybdate complex using a 1:1 butanol-chloroform mixture. Smith and McCallum (40) used ferric chloride to precipitate the phosphate ions at pH 3-4 but, as this required very careful control of the pH and necessitated the complete removal of any excess ferric ions, Padhye (41) recommended the precipitation of the phosphate using zirconium nitrate. However, this method is not applicable to sulphate determination since zirconium forms stable complexes with the sulphate anion and Palaty (42) recommends the removal of fluoride and phosphate ions as the insoluble lanthanum salts.

There can be little doubt, however, that ion exchange is the most satisfactory method of removing interfering cations and this is confirmed by its frequent use in procedures given in the literature.

1.5 Storage of Solutions.

Goetz and his co-workers (21) found that EDTA solution concentrations changed with time but found that at pH 5 they were reasonably stable in one gallon bottles over a period of up to four months. Bruno Riva (43) stored all standard solutions in polythene bottles to avoid solution of calcium from the glass of aspirators. Flaschka and Sadek (44) discussed the effects of storing solutions in glass and recommended storage in plastic bottles or hard-glass bottles which had had 1% versenate in 2% sodium hydroxide boiled in them, followed by thorough washing with distilled water.

As far as possible, all solutions used in the work recorded in this thesis were kept in polythene bottles.

1.6 Critical Review of Published Complexometric Methods using Disodiumethylenediaminetetraacetate.

In 1950, Munger, Nippler and Ingols (9) applied the complexometric method of alkali-earth determination by means of EDTA to the determination of the sulphate ion. After determining the hardness with EDTA and the alkalinity with HCl solution, standard acid, equivalent to the alkalinity, was added to a 50 ml. sample to destroy carbonates. The solution was then boiled, 0.02 N $BaCl_2$ solution was added according to the estimated sulphur content, the solution was

again boiled for a few seconds and then cooled. A buffer solution (pH 10) and Eriochrome Black T indicator solution were then added and the solution titrated with standard EDTA solution. The first end-point was not used but a small quantity of standard magnesium solution was added and the solution titrated to the second end-point. The sulphur content was hence determined by calculation. The method was, however, subject to a number of interferences due to foreign ions and the end-point was still by no means easily distinguishable.

In 1953, Langford (34) adapted this method to the determination of sulphate in nickel-plating solutions, using a solution containing 0.2 N $BaCl_2$ (to precipitate the sulphate) and 0.1 N $MgCl_2$ (to improve the end-point). KCN was used to complex heavy metals. Tettweiler and Pilz (33) added zinc-complexonate to the solution before titrating to the end-point. Sijderius (45), in order to avoid the addition of a fixed amount of magnesium solution, added dipotassium magnesium EDTA to the buffer solution used in the method. He removed calcium and magnesium by ion exchange and found it necessary to age the $BaSO_4$ precipitate overnight since low values for the sulphur content were obtained if the titration was carried out immediately after the precipitation. This considerably lengthened the procedure. Fitzgerald (46) added to his buffer solution magnesium chloride solution which he initially sequestered with EDTA.

In 1954, Belcher, Gibbons and West (10) described a procedure in which the barium sulphate precipitate was filtered off using a paper-pulp pad after the precipitate had aged overnight. The pad was well washed, after which it was transferred to a flask, the $BaSO_4$

dissolved in ammoniacal EDTA solution and the excess EDTA back-titrated with standard magnesium chloride solution to a clear red end-point. In the same year, Jackson (36) suggested that, in place of the paper-pulp pad, a 21 mm. circle of No. 42 Whatman filter paper be used in a Gooch crucible, as this paper would readily be disintegrated in the boiling ammoniacal EDTA solution. Mukai and Goto (37), in 1957, used metal phthalein indicator in the titration of the dissolved barium sulphate precipitate.

In a later paper, Belcher, Gibbons and West (11) filtered off the barium sulphate precipitate by means of a sintered glass crucible and, after thorough washing and after the addition of a two-fold excess of EDTA, titrated the filtrate with magnesium chloride solution in the manner described in their earlier paper (10).

In view of the uncertainty of the colour-change with the Eriochrome Black T indicator, McCallum (29) proposed the use of an entirely new indicator comprising Phthalein Purple, Methyl Red and Diamine Green B (dianil green) in water. After precipitation of the barium sulphate and ageing for at least an hour, the solution is neutralised and alcohol added to make the solution 50% alcoholic. After addition of 5 ml. concentrated ammonia solution and some indicator solution, the solution is back-titrated with standard EDTA to the sharp colour-change from deep blue to permanent yellow-green. A very clear end-point was obtained and the use of added magnesium was avoided. One of the main disadvantages was the use of large quantities of ethanol in the method. Another is the short "shelf-life" of the indicator, given by McCallum as one week.

In 1958, Sporek (27) used lead nitrate to precipitate the sulphate, decreasing the solubility of the lead sulphate by working in 50% isopropyl alcohol-aqueous solution. The precipitate was dissolved in a fritted-disc filter funnel by means of ammoniacal EDTA and the excess EDTA back-titrated with zinc chloride solution. A solid mixture of 0.1% Eriochrome Black T indicator in ammonium chloride was used. His results indicated that, whereas there was little or no interference from reasonable amounts of arsenates, iron and uranium, the presence of phosphate resulted in high values for the sulphur-content. His results for uranate-containing ores were also high. The main advantage he claimed was that the method required from 30 to 45 minutes as opposed to the normal 1 to 2 days. Isopropyl alcohol was used in the method since, the higher the alcohol in the homologous series, the more it reduced the solubility of the $PbSO_4$.

To replace ammonia in the method involving the dissolution of the $BaSO_4$ precipitate, Rumler, Herbolsheimer and Wolf (38) advocated the use of mono- or tri-ethanolamine or sodium hydroxide with EDTA to dissolve the precipitate in order to speed up the procedure. In all these methods, however, the difficulty of dissolving the precipitate quantitatively and readily is the main drawback.

In the same year, 1959, Bakacs-Polgar and Szekeres (47) described a procedure for determining phosphorus and sulphur in the same sample. They worked in aqueous alcoholic medium at pH 10 in the presence of Eriochrome Black T as indicator. Initially, the phosphate was titrated with magnesium chloride solution when the phosphate precipitated as $NH_4.MgPO_4.6H_2O$. The first drop of excess standard

magnesium chloride solution was detected by the red-violet colour of the indicator. The original blue colour of the indicator was restored by means of a few drops of EDTA solution after which the sulphate was titrated with a barium chloride solution. At the end-point the excess barium ions replaced the magnesium ions from the magnesium complexone and the indicator showed a red-violet colour. There were numerous precautions to be observed with regard to quantities of reagents and, notwithstanding the observance of these precautions, the present author was unable to achieve any results with this method.

Recently, the same authors (48) have described a method for the simultaneous determination of phosphate and sulphate ions in the presence of metal contaminants. Ferric and aluminium ions were masked with DCTA (DCYTA, see page 5) at pH 2-3. The pH was raised to 10-11, Eriochrome Black T was added and the excess EDTA and DCTA were eliminated with standard 0.1 M $MgCl_2$ solution. Ethanol was added and the phosphate titrated with the standard magnesium chloride until a stable purple-violet colour appeared. The authors claimed that precipitation occurred immediately magnesium ions were added and that the titration might be carried out fairly rapidly. The blue colour was then restored to the solution and the titration of the sulphate carried out as before.

The same authors have also published a method for determining phosphate and sulphate ions in the presence of each other in superphosphate fertilizers (50). This procedure appears to be the same as that described in the preceding paragraph.

A number of other workers in the field of complexometric

analysis have made minor modifications in the methods. These mainly concern changes in the concentration of reagents used, time of digestion of the $BaSO_4$ precipitate, and the quantity of magnesium ions to be added to the solution. In general, the more dilute the standard solutions used, the greater the accuracy claimed, although a limit was set by the increasing difficulty of end-point detection as the dilution increased. Most workers have advocated ageing the precipitate overnight if possible as this resulted in analysis values for the sulphur-content closer to the theoretical values.

2. THE METHOD OF MUNGER, NIPPLER AND INGOLS.

Munger, Nippler and Ingols published, in 1950, a method of determining the sulphate ion complexometrically using barium ions and a standard disodium dihydrogenethylenediaminetetraacetate solution. A description of the method follows:-

REAGENTS:

Standard Versenate Solution.

An approximately 0.02 N solution of the disodium salt of ethylenediaminetetraacetic acid is used. The reagent that includes magnesium chloride may be used (18).

Buffer Solution.

A solution is made of 8.25 g. ammonium chloride and 113 ml. concentrated reagent grade ammonium hydroxide in 1 litre. This provides a solution of pH 10 when 10 ml. are added to 50 ml. of sample.

Barium Chloride Solution.

An approximately 0.02 N solution of the salt is made and the normality accurately determined.

Calcium Chloride Solution.

A 0.020 N solution is prepared from calcium carbonate as described in "Standard Methods for Estimation of Water and Sewage" (1). This is the primary standard for the versenate, barium and magnesium chloride solutions.

Magnesium Chloride Solution.

A 0.02 N solution is prepared.

Standard Hydrochloric Acid Solution.

A 0.02 N solution is prepared.

Indicator.

A 0.4% solution of Eriochromeschwartz T in ethanol is prepared.

PROCEDURE:

Initially the hardness is determined with versenate as described by Biedermann and Schwarzenbach (14, 15) and modified by Diehl, Goetz and Hach (18). The alkalinity is determined with the standard hydrochloric acid solution and not with the more usual sulphuric acid solution. To a third aliquot of 50 ml. sample, standard acid equivalent to the alkalinity (or slightly more) is added in order to destroy carbonates. The sample is boiled and 5-10 ml. of the standard barium chloride is added, the amount added depending on the estimated sulphate content of the sample. The mixture is allowed to boil for a few seconds. The flask is then cooled, 10 ml. of buffer solution is added and also 5 drops of indicator solution. The solution is then titrated with the standard versenate solution.

The first end-point may not be used, because its accuracy will be poor even when the standard solution containing some magnesium ion is used (18). The addition of a small amount of standard magnesium ion solution and a second end-point with extra versenate solution gives good accuracy since the indicator is more sensitive to the magnesium ion. In order to minimise the end-point error, it is recommended that the end-point be approached to the same colour-change used for the determination of the hardness.

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The authors observed that, since the barium solution was

added to the solution in excess in the first step, it was possible to precipitate the barium sulphate completely, even at low sulphate concentrations, before making the titration for the excess barium ions. They also found it unnecessary to remove the barium sulphate precipitate before titrating the excess barium ions and thus the titration step could be started very shortly after beginning the procedure.

They added that the method would be particularly useful in laboratories where the versenate procedure for determining hardness was used, since the value of the total calcium plus magnesium content was required for calculating the sulphate concentration.

3. EXPERIMENTAL STUDIES ON THE COMPLEXOMETRIC (EDTA) METHOD.

3.1 The Preparation of a Standard Sulphate Solution.

Most workers in the field of sulphate determination have found A.R. potassium sulphate most suitable for the preparation of standard sulphate solutions. Accordingly, 4 litres of a solution containing 250 p.p.m. sulphur was prepared by dissolving 5·435 g. A.R. K_2SO_4 in 2 litres solution and diluting to 4 litres, sulphur-free deionised distilled water being used throughout. This solution was then analysed gravimetrically by precipitation as barium sulphate, 200 ml. aliquots being used for the analyses. The results are recorded in Table I.

TABLE I

Gravimetric Analysis of Sulphate Stock Solution

	A	B	C	D
Volume of aliquot (ml.)	200	200	200	200
Weight of barium sulphate (g.)	0.3644	0.3643	0.3650	0.3643
Concentration of sulphur (p.p.m.)	249.8	249.8	250.2	249.8

Mean: 249.9 p.p.m. S

Since these results, within the limits of accuracy of the procedure, showed this method of preparing a standard sulphate solution to be accurate, the prepared stock solution was used as a standard solution in all subsequent experimental studies.

Two diluted standard solutions were used:

(a) 150 ml. diluted to 2 litres to provide a solution containing 18.75 p.p.m. sulphur.

(b) 300 ml. diluted similarly to give a 37.5 p.p.m. S solution.

3.2 The Storage of Solutions.

Research on the storage of solutions has been mentioned on page 11. In view of the very slight changes in concentration reported by other workers, the EDTA solution used in the analysis was stored in a pyrex glass aspirator. At intervals, checks on the concentration were carried out. No deterioration in the solutions was noticed.

All other solutions, with the exception of indicator solutions, were stored in polythene bottles, conforming with established practice in trace element analysis.

Indicator solutions were stored in 20 ml. pyrex bottles with ground-glass stoppers closed by a rubber teat. (See fig. 2)

3.3 The Influence of Magnesium Ion Concentration on End-point Detection.

In view of the difficulty of detecting the end-point in the titration of the excess barium ions (after precipitation of the sulphate) with EDTA, since the colour-change is from mauve or bluish-purple to blue, most workers either added magnesium ions in the EDTA solution or in the buffer solution, or alternatively added magnesium ions in a fixed amount before titrating to the final end-point.

The addition of magnesium ions in the other reagents having been found unsatisfactory, an investigation was carried out to find the most suitable ratio of magnesium to barium ions for optimum end-point detection. Riva (43) found that a barium to magnesium ratio of 2 : 1, 1 : 1 or 1 : 2 was satisfactory.

In the preliminary work, 0.01 M solutions of versenate (disodium ethylenediaminetetraacetate), barium chloride and magnesium ammonium chloride were used. This magnesium salt was used as it was

not nearly as hygroscopic as magnesium chloride. It could thus be weighed out satisfactorily to give an approximately 0.01 M solution.

The solutions were prepared and standardised as follows:

Magnesium Sulphate solution (i).

An exactly 0.010 M solution of magnesium sulphate was prepared from dried "Specpure" $MgSO_4.7H_2O$.

Standard Versenate solution (ii).

An approximately 0.01 M solution of disodium ethylenediaminetetraacetate was prepared from the A.R. salt. The solution was standardised against the magnesium sulphate solution (i).

Standard Magnesium solution (iii).

An approximately 0.01 M solution was prepared from A.R. $MgCl_2.NH_4Cl.\ 6H_2O$, dried as far as possible. The solution was standardised against the EDTA solution (ii).

Standard Barium Chloride solution (iv).

An approximately 0.01 M solution was prepared from A.R. $BaCl_2.2H_2O$. The concentration was calculated, assuming 100% purity for the dried salt and confirmed by titration against the EDTA solution (ii) in the presence of standard magnesium solution (iii) in a magnesium to barium ratio of approximately 1:1.

Buffer solution (v).

A pH 10 buffer solution was prepared containing 33.7 g. ammonium chloride (A.R.) and 285 ml. conc. ammonium hydroxide solution (A.R.) in 500 ml. buffer solution. 5 ml. of this buffer solution was used in each of the above standardisations.

Indicator (vi).

A solid mixture of 0.1 g. Eriochrome Black T indicator and 100 g. A.R. sodium chloride, well-mixed and powdered. Approximately 0.25 g. of the solid indicator mixture was used in each of the above standardisations.

A solution containing varying volumes of barium and magnesium solutions was then titrated against standard EDTA solution. For each series of titrations, 5 ml. buffer solution was added and approximately 0.25 - 0.3 g. indicator mixture. The titration was continued until the last visible trace of pink disappeared from the solution, leaving a pure blue solution.

SERIES A: The magnesium concentration was maintained equal to or slightly below that of the barium.

SERIES B: The magnesium ion concentration was maintained constant, a small amount viz. 5 ml. being used while the barium concentration was increased. The barium to magnesium concentration ratios varied from 2:1 to 8:1.

SERIES C: The magnesium ion concentration was again maintained constant, 25 ml. being used, while the barium concentration was increased. The barium to magnesium concentration ratios varied from 1:4 to 1:1.

Additional Observations.

In series B, the difference between the volume of EDTA calculated and the volume used increased as the volume of barium increased i.e. as the Ba : Mg ratio increased. In series C, the difference remained fairly constant. It would thus appear that a ratio of

1 : 1 provides the optimum end-point conditions, while a magnesium ion concentration less than the barium ion concentration should be avoided, the end-point being particularly difficult to detect for very low magnesium ion concentrations.

TABLE II

OPTIMUM RATIO OF MAGNESIUM TO BARIUM

Series	Number of analyses	Mean (volume of EDTA calculated - volume used)	Maximum deviation (positive)	Maximum deviation (negative)
A	16	- 0·02 ml.	+ 0·06 ml.	- 0·08 ml.
B	11	+ 0·32 ml.	+ 0·76 ml.	NIL
C	10	- 0·15 ml.	NIL	- 0·20 ml.

3.4 The Influence of pH on End-point Detection.

It was found essential to neutralise any acid present in solution before adding buffer solution. However, in spite of this, the end-point, even with optimum Ba : Mg proportions, proved to be very difficult to detect accurately when a pH 10 buffer was used. With care, with constant illumination and a constant white background, it was found possible to obtain reasonably accurate end-points after a little practice. Results obtained while working at pH 10 will be found in Table III on page 26 (series D, E and F).

When 5 ml. of a pH 11 buffer solution (containing 6.75 g. A.R. ammonium chloride and 500 ml. A.R. concentrated ammonium hydroxide solution made up to 1 litre solution) was used in each titration, the end-point was found to be considerably improved. There was little change in the accuracy. This buffer was used in all later work.

3.5 The Titration in the Presence and Absence of the $BaSO_4$ Precipitate.

(i) In the presence of the precipitate (series D, E, F and G).

The following procedure was followed:

The aliquot was brought to the boil in a 250 ml. beaker, 10 ml. standard 0.01 M barium chloride solution was added by means of a pipette and the solution boiled for a further 5 minutes. The solution was then cooled, 5 ml. buffer solution was added followed by a measured volume of standard approximately 0.01 M Mg^{++} solution. Approximately 0.25 g. eriochrome black T indicator mixture was added and the solution titrated with a standard approximately 0.01 M EDTA solution.

From the EDTA equivalents of the volumes of Ba^{++} and Mg^{++} solutions added and the volume of EDTA solution used, the volume of EDTA solution equivalent to the sulphate content of the aliquot was calculated and hence the weight of sulphur in the aliquot:

$$\text{Weight of sulphur (mg.)} = \frac{\text{vol. EDTA equiv. to total sulphate}}{\text{vol. EDTA equiv. to 1 ml. } Ba^{++} \text{ soln.}} \times \frac{32{\cdot}06}{100}$$

where the volume EDTA in the numerator is the theoretical volume of EDTA equivalent to the volumes of Ba^{++} and Mg^{++} solutions added, less the volume of EDTA solution used in the titration.

(ii) In the absence of the precipitate (series H, J, K and L).

The following procedure was followed:

The aliquot was brought to the boil in a 250 ml. beaker, 9 ml. standard approximately 0.01 M Barium chloride solution was run into a 50 ml. beaker from a micro-burette and also brought to boiling point. The hot barium chloride solution was carefully washed into the

250 ml. beaker and the solution boiled for a further 5 minutes. After thorough cooling, the solution was filtered through a No. 4 sintered glass crucible (series H, J and K) or Gooch crucible with asbestos pad (series L) into a 250 ml. Buchner filter flask. The precipitate was carefully washed with water and the filtrate and washings titrated with standard approximately 0.01 M EDTA solution as described in (i).

In series K, after reaching the end-point, a further 0.5 ml. Mg^{++} solution was added and the solution again titrated to the end-point. In series L, three end-points were obtained viz. after the addition of 2.5, 3.0 and 3.5 ml. Mg^{++} solution respectively. For each aliquot, a mean sulphur-content was calculated.

TABLE III

COMPLEXOMETRIC ANALYSIS OF DILUTE STANDARD SULPHATE SOLUTION.

Sulphur content of soln. = 18.75 p.p.m.

Series	Number of determinations.	Size of aliquot	pH of solution	Vol. of Mg soln.	Vol. of Ba soln.	Mean wt. of S (mg.)	Mean error %
D	6	100 ml.	10	3.0 ml.	10 ml.	1.857	-1.0
E	6	50 ml.	10	3.0 ml.	10 ml.	0.945	+0.8
F	3	50 ml.	10	3.5 ml.	10 ml.	0.929	-0.9
G	6	50 ml.	11	3.5 ml.	10 ml.	0.932	-0.6
H	12	50 ml.	11	3.5 ml.	9 ml.	0.943	+0.6
J	5	50 ml.	11	3.0 ml.	9 ml.	0.948	+1.1
K	10	50 ml.	11	3.0, 3.5 ml.	9 ml.	0.937	-0.1
L	28	50 ml.	11	3.0, 2.5, 3.5 ml.	9 ml.	0.920	-1.9

disappearance of any pink colour), the colour was found to change back slowly to a dull purple, further EDTA additions proving necessary to restore the pure blue colour to the solution.

The errors resulting from the presence of phosphate ions in solution are recorded in Table V and are also shown graphically in fig. 1.

TABLE V

ANALYSES OF STANDARD SULPHATE SOLUTIONS CONTAINING PHOSPHATE.

Sulphur content of solution = 18·75 p.p.m.
Aliquot size = 50 ml.

Series	Number of determinations	Vol. PO_4^{3-} soln. (ml.)	Wt. of P (mg.)	Mean wt. of S (mg.)	Mean Error %
P	6	10	0·030	1·000	6·7
Q	6	20	0·060	1·029	9·8
R	6	35	0·105	1·042	11·1
S	6	50	0·150	1·055	12·5

3.8 Attempts at Removal of Phosphate Interference.

(i) Anion exchange after precipitation of the sulphate as $BaSO_4$.

The column used was 20 × 50 mm. Amberlite IR4B ; (Cl^- ; 20 - 50 mesh).

This method was unsuccessful, both when the precipitate was filtered off using sintered glass crucibles and when cotton-wool plugs above the resin column were used. This was almost certainly due to the fact that the phosphate interference was caused by precipitation of barium phosphate; barium was thus removed from solution by the phosphate before the solution was passed through the column

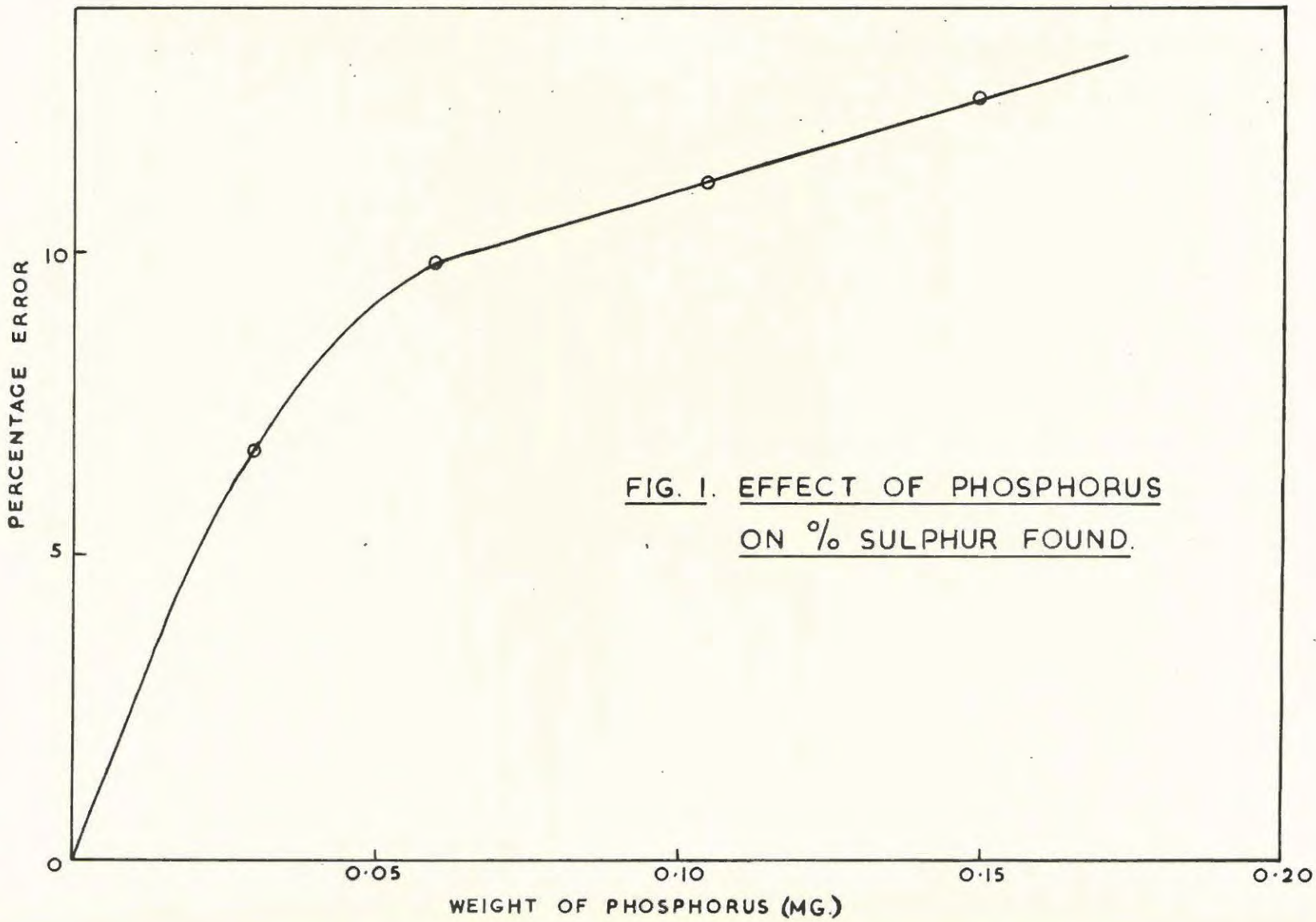


FIG. 1. EFFECT OF PHOSPHORUS ON % SULPHUR FOUND.

and hence the ion exchange had little or no effect.

(ii) Precipitation of the phosphate by means of ferric chloride soln.

The use of a 1% ferric chloride solution to precipitate the phosphate, followed by filtration through Whatman No. 31 filter paper also proved unsuccessful. This was probably due to a number of factors, among which may be mentioned the very low concentration of phosphate ions, adsorption errors with the phosphate precipitate and the effect of excess ferric ions in solution after filtration.

(iii) Precipitation by means of zirconium chloride solution.

To each 50 ml. sample was added 1 ml. 2% zirconium chloride solution (prepared from zirconium carbonate and hydrochloric acid). To complex the excess zirconium ions, 4% ammonium citrate solution was added. With insufficient citrate, the excess zirconium ions formed zirconium hydroxide on making the solution alkaline with the buffer solution. This precipitate adsorbed a certain amount of the indicator and remained pink even after the end-point had been reached, even though the rest of the solution changed to blue at the equivalence point. Thus the colour-change was missed during the constant shaking of the solution. When an excess of citrate was added, the entire solution remained pink, even beyond the equivalence point. The unsuitability of zirconium ions for the removal of phosphate ions had also been confirmed by Palaty (42).

(iv) Adsorption of the PO_4^{3-} ions on ferric ions on a cation exchanger.

A tentative attempt was made to remove the phosphate ions by passing the solution through a column of Nalcite HCR, previously

saturated with ferric ions, which, it was hoped, would adsorb the phosphate ions. The attempt was unsuccessful.

(v) Precipitation of the phosphate ions with uranyl acetate solution.

A 0·2% uranyl acetate solution was added to the phosphate-containing sulphate solution but, although the uranyl phosphate was satisfactorily precipitated, the excess uranyl ions interfered and the errors in the method were only slightly less than those obtained with untreated solutions. The removal of the uranyl phosphate precipitate by filtration through Whatman No. 42 filter paper or No. 4 sintered glass crucibles resulted in no improvement. The excess uranyl ions showed no tendency to be adsorbed onto a cation exchanger such as Nalcite HCR. Various other methods of complexing the excess uranyl ions (49), which included treatment with solutions of thallous acetate, ammonium chloride, o- hydroxy quinoline and hexamethylene tetramine, proved ineffective, as also did an attempt to complex the ions using a potassium iodate solution. In some cases the indicator appeared to be oxidised or partially destroyed by the reagents in solution; in other cases, the error was partially reduced but results were not reproducible.

(vi) Extraction of the phosphate as the phospho-molybdo complex (39).

An attempt was made to remove the phosphate ions by the method of Collier (39). An aliquot of the phosphate-containing sulphate solution was pipetted into a 125 ml. separatory funnel, 2 ml. concentrated hydrochloric acid was added for each 40 ml. of sample, 20 ml. of 1 : 1 n-butanol - chloroform mixture was added followed by 10 ml. of 20% ammonium molybdate solution. The funnel was immediately stoppered

and shaken vigorously for 1 minute, after which it was allowed to stand for 1 minute. The yellow bottom layer was drained off and the extraction repeated using 20 ml. and 10 ml. of the solvent mixture. The aqueous layer was then treated with barium chloride as before but the added reagents interfered, a blue colour developing in the solution on boiling and no end-point being obtainable in the titration. Variations in the extraction procedure, including substitution of amyl alcohol for n-butanol and carbon tetrachloride for chloroform and carrying out an additional extraction with chloroform or carbon tetrachloride alone, failed to produce better results and this extraction procedure had to be abandoned.

(vii) Initial determination of phosphate followed by sulphate determination.

As already mentioned (page 14), attempts to analyse phosphate-containing solutions by the method of Bakacs-Polgar and Szekeres (47) were unsuccessful.

4. THE MODIFIED EDTA COMPLEXOMETRIC METHOD FOR DETERMINATION OF SULPHUR IN SOLUTIONS NOT CONTAINING INTERFERING ANIONS.

REAGENTS.

(i) Water:

All water used in preparing standard solutions, buffer solutions and any other solutions used in the analysis, should be deionised distilled water.

(ii) Standard Magnesium Sulphate Solution:

An exactly 0·010 M solution of magnesium sulphate is prepared from dried "Specpure" $MgSO_4.7H_2O$ crystals.

(iii) Standard Magnesium Solution:

An approximately 0·01 M solution is prepared from A.R. $MgCl_2.NH_4Cl.6H_2O$ crystals, dried as far as possible. The solution is standardised against the EDTA solution (iv).

(iv) Standard Versenate Solution:

An approximately 0·01 M solution of disodium ethylenediaminetetraacetate is prepared from the A.R. salt. The solution is standardised against the standard magnesium sulphate solution (ii).

(v) Standard Barium Chloride Solution:

An approximately 0·01 M solution is prepared from the A.R. salt. Provided the dried salt is used, the concentration may be calculated from the exact weight taken and may be checked by titration against the standard versenate solution (iv) in the presence of standard magnesium solution (iii) in a magnesium to barium ratio of approximately 1 : 1.

(vi) Buffer Solution (pH 11):

6·75 g. A.R. ammonium chloride and 500 ml. A.R.

concentrated ammonium hydroxide solution is made up to 1 litre with deionised distilled water.

(vii) Indicator solution:

A solution is made of approximately 0·1 g. solid Eriochrome Black T indicator in 25 ml. absolute ethyl alcohol. The filtered solution is stored in a small pyrex glass bottle having a ground-glass stopper fitted with a dropper which is closed by a rubber teat. The bottle is kept in a light-tight wooden container (see fig. 2).

(viii) Cation Exchange Resin:

The resin used is a column 12 × 100 mm. of Nalcite HCR (Dowex 50), (H^+ ; 16 - 40 mesh). The resin is prepared as follows: About 50 g. of the resin is shaken with 100 ml. 4 N hydrochloric acid and allowed to stand for $\frac{1}{2}$ an hour. This is repeated with a further three 100 ml. portions of 4 N hydrochloric acid, allowing the resin to stand for 2 hours in contact with the last portion. The resin is finally washed with water by decantation until the supernatant water is free from any yellow coloration.

STORAGE OF REAGENTS.

The versene solution is stored in a pyrex glass aspirator since, kept thus, it appears to suffer very little change in concentration over a considerable period. Unless otherwise stated, all other reagent solutions should be stored in polythene bottles, previously cleaned thoroughly and well rinsed out with deionised distilled water.

GLASSWARE.

All glassware used should be cleaned thoroughly with 'Teepol' solution and, after this and between each analysis, several times with deionised distilled water. Only finest quality burette grease should be used for burette and exchange column stopcocks.

PROCEDURE.

For the cation exchange, pyrex glass columns, 12 cm. long and 1·2 cm. internal diameter, with a reservoir at the top, and a glass stopcock with 2 mm. delivery bore, are used (see fig. 3). The resin is packed into the column as follows:- A small glass-wool plug is pushed to the bottom by means of a long glass rod. A slurry of the resin is poured into the column and tapped down into a firm bed with the glass rod, a column 10 cm. long being formed. A second glass-wool plug is pushed down on top of the resin bed. The resin is washed with 200 ml. water with the stopcock fully opened, the level of water never being allowed to drop below the level of the top glass-wool plug, in order to prevent air bubbles from entering the resin bed.

An aliquot containing between 0·8 and 1·6 milligrams sulphur is pipetted carefully into the reservoir of the exchange column and leached into a 200 ml. erlenmeyer flask at the rate of about 2 drops per second. The resin is washed with a number of small portions of water (approximately 5 ml. portions) until 40 ml. water has been used. After each addition, the water level is allowed to drop to the level of the glass-wool plug before the next addition is made. The column is now ready for the next sample. After 6 - 8 samples, the column should be regenerated by leaching 100 ml. 4 N hydrochloric acid through the

FIG. 2. INDICATOR STORAGE.

FIG. 3. CATION EXCHANGE COLUMNS.

column and washing the resin free of acid with about 100 ml. water, added in small portions as before.

From a micro-burette, 9.00 ml. standard barium chloride solution (v) is run into a 50 ml. beaker. The beaker and erlenmeyer flask are heated on an electric hot-plate until the solutions are almost at boiling point when the barium chloride solution is added rapidly and quantitatively to the sulphate solution and the beaker washed out well into the erlenmeyer flask with small portions of deionised distilled water from a polythene wash-bottle. The solution is allowed to simmer gently for a minimum period of five minutes (preferably longer) and cooled to between 30° and 40°C. To the cooled solution, 5 ml. pH 11 buffer solution (vi) is added, followed by 2·50 ml. standard magnesium solution (iii) from a micro-burette. Ethanolic Eriochrome Black T solution is added, the number of drops used depending on the drop-size. A little more than 0·5 ml. indicator solution is generally required. The solution is then titrated with standard versenate solution (iv) from a micro-burette until the last trace of pink colour has disappeared from the solution, leaving a pure-blue solution. The volume of versenate solution added is noted, a further 0·50 ml. standard magnesium solution (iii) is added and the solution again titrated to the end-point with versenate solution. Finally, a third 0·50 ml. standard magnesium solution is added and the solution titrated to the end-point. Throughout the titration, the flask is constantly swirled.

From the volumes of barium chloride, magnesium ammonium chloride and versenate solutions, and the previously calculated EDTA

equivalents of the barium and magnesium solutions, the sulphur content of the sample aliquot may be calculated as described on page 25.

DISCUSSION.

The main modifications introduced as compared with the method of Munger, Nippler and Ingols are as follows:-

(1) A time of boiling the solution (after barium addition) of longer than a few seconds is advocated, a minimum of five minutes being advisable.

(2) The solution is cooled down only to between 30° and 40°C. since the end-point colour-change is clearer and more rapid at this temperature.

(3) A solution of magnesium ammonium chloride is used in place of magnesium chloride owing to the difficulty of preparing the latter salt. Magnesium ammonium chloride, although slightly hygroscopic, is very much easier to handle and the ammonium chloride has no harmful effect in the titration, the latter salt being used in the buffer solution.

(4) The titration is carried out at a pH of 11 rather than 10 as the colour-change of the indicator is clearer at this pH.

(5) Three end-points are obtained for each sample, a mean value for the sulphur-content being found. This reduces the errors in the titration, including recognition of the end-point.

ADDITIONAL NOTE ON THE CATION EXCHANGE RESIN COLUMN.

The total capacity of a column of Nalcite HCR prepared in the above way was determined as follows:-

100 ml. 5% NaCl solution was leached through the column, the eluate being titrated against standard sodium hydroxide solution. The total exchange capacity was found to be 17·5 meq. cation.

An exchange isoplane or breakthrough curve was plotted for the column, a 0·04 N sodium chloride solution being leached through the column and 3 ml. portions of the effluent tested for sodium content

using an EEL flame photometer. The curve is shown in fig. 4. Assuming the first 10 ml. effluent to be water originally in the column, the total capacity of the resin was calculated to be 18·5 meq. cation and the breakthrough capacity to be 7·6 meq. cation. Since the maximum concentration of cations in a plant solution is usually of the order of 40 meq./litre, the resin may be used for 200 ml. plant solution before regeneration is necessary.

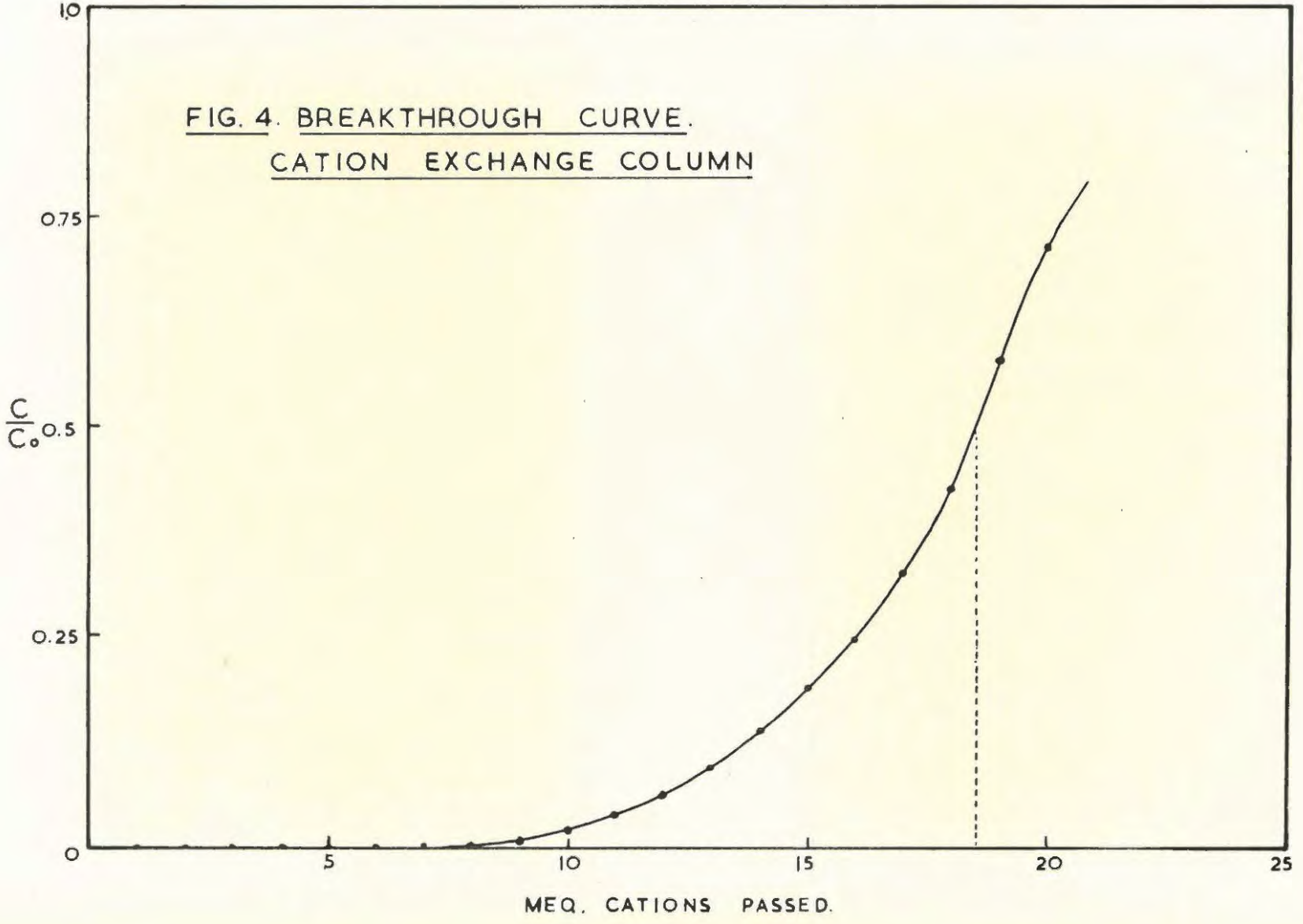


FIG. 4. BREAKTHROUGH CURVE.
CATION EXCHANGE COLUMN

5. STATISTICAL STUDY OF THE PRECISION OF THE MODIFIED EDTA METHOD.

To test the precision of the modified EDTA method for the determination of sulphate in solutions not containing interfering anions, thirty-eight analyses were carried out on a solution containing 18·75 p.p.m. sulphur as sulphate. The procedure followed was as outlined in Section 4. The aliquot size used in each analysis was 50 ml. The results were examined statistically as shown in Table VI.

The results of a further thirty-six analyses carried out with the removal of the barium sulphate precipitate by means of a No. 4 sintered glass crucible are also given and compared statistically with those carried out in the presence of the precipitate. It may be mentioned here that, after use, the crucibles were cleaned using a hot solution of ammoniacal EDTA.

It will be noticed that the mean of the results of analyses in which the titration was carried out in the presence of the precipitate was only 0·12% below the theoretical value. The difference in precision of the two methods justifies the carrying out of the titration in the presence of the precipitate.

In the first series of analyses, 71% of the results were within 1% of the theoretical value whereas, in the second series, only 58% of the results were within 1% of the theoretical value. This would appear to indicate that the filtration step increases the possibility of errors in the determinations.

TABLE VI

ANALYSIS No.	IN PRESENCE OF PRECIPITATE		IN ABSENCE OF PRECIPITATE	
	µg. S	$(y - m)^2$	µg. S	$(y - m)^2$
1	940·5	16·81	933·7	79·21
2	937·5	1·21	991·0	2342·56
3	916·5	396·01	938·3	18·49
4	940·5	16·81	925·5	292·41
5	918·5	320·41	913·1	870·25
6	930·5	34·81	944·8	4·84
7	945·0	37·96	947·9	28·09
8	916·5	396·01	963·2	424·36
9	909·5	723·61	968·7	681·21
10	926·0	108·16	949·1	42·25
11	944·5	65·61	935·3	53·29
12	937·0	0·36	947·3	22·09
13	926·0	108·16	938·3	18·49
14	937·0	0·36	937·6	25·00
15	922·0	207·36	928·7	193·21
16	938·4	4·00	944·1	2·25
17	935·0	1·96	951·0	70·56
18	936·5	0·01	935·5	50·41
19	947·9	132·25	937·0	31·36
20	945·8	88·36	949·2	43·56
21	943·6	51·84	925·0	309·76
22	942·3	34·81	942·5	0·01
23	944·8	70·56	983·0	1632·16
24	941·2	23·04	948·5	34·81
25	937·6	1·44	942·8	0·04
26	934·4	4·00	919·5	533·61
27	932·3	16·81	941·0	2·56
28	937·6	1·44	931·3	127·69
29	941·2	23·04	938·9	13·69
30	937·6	1·44	939·9	7·29
31	948·2	139·24	937·6	25·00
32	936·9	0·25	936·9	32·49
33	947·5	123·21	938·7	15·21
34	938·7	5·29	939·7	8·41
35	939·7	10·89	947·5	24·01
36	942·3	34·81	942·3	0·09
37	939·7	10·89		
38	947·5	123·21		
TOTAL	35584·2	3372·44	33934·4	8060·72
MEAN	936·4		942·6	

Std. Deviation: 9·55 µg. Sulphur — 15·18 µg. Sulphur

% Std. Deviation: 1·02 %. — 1·60 %.

PART II

THE METHYLENE-BLUE METHOD FOR THE SPECTROPHOTOMETRIC DETERMINATION OF SULPHUR.

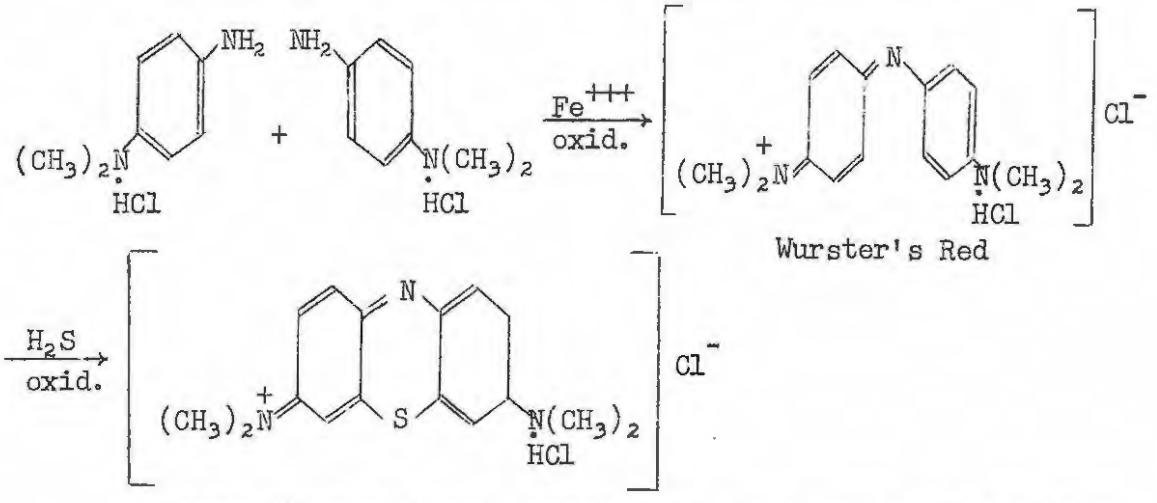
1. REVIEW OF PUBLICATIONS ON THIS METHOD.

1.1 The Colour Reaction.

In 1883, Emil Fischer (51) suggested that Caro's Methylene Blue (MB) reaction be used for the detection of small quantities of hydrogen sulphide. Lindsay (52) was the first to apply the method practically for the estimation of sulphur in pig-iron. Almy (53) later modified the method of Mecklenburg and Rosenkränzer (54) for determining sulphur in proteinaceous foods.

The solution containing hydrogen sulphide is mixed with an acidic solution of p-amino-N,N-dimethylaniline (or N,N-dimethyl-p-phenylenediamine) and ferric iron is added. The solution becomes red due to the formation of an intermediate compound but changes to blue as the MB is formed.

The reaction believed to take place is:-



Wurster's Red

Among other products, sulphide green, leuco methylene-blue and methylene-red are said to be formed.

Rabinowitch and Epstein (55) investigated the molecular state of MB and thionine. They found that the absorption curve for MB was made up by the superposition of two bands, one with a maximum at 656·5 mμ and the other with a maximum at 600 mμ, the latter being more prominent in more concentrated solutions. This could be explained as due to the formation, in concentrated solutions, of dimeric ions $(MB)_2^{++}$. They also found that the transmission decreased with rise in temperature, the effect on the 656·5 mμ band being the greater. This would be due to the dissociation of the dimeric ions.

In 1929, St. Lorant (56) developed a method for the reduction of microgram amounts of sulphate to hydrogen sulphide which he then determined colorimetrically as MB.

In 1948, Kosior (57) applied the method to determine hydrogen sulphide in natural gas, measuring the colour by comparison with standards or using a photoelectric colorimeter. He found that the presence of thiosulphate had a two-fold effect on the MB formation; a fine colloidal precipitate (probably sulphur) was produced on mixing the reagents and this prevented the formation of MB unless it was removed; also, the thiosulphate itself formed a MB colour when mixed with the reagents. A year later, Fogo and Popowsky (58) used modern spectrophotometric instruments in their measurements.

Sands et al. (59) in their research for the U.S. Bureau of Mines, investigated the MB method as an ultrasensitive technique for determining hydrogen sulphide in gases. In their report they gave the most favourable conditions for the colour reaction.

Roth (60), and Johnson and Nishita (7) further developed

St. Lorant's method, the latter authors adapting the procedure specifically for the determination of sulphur in plant materials, soils and irrigation waters.

In the same year, Budd and Bewick (61) outlined a procedure for determining sulphide and reducible sulphur in alkalis by means of the MB reaction.

In 1953, Treiber et al. (62) stated that pure fresh diamine dissolved in water to give a solution having a pale brown colour. A red colour was then produced on the addition of ferric chloride but not if there was a considerable quantity of hydrochloric acid present. Most commercial preparations, however, darkened considerably on exposure to light and air and gave a product which had an orange-red colour in solution. The maximum absorption of this solution, which was little influenced by hydrochloric acid, occurred at 503 mμ. As alternative reagents, they suggested an iodine compound of MB as used by Kuhlberg (63) or "Bindshedler's green" as used by Wright et al. (64).

In 1958, Freney (65) described a procedure for the determination of water-soluble sulphate in soils, based on the method of Johnson and Nishita (7). He had found that their method was not specific for sulphate, since other forms of sulphur were also reduced. He modified the method to include a step in which sulphates were removed from other sulphur compounds.

Iismaa (66) modified the method of Johnson and Nishita for plant materials by replacing the 'wet combustion' procedure by a hydrogen peroxide oxidation.

During 1959, Lilly Gustafsson made an intensive study of

the MB colour reaction, both from the point of view of the reduction procedure and the colour reaction (67, 68). She studied the effects of acidity, variations in the concentrations of the reagents, temperature, traces of heavy metals, and also the susceptibility of the sulphide in zinc acetate solution to air oxidation, the stability of the colour, the relation to Beer's Law and the yield of the MB reaction.

In 1960, Tyou and Humblet (69) published a method for determining sulphur in steel, pure iron and cobalt, which employed the MB reaction, while McKinley and Jones (70) made use of the reaction for determining sulphate in chromium plating solutions.

1.2 The Reduction of Sulphur to Hydrogen Sulphide.

Where sulphur in forms other than sulphide is to be determined, it must initially be reduced quantitatively to hydrogen sulphide which is then absorbed in a suitable solution prior to the application of the colour reaction. Considerable research has been carried out to find the most suitable reduction mixture.

Except in a few isolated instances where other reducing agents have been employed (as for example an ammoniacal solution of a silver salt by Sheppard and Hudson (71); also, stannous chloride and hydrochloric acid with aluminium strips by Budd and Bewick (61)), most authors have employed mixtures containing two or more of the following reagents: Hydriodic acid, hydrochloric acid, formic acid, acetic acid, hypophosphorus acid or its sodium and potassium salts, and red phosphorus. The mixtures used varied not only in their constituents but in the relative quantities of these constituents.

St. Lorant (56) appears to have been the first to make use of hydriodic acid as a reducing agent, employing 100 ml. 50% HI together with 75 ml. formic acid and 15 g. red phosphorus. The red phosphorus served to reduce any iodine to hydrogen iodide. More recently, Luke (72) employed a reducing mixture of hydriodic acid, hydrochloric acid and hypophosphorus acid, while Roth (60) replaced the red phosphorus in St. Lorant's mixture by 1 g. potassium hypophosphite. Gustafsson (68) investigated the reducing action of seven different reducing mixtures viz. HI + P ; HI + P + HCOOH ; HI + P + HAc ; HI + P + H_3PO_2 ; HI + H_3PO_2 + HCOOH ; HI + NaH_2PO_2 + HAc ; HI + H_3PO_2. In addition, the proportions of the constituents were varied. As a result of her experiments, she found that the reagent consisting of 100 ml. hydriodic acid (S.G. 1·7), 25 ml. acetic acid and 2·5 g. sodium hypophosphite gave the best precision and the lowest blanks. She recommended boiling the mixture under reflux for 1 hour, bubbling a stream of nitrogen (about 50 ml. per minute) through the solution. In this way traces of sulphur were removed and free iodine was reduced to hydrogen iodide. She also recommended storing the mixture in the same flask, closed by means of a glass stopper, in subdued light.

In 1956, in a personal communication to Bethge, Johnson and Arkley advocated the use of a reduction mixture containing 300 ml. hydriodic acid (S.G. 1·7), 75 ml. hypophosphorous acid and 150 ml. 90% formic acid. The initial boiling of this mixture was carried out for only 10 minutes. In addition, the mixture was not regenerated after use owing to the danger of phosphine formation. McKinley and Jones (70) in their method for determining sulphate in chromium plating solutions

have used a mixture of 200 ml. hydriodic acid (S.G. 1·5 - 1·7), 200 ml. hydrochloric acid (S.G. 1·18) and 50 ml. hypophosphorous acid (50%), refluxed for 1 hour and stored in a brown glass bottle in a refrigerator.

Apart from the action of formic or acetic acid in destroying oxidising agents, the chief reactions involved are as follows:-

$$MSO_4 + 10\ HI \rightarrow MI_2 + 4\ I_2 + 4\ H_2O + H_2S$$

$$2\ I_2 + PO_2^{3-} + 2\ H_2O \rightarrow 4\ HI + PO_4^{3-}$$

1.3 The Washing of the Gas.

St. Lorant (56) prevented the carrying-over of hydrogen iodide from the reduction flask into the absorber by means of a wash-bottle containing a solution of sublimed pyrogallol and sodium dihydrogen phosphate. He claimed that, in spite of the solution turning brownish, it nevertheless maintained its usefulness month-long. Johnson and Nishita (7) preferred, however, to prepare the wash-solution fresh daily.

Gustafsson (68) found that very little hydrogen iodide and iodine was carried over during the distillation and stated that deionised distilled water could be substituted for the wash-solution of pyrogallol and NaH_2PO_4 provided it was replaced after about six successive determinations.

Tyou and Humblet (69) used a wash-solution of 0·2 N hydrochloric acid and 0·1% hydriodic acid.

1.4 Carrier Gas.

St. Lorant (56) used hydrogen, carbon dioxide and nitrogen as

carrier gases and claimed that even ordinary air was usable. However, carbon dioxide was not recommended as carrier gas as a considerable pressure developed as a result of acidification. He preferred to use nitrogen gas from a cylinder of the gas. The gas was purified by passage through a 2% solution of potassium permanganate containing a large amount of mercuric chloride.

Budd and Bewick (61) stressed on the other hand that it was essential to sweep all traces of oxygen out of the apparatus before carrying out the reduction procedure and advocated the rapid passage of nitrogen through the apparatus for 10 minutes before commencing the reduction. Gustafsson (68), while ruling out the use of air as a carrier gas owing to the rapid formation of free iodine in the apparatus by the oxidation of hydrogen iodide, nevertheless claimed that the complete removal of air was unnecessary.

During the reduction, varying flow-rates of carrier gas have been recommended, from 40 - 50 ml. per minute (73) to 150 - 200 ml. per minute (68). A rate between 100 and 200 ml. per minute seemed generally acceptable, lower rates necessitating excessively long reduction times while higher rates resulted in hydrogen iodide being carried over into the absorber. The reduction times, depending as they did on the carrier gas flow-rate, varied from 10 minutes (68) to 1 hour (7).

1.5 Cleaning the Apparatus.

When not in use for a period longer than a few minutes, iodine droplets were found to condense on the walls of the condenser due to oxidation of the hydrogen iodide. Gustafsson (68) stated that in this case, the apparatus should be washed out with distilled

water before re-use. Most researchers washed the distillation apparatus with distilled water daily before use. St. Lorant (56) instructed that the inner walls of the condenser should be dried after washing. Hogan and Breen (74) described a procedure for washing a battery of six distillation apparatuses thoroughly in about 5 minutes by drawing distilled water through the condenser section in situ while the gas-washing column was being recharged.

Most researchers have found that the first reduction on any day, or after washing the apparatus, gave a low value for the sulphur content. Johnson and Nishita (7) gave the error as about 5%. It appeared that the apparatus required to be "conditioned" before use and it has been recommended that a preliminary run be carried out with a sample containing a small amount of reducible sulphur, the development and measurement of the colour being unnecessary (7, 68, 69). Gustafsson attributed the error in the first determination of a series to absorption of hydrogen sulphide in the apparatus in spite of precautions taken to keep the glass walls and water in the washing column free from traces of metals.

1.6 The Absorption Step.

Prior to determination of hydrogen sulphide either by the MB method or iodimetrically, the quantitative absorption of the gas is essential. In the MB method, it is also essential that the hydrogen sulphide be readily liberated from the absorption solution in order to ensure quantitative reaction with the colour reagents.

To absorb the gas, Almy (53) used two flasks, the first

containing 20 ml. and the second 30 ml. 0·6% zinc acetate solution. St. Lorant (56) used a solution containing 50 g. zinc acetate, 10 g. sodium acetate and 0·05 g. sodium chloride in 1 litre of solution, 20 ml. being used in each 50 ml. absorption flask. Roth (60) also used this absorber.

Sheppard and Hudson (71) used 130 ml. 1% zinc acetate solution and 5 ml. 10% sodium hydroxide solution in a 250 ml. absorption tube. Kosior (57) and Fogo and Popowsky (58) also used alkaline solutions of zinc acetate. Tyou and Humblet (69) acidified the stock zinc acetate solution with 2 - 3 drops of acetic acid per litre to avoid the precipitation of hydroxides on keeping.

Sands et al. (59) stated that the use of an alkaline solution was inadvisable due to its rapid oxidation by the air. They found that neutral or alkaline cadmium acetate or cadmium chloride solutions, while giving complete absorption of the hydrogen sulphide, did not lend themselves to dilution due to the formation of a heavier and more rapidly settling precipitate than zinc acetate, which gave an almost colloidal precipitate. In fact, they found that if the zinc sulphide precipitate was perceptible at all, it invariably meant that the resulting colour would be beyond the useful range of the method. In their own work, they used a 2% zinc acetate solution in order to give increased assurance of complete absorption. Each litre of solution was acidified with about 3 drops of acetic acid.

Jacobs et al. (75) nevertheless preferred to use a solution containing 4·3 g. cadmium sulphate and 0·3 g. sodium hydroxide in 1 litre of absorber solution.

Johnson and Nishita (7) used a solution containing 50 g. zinc acetate and 12·5 g. sodium acetate per litre of solution, filtered after making to volume. Gustafsson (67) advocated the use of a solution 0·25 M with respect to zinc acetate and 0·10 M with respect to sodium acetate. Traces of heavy metals were removed by precipitation as sulphides by the addition of 2 ml. 0·05 M sodium sulphide solution to 1 litre of the absorber solution. The zinc acetate solution after standing overnight and swirling of the precipitate, was filtered.

1.7 The Colour Development.

In Table VII a summary is given of the reagents used in the MB colour development and their concentrations. From this table it may be gathered that considerable variation was found permissable, the main criterion being constancy of pH and temperature in each determination.

It was found necessary to add the amine before the ferric ions and to mix the contents of the flask thoroughly after each addition. Agitation of the flask, following the addition of the amine, resulted in part of the hydrogen sulphide going into the gaseous phase and, unless great care was excercised, part of this might be lost when the stopper was removed for the ferric ion addition. Vigorous shaking after the addition of the ferric ions was also found to be essential to ensure reaction of all the hydrogen sulphide, including that in the gaseous phase. The use of fast-delivery pipettes to minimise losses of hydrogen sulphide was advised, the accuracy of the volume added not being critical (58). Fairly large variations in concentrations produced only slight effects.

TABLE VII

METHYLENE BLUE COLOUR REAGENTS AND CONCENTRATIONS

AUTHOR	DIAMINE REAGENT			FERRIC REAGENT		
	wt./litre	vol. acid/l.	reagent/ 100 ml.	wt./litre	vol. acid/l.	reagent/ 100 ml.
Almy (53)	0·4 g. C	500 ml. E	5·0 ml.	5·4 g. G	100 ml. E	1·0 ml.
St. Lorant (56)	0·5 g. B	200 ml. D	15·0 ml.	125 g. F	25 ml. D	4·0 ml.
Kosior (57)	1·0 g. C	500 ml. E	12·5 ml.	2·7 g. G	-	2·0 ml.
Fogo, Popowsky (58)	1·0 g. B	487 ml. E	10·0 ml.	6·12 g. G	106·2 ml. E	2·0 ml.
Roth (60)	0·5 g. B	200 ml. D	15·0 ml.	125 g. F	25 ml. D	4·0 ml.
Johnson, Nishita (7)	1·0 g. B	200 ml. D	10·0 ml.	125 g. F	25 ml. D	2·0 ml.
Sands et al. (59)	1·0 g. B	667 ml. D	10·0 ml.	27 g. G	500 ml. E	2·0 ml.
Budd, Bewick (61)	6·8 g. B	500 ml. D	3·0 ml.	1000 g. G	-	5 drops
Sheppard, Hudson (71)	1·0 g. B	500 ml. E	10·0 ml.	5·4 g. G	-	2·0 ml.
Jacobs et al. (75)	3·75 g. A	500 ml. D	1·2 ml.	1000 g. F	-	2 drops
Gustafsson (67)	0·93 g. B	186·8 ml. D	10·0 ml.	66·5 g. F	27·25 ml. D	2·0 ml.
Tyou, Humblet (69)	1·0 g. C	460 ml. E	10·0 ml.	6 - 7 g. G	100 ml. E	2·0 ml.

A = diamine, B = diamine sulphate, C = diamine hydrochloride, D = H_2SO_4, E = HCl,
F = ferric ammonium sulphate, G = ferric chloride.

Almy (53) stressed that all solutions should be within 0.5°C when developing the colour while Fogo and Popowsky (58) adjusted the temperature of their solutions to 24 ± 3°C. Sands et al. (59) cooled their solutions to 10°C as this was found to give maximum colour intensity. Changes in temperature after colour development had no effect on the intensity.

In general, it was found that the final stable colour was reached sooner when more concentrated reagents were used. Hence, Almy (53), using comparatively dilute solutions, found that two hours was required for the maximum colour intensity to be reached, whereas Johnson and Nishita (7) stated that the absorption might be measured ten minutes after making the solutions to volume.

The colour, once fully developed, was found to be relatively stable. Fogo and Popowsky (58) recommended that the colour be read not later than 20 hours after development, while Johnson and Nishita (7) gave the maximum as 24 hours. Gustafsson (67) stated that the colour should be read the same day.

Since a certain amount of zinc sulphide adhered to the detachable absorption delivery tube, a number of authors have recommended that the absorption tubes be detached and allowed to remain in the flasks during colour development. Gustafsson (68) added that the amine solution should be pipetted through the delivery tubes so that they are well rinsed. Before making the solutions to the mark, the delivery tubes were removed by means of tweezers and washed well (60, 76). Alternatively, they were left in the flasks while the solutions were made to the mark, provided that the tubes had the same internal and

external diameters and that the graduation marks on the volumetric flasks were at the same level (7).

Sands et al. (59), after studying the variables affecting the colour reaction, arrived at the conclusion that the use of the amine sulphate rather than the hydrochloride was preferable. Reports on the stability of the amine and ferric reagent solutions have been conflicting but most authors have found the solutions to be stable for several weeks.

1.8 Sensitivity and Relation to Beer's Law.

In the earliest work, visual comparisons were used and accordingly, as photoelectric measuring instruments came into more general use, the reported sensitivities increased.

Kosior (57) stated that for a natural gas sample of 1 cubic foot, an amount of hydrogen sulphide as low as 0·2 grain per 100 cubic foot of gas could be measured quantitatively. This sensitivity might readily be improved still further by making use of increased volumes of gas in the analyses. Should the MB colour be too intense, dilution with distilled water to bring the intensity within the prepared range of standards was found not to impair the accuracy. A year later, Sands et al. (59), in their report, gave the lower limit of sensitivity as 0·001 grain hydrogen sulphide per 100 cubic foot of gas for a sample volume of 1 cubic foot.

Fogo and Popowsky (58) gave the range of sulphur which could be determined as 3·5 to 500 µg. sulphur per 250 ml. absorption solution. They stated that the sensitivity might be increased still further by decreasing the volume of the absorption solution.

Johnson and Nishita (7) found that MB solutions obeyed Beer's Law in the range from 1 to 50 µg. sulphur per 100 ml. and that the range might be increased to 300 µg. sulphur by diluting 10 ml. of the intensely coloured MB solutions (from samples containing above 50 µg. sulphur) to 100 ml. by the addition of 10 ml. amine reagent, 2 ml. ferric reagent and distilled water to volume, thus showing that the deviations were due to deviations from Beer's Law and not decrease in the yield of MB. In their work, a Beckmann Model B spectrophotometer and 1-cm. cells were used. At about the same time, Roth (60) stated that the greatest accuracy was obtained when the sample tested contained between 10 and 40 µg. sulphur, while Iismaa (66) advocated the use of plant samples containing 20 to 70 µg. sulphur.

Budd and Bewick (61), in 1952, gave the lower and upper limits for determining sulphide and reducible sulphur in alkalis as 0·2 and 100 p.p.m. respectively. However, they used a calibration curve having the range 0 - 40 µg. sulphur.

In 1958, Freney (65), in his application of the method of Johnson and Nishita to the determination of water-soluble sulphate in soils, found that the MB solutions obeyed Beer's Law over the range from 1 - 80 µg. sulphur.

In 1960, Gustafsson (67, 68), in her investigation of the MB method for determining sulphate, found a noticeable decrease in specific extinction at 667 mµ for concentrations larger than about 20 to 25 µg. sulphur per 100 ml., the deviations above these concentrations being greater than the experimental errors. She also confirmed Johnson and Nishita's method for diluting more concentrated solutions.

1.9 Critical Review of the Procedures.

The MB method appears from the literature to have been applied to the determination of reducible sulphur in practically every type of material, including as it does the determination of sulphur in such diverse substances as alkalis, plating solutions, plant materials, soils and blood. In a recent volume on methods of determining non-metals colorimetrically (77), no fewer than five of the thirteen methods for sulphur determination involve the MB reaction.

In view of the peculiar requirements of plant analysis and since their work appears to have been the first intensive research into the application of the MB method to plant materials, the method of Johnson and Nishita (7) has been selected as the basis of the present investigations. In addition, their method utilises the wet-ashing technique which is also favoured by the present author. However, many later modifications, and in particular those of Gustafsson (67, 68) and Hogan and Breen (74), have been applied.

2. THE METHOD OF JOHNSON AND NISHITA.

In 1952, Johnson and Nishita published their method for the micro-estimation of sulphur in plant materials, soils and irrigation waters, using the methylene-blue colour reaction.

A description of the method follows:-

REAGENTS:

Reducing Mixture.

This consists of 15 g. reagent grade red phosphorus, 100 ml. hydriodic acid (S.G. 1·7, methoxyl grade), and 75 ml. 90% formic acid (reagent grade). Details of the preparation are given in the original paper (7) and also in "Colorimetric Determination of Non-metals" (77). Stability and regeneration are also discussed.

Nitrogen purification solution.

Add 5 - 10 g. mercuric chloride to 100 ml. 2% potassium permanganate solution.

Pyrogallol - sodium phosphate solution.

Dissolve 10 g. sodium dihydrogen phosphate and 10 g. pyrogallol in 100 ml. sulphur-free distilled water with the aid of a stream of nitrogen bubbling through the solution. Prepare fresh daily.

Sulphur-free distilled water.

If necessary, distil from alkaline permanganate in an all-glass still.

Zinc acetate - sodium acetate solution.

Dissolve 50 g. zinc acetate dihydrate and 12·5 g. sodium acetate dihydrate in sulphur-free distilled water; dilute to 1 litre and filter.

Aminodimethylaniline solution.

Dissolve 2 g. p-amino-dimethylaniline sulphate (Eastman Kodak No. 1333) in 1500 ml. distilled water. Add 400 ml. concentrated sulphuric acid, cool and dilute to 2 litres.

Ferric ammonium sulphate solution.

Dissolve 25 g. ferric ammonium sulphate in 5 ml. reagent grade concentrated sulphuric acid and 195 ml. distilled water.

Standard sulphate solution.

Dissolve 5·434 g. reagent grade potassium sulphate in 1 litre. This contains 1 mg. sulphur per ml. and may be further diluted as required to give working solutions of desired concentrations.

Sulphur-free ground joint lubricant.

Mix approximately 5 g. Dow-Corning silicone stopcock lubricant with 10 ml. of an equal volume of hydriodic acid and hypophosphorus acid. Heat to boiling with frequent stirring for about 45 minutes, pour off the acids and wash the lubricant thoroughly with sulphur-free water. Lubricate all joints with a minimal amount of this lubricant.

PROCEDURE:

A procedure is described for samples containing a considerable amount of nitrate. The sulphur is precipitated as barium sulphate by the addition of ethanol and barium chloride solution. The solution is centrifuged and the precipitate, together with any insoluble plant residue, is transferred quantitatively to the digestion flask, after which it is treated as for samples containing not more

than 6 mg. nitrate.

When little or no nitrate is present in the sample, the procedure is as follows: The gas-washing vessel is charged with 10 ml. pyrogallol - sodium phosphate solution and 10 ml. zinc acetate - sodium acetate solution and 70 ml. sulphur-free distilled water is placed in a 100 ml. volumetric flask which acts as a receiver. The gas is bubbled into this flask through a glass tube connected to the top of the gas-washing column by a short length of sulphur-free rubber tubing (boiled in dilute alkali and washed). An aliquot of the sample or standard, evaporated down if necessary, so that the volume does not exceed 2 ml., is introduced into the digestion flask. The aliquot should not contain more than 300 micrograms sulphur. 4 ml. of recently agitated reducing mixture is introduced into the digestion flask which is quickly attached to the reflux condenser, the upper end of which is connected by means of a U-shaped glass tube to the gas-washing vessel. The nitrogen flow is started, using nitrogen washed by bubbling through the $HgCl_2$ - $KMnO_4$ solution. The flow-rate is adjusted to 100 - 200 ml. per minute and the heating begun, a low boil being maintained for 1 hour. The volumetric flask is removed, leaving the connecting glass tube in it so that no adhering zinc sulphide is lost. 10 ml. of the p-amino-dimethylaniline solution is quickly added, the flask stoppered, the contents mixed, 2 ml. ferric solution added, the flask restoppered and the contents again mixed. The solution is made to volume and thoroughly mixed. After 10 minutes but before 24 hours, the absorbance is read at 670 mμ or by means of a suitable filter photometer.

A HNO_3 - $HClO_4$ wet digestion procedure is described in some detail to ensure that the solution is sufficiently low in NO_3^- and ClO_4^- to prevent interference in the sulphate reduction and MB synthesis.

3. EXPERIMENTAL STUDIES ON THE METHYLENE BLUE (MB) METHOD.

3.1 The Preparation of a Standard Sulphate Solution.

The 250 p.p.m. stock solution of potassium sulphate prepared for use in the EDTA method was also used in these studies.

Two diluted standard solutions were used:

(a) 400 ml. diluted to 1 litre, to contain 100 p.p.m. sulphur.

(b) 200 ml. solution (a) further diluted to 1 litre to provide a solution containing 20 p.p.m. sulphur.

All standard solutions were stored in polythene bottles.

3.2 Storage of Solutions.

With the exception of the standard solutions of potassium sulphate and the reducing mixture, all other solutions required to be kept for any length of time were stored in the volumetric flasks in which they were prepared and showed no deterioration on keeping.

The sulphur- and copper-free water used in the studies was stored in a large polythene bottle.

The reduction mixture was stored in a glass-stoppered amber-glass bottle and appeared to suffer no deterioration even after a period of four months.

3.3 Transmission Curves for Methylene Blue and the Reagent Solutions.

Using a Uvispek spectrophotometer with 4 cm. cells, transmission curves were plotted for a solution of commercial methylene blue (fig. 5), a solution containing all the methylene blue reagents but no sulphide and hence no blue colour (fig. 6), and a solution prepared by reducing a sample containing approximately 20 µg. sulphur,

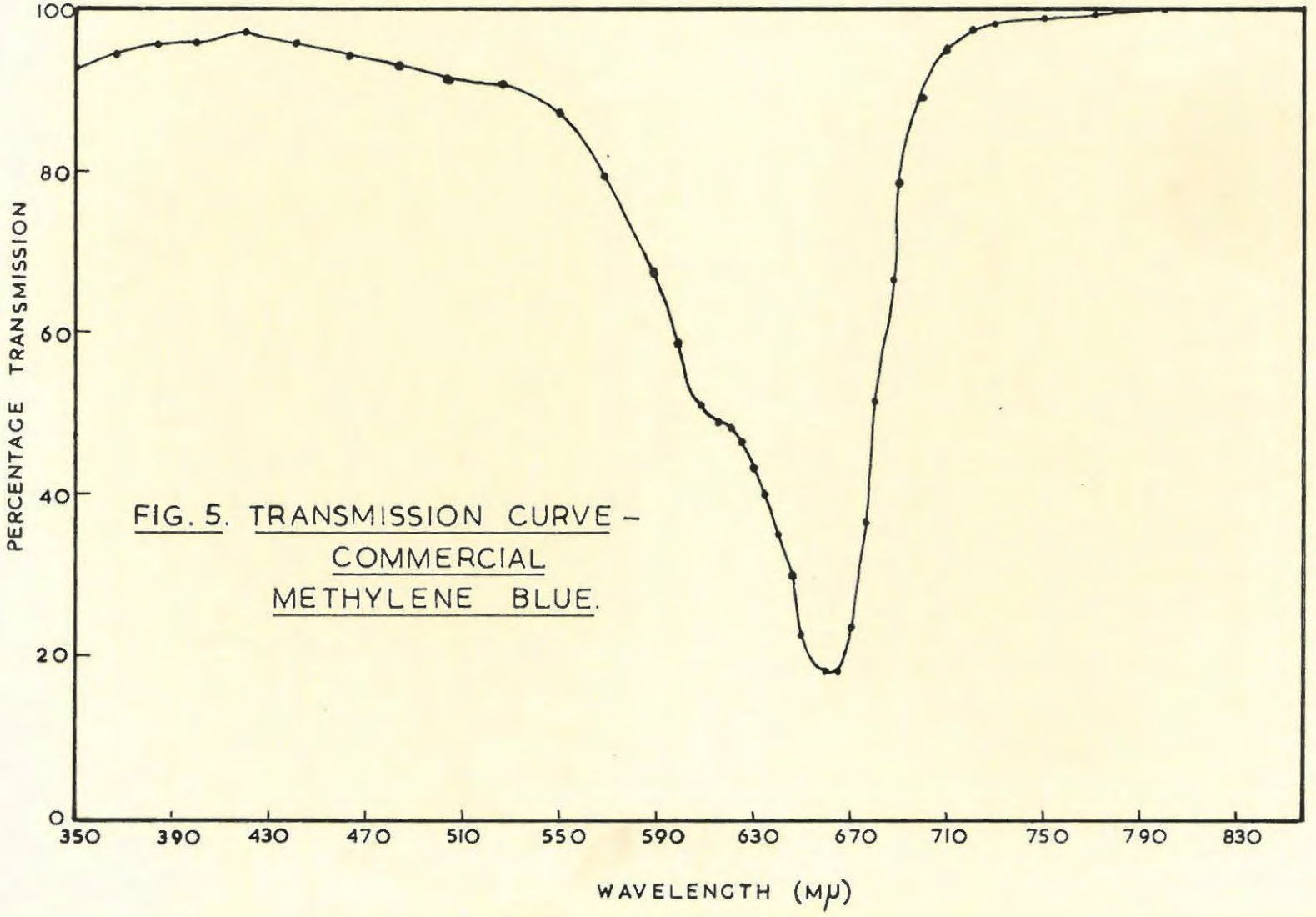


FIG. 5. TRANSMISSION CURVE – COMMERCIAL METHYLENE BLUE.

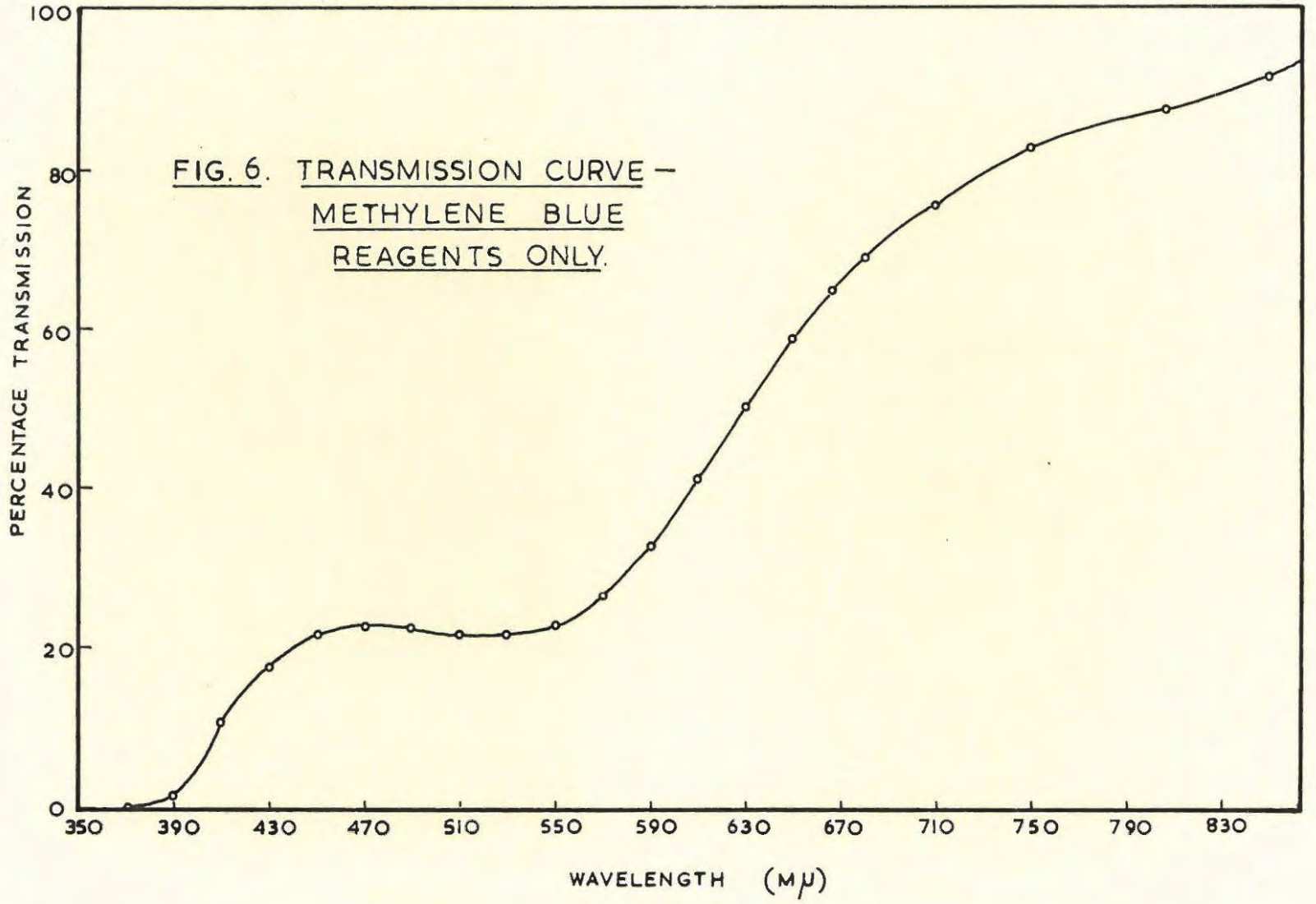


FIG. 6. TRANSMISSION CURVE — METHYLENE BLUE REAGENTS ONLY.

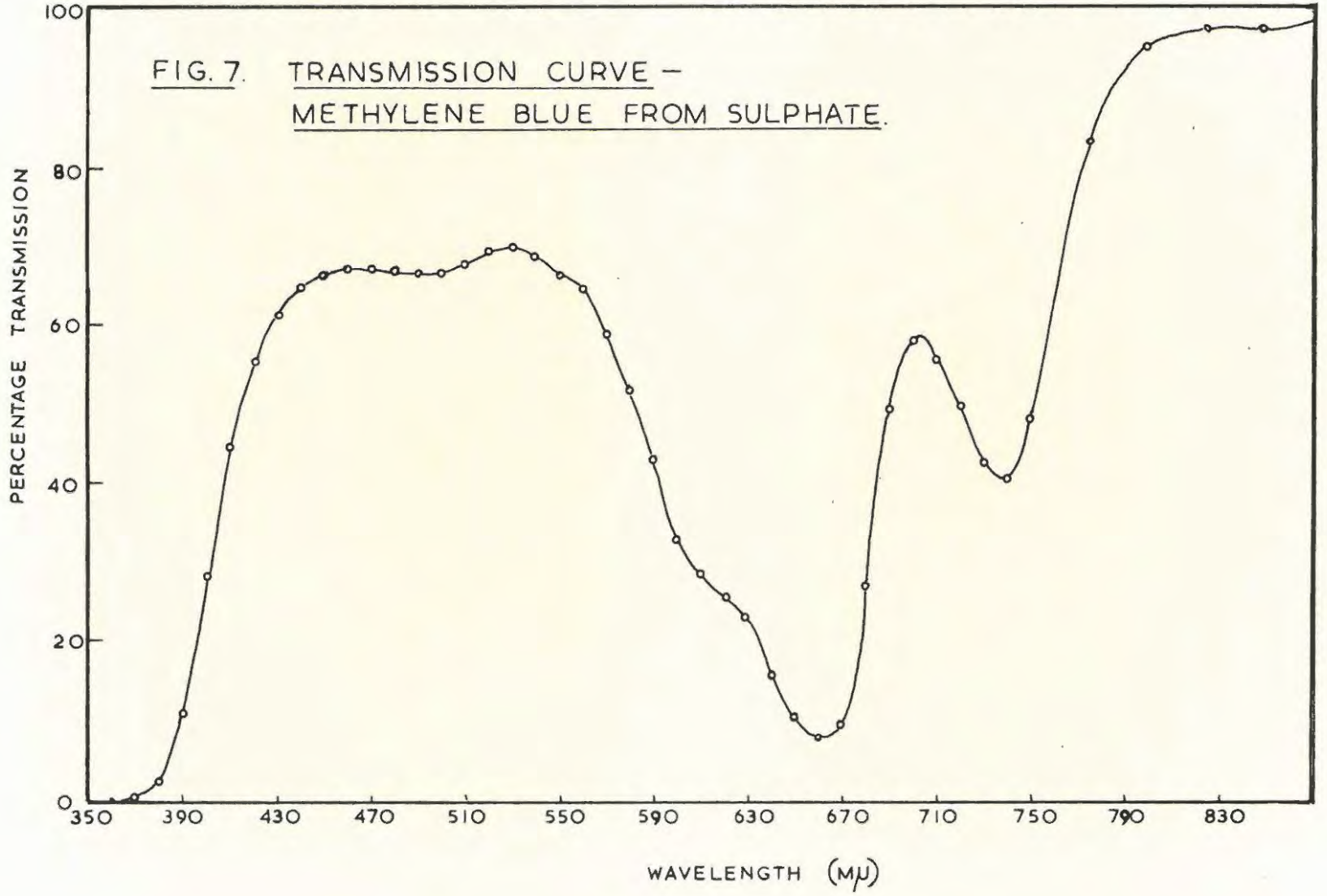


FIG. 7. TRANSMISSION CURVE –
METHYLENE BLUE FROM SULPHATE.
PERCENTAGE TRANSMISSION
100
80
60
40
20
0
350
390
430
470
510
550
590
630
670
710
750
790
830
WAVELENGTH (mμ)

absorbing the hydrogen sulphide and developing the colour in the normal manner (fig. 7). This last transmission curve corresponds almost exactly with the extinction curve obtained by Gustafsson 67).

The differences between the curves in fig. 5 and fig. 7 are almost certainly due to the other coloured compounds formed in the reaction and already mentioned in section 1.1, and which are responsible for the transmission curve in fig. 6.

From fig. 7 it was deduced that the maximum absorption occurred at about 667 mμ but that, for intensely coloured solutions, the absorption maximum at 740 mμ might also be used. Since the maximum at 667 mμ is so sharp, careful adjustment of the instrument is required to ensure that measurements are made at the exact wavelength of maximum absorption, since absorbance readings diminish rapidly on either side of this peak.

3.4 Experimental Studies on the Reduction Procedure.

3.41 The Reduction Mixture.

Reduction mixtures of hydriodic acid, formic acid and red phosphorus (7), hydriodic acid, formic acid and hypophosphorous acid (74) and hydriodic acid, acetic acid and sodium hypophosphite (68), were used.

The mixture containing red phosphorus, after an initial trial, was discarded owing to the difficulty experienced in dispensing the reagent satisfactorily. The present author found the mixture which contained formic acid preferable to that containing acetic acid since, in his estimation, the volatile acid vapours from the hot solution in

the former case were less unpleasant than in the latter case. From the practical viewpoint, however, both mixtures were found to be equally efficacious.

Attempts at regenerating the mixture or distilling off the unused hydriodic acid fractionally proved unsuccessful owing to the decomposition of the sodium hypophosphite or hypophosphorous acid, resulting in the formation of phosphine, readily detectable by its odour. On using reduction mixtures in which this phosphine was present as a dissolved impurity, seemingly random copious white fumes were found to be evolved and serious negative errors resulted (Table VIII).

TABLE VIII

Errors observed when phosphine was present in the reduction mixtures.

Sulphur content (µg. S)	4·00	8·00	12·00	16·00	20·00
Sulphur found (µg. S)	1·20	4·00	11·65	7·40	17·90
	2·95	7·45	7·10	5·50	11·20
	2·40	7·00	11·50	14·70	12·40
	2·80	5·05	11·40	13·50	16·30

It will be noticed from the Table that the magnitude of the errors varied considerably. There was found to be some correlation, however, between the magnitude of the error and the quantity of white fumes observed in the distillation apparatus. These fumes were found to be absorbed by neither the wash solution of pyrogallol or pure water, nor the acetate absorption solutions. Various methods were employed in an effort to remove the phosphine from the reduction mixtures but no

successful method was found. In addition, varying the heating of the reduction flask and adjusting the heating mantle, with its asbestos guard, in order to minimise the possibility of overheating the walls of the flask, proved to have little or no effect on the evolution of these fumes.

Provided that the amount of nitric acid, perchloric acid and other oxidising agents in the sample aliquots used was not too high, it proved possible to use the reduction mixture for two or even three determinations without reheating or any other form of regeneration and without loss in efficiency.

Where the cost of the reduction mixture is of importance, the use of the mixture containing red phosphorus is advisable owing to the possibility of its regeneration.

The present author's researches confirmed Gustafsson's findings that dilution of the reducing agent seriously reduced its efficiency (68). Even with sample aliquots of only 0·2 ml., errors of up to 5% resulted (see Table IX).

TABLE IX

Effect of diluting the reduction mixture.

Sulphur Content (µg. sulphur)		16·53	10·83
Sulphur found (sample heated to dryness (µg. S)		16·50	10·81
Sulphur found (not heated to dryness) (µg. S)	(i)	16·06	10·52
	(ii)	15·67	10·46
Percentage error	(i)	- 2·8	- 2·9
	(ii)	- 5·2	- 3·4

For all subsequent determinations, the samples were heated to dryness in an oven at about 120°C.

Throughout the present author's researches, the reduction mixture was stored in a brown glass bottle fitted with a ground glass stopper. No noticeable deterioration was observed after a period of over one month.

3.42 Reduction Time and Nitrogen Flow-rate.

The time required for the complete reduction of all the available sulphur in the sample aliquot was found to be largely dependent on the flow-rate of the nitrogen carrier gas. When using a flow-rate of about 50 ml./ min., between 30 minutes and an hour was found necessary; however, when the flow-rate was increased to 100 ml./min., the reduction and absorption was completed within 10 minutes. A further 5 minutes was allowed, however. The more rapid flow-rate was found to give greater reproducibility of results.

3.5 Experimental Studies on the Absorption Procedure and Colour Development.

3.51 Variations in the compositions of the absorbing solutions.

Zinc acetate - sodium acetate solution, as prescribed by Johnson and Nishita (7), was employed for most of the research work carried out and proved entirely satisfactory, both with regard to quantitative absorption of the hydrogen sulphide and stability at room temperatures. On the addition of the acid amino-reagent, quantitative recovery of the hydrogen sulphide was also obtained. The zinc acetate - sodium hydroxide solution as prescribed by Tyou

and Humblet (69) was also tested and found to be equally satisfactory. However, the 12% aqueous solution of sodium hydroxide was found to be less satisfactory to store and dispense and, in addition, time is saved in dispensing the single ZnAc - NaAc reagent as opposed to the two solutions required for the ZnAc - NaOH reagent.

The addition of a few drops of glacial acetic acid when preparing the stock absorption solution was definitely found to be advantageous in preventing, or at any rate delaying, the precipitation of hydroxides in the solution on keeping.

3.52 Concentration of the reagents.

The concentration of the zinc acetate and sodium acetate in the absorption solution as prescribed by Johnson and Nishita (7), viz. 0·5 g. zinc acetate and 0·125 g. sodium acetate in the 100 ml. absorption flask, were found to be entirely adequate and the considerably higher concentration suggested by St. Lorant (56) and confirmed by Roth (60) would appear to be unnecessary.

Time did not permit an investigation to be made into the effects of altering the concentrations of the amine reagent constituents and the resulting pH effects. It may however be mentioned that, since no fresh p-amino dimethylaniline sulphate was available, a solution of p-amino dimethyl aniline in sulphuric acid solution was used. This solution proved satisfactory and, although fresh supplies of the reagent solution were prepared three times, no special care being taken to prepare the solutions exactly alike, no noticeable difference in the absorbance measurements was detected. It would thus appear that

a limited error in making up the reagent solutions is allowable.

3.53 The addition of the colour reagents.

The addition of the colour reagents proved to be one of the most sensitive factors influencing the accuracy of the method. The amine reagent and the ferric reagent must be added successively; however, the sulphuric acid in the amine reagent liberated hydrogen sulphide from the zinc sulphide and, during mixing, part of this gas entered the space above the solution and, on adding the ferric reagent, a certain amount of the gas escaped into the air and low sulphur recoveries resulted.

Washing of the detachable glass tube and its removal before the colour development was completed also increased the possibility of hydrogen sulphide losses in this way. Allowing the glass tube to remain in the flask while making the solution to the mark was found to suffer from the disadvantage that it necessitated the use of lengths of glass tubing of identical internal and external dimensions, and also the use of volumetric flasks having their volume markings at identical positions on the flask necks so that, for each solution, the same volume of glass tube was submerged. The following procedure, described by Kilmer and Nearpass (76), was thus followed:

Ten minutes after the addition of the colour reagents, the glass tubes were removed by means of tweezers, washed well and the solutions made to the mark. Nevertheless, care was required while shaking the flasks since, unless the detachable glass tubes fitted exactly into the flasks, their movement inside the flasks tended to

result in the chipping off of small fragments of glass from the tubes and, in one case, the glass stopper used, being hollow and of relatively thin glass, was fractured.

For the addition of the reagents, fast-delivery pipettes were used, the most effective procedure proving to be the addition of the amine reagent through the detached glass tube so that the acid solution formed a layer at the bottom of the flask and mixing only occurred after stoppering the flask. The flask was then thoroughly swirled and the ferric reagent rapidly added. Blowing to speed up the pipette delivery was tried but cannot be advocated since the hydrogen sulphide was found to be readily lost when blowing was employed. Each flask was then thoroughly shaken for 30 seconds to 1 minute before removing the tubes and making to the mark.

3.54 The effect of temperature on colour development.

Time did not permit a thorough statistical investigation to be carried out on the effects of temperature changes on the extinction of MB solutions. However, preliminary tests showed slight variations in the temperature during the colour development to affect seriously the absorbance of the MB solutions and all later work was carried out in a constant-temperature room at 24°C ± 0·5°C. The actual spectrophotometric measurements, however, were carried out at various room temperatures between 15° and 23°C.

The findings of Gustafsson (67) were largely confirmed and experiment showed that a temperature deviation of about 1° from 24°C. resulted in a mean error of approximately 0·3% in the % sulphur. The

temperature of all the reagents and solutions should thus be controlled within about 1°C during the colour development. After the maximum colour has been attained at that temperature, variations in the temperature were found to have little or no effect.

3.55 Washing the hydrogen sulphide and cleaning the distillation apparatus.

In the initial research work, the pyrogallol wash-solution described by Johnson and Nishita (7) was employed and renewed daily. Later, the use of deionised distilled water was tested and found to be equally effective in removing traces of acid carried over by the nitrogen, provided the water was replaced after six to ten successive reductions. No yellow iodine coloration was ever observed in this water.

After use, the water was run out, the stopcock closed, a beaker of deionised distilled water placed under the condenser in place of the reduction flask, and the water sucked through the condenser and wash-column by means of a length of tubing attached to the outlet tube of the wash-column at the point where the detachable tube to the absorption flask was normally attached. This procedure was repeated four times, the rinsing solution being drained off after each washing except the final washing when about 15 ml. was allowed to remain in the wash-column for the following determinations. The inner surface of the condenser was allowed to drain overnight until dry. This washing procedure eliminated the necessity for dismantling the apparatus.

/ 3.56 The stability of the colour.

3.56 The stability of the colour.

The colour intensity of MB solutions was found to decrease fairly rapidly if exposed to light but, kept in the dark, the absorbance of solutions was found to be unaltered even after 48 hours. Under normal circumstances, however, it should prove possible to measure the absorbance within 12 hours.

3.57 Relation to Beer's Law.

Fig. 8 shows the relation to Beer's Law of solutions of methylene blue prepared according to the usual procedure, from samples containing from 0 to 100 µg. sulphur. Measurements were made using 1 cm. cells. A Hilger Uvispek Spectrophotometer (fig. 9) was used at a wavelength of 667 mµ. From the calibration curve, it was readily deduced that for aliquots containing more than about 25 µg. sulphur, the methylene blue colour showed a marked deviation from Beer's Law. Hence, the range from 0 - 20 µg. sulphur was used for later determinations.

Johnson and Nishita's method (7) for the dilution of more concentrated MB solutions was found to bring such solutions within the range in which Beer's Law was obeyed. Where possible, however, it was found very much more convenient to choose aliquots of plant solution which would contain less than 20 µg. sulphur.

3.58 Magnitude of blanks.

Blank readings were obtained by carrying out the complete procedure, omitting only the sulphur-containing aliquot of sample. Throughout the determinations, Fisher Colloseal lubricant was used for all ground-glass joints in place of the sulphur-free silicone lubricant

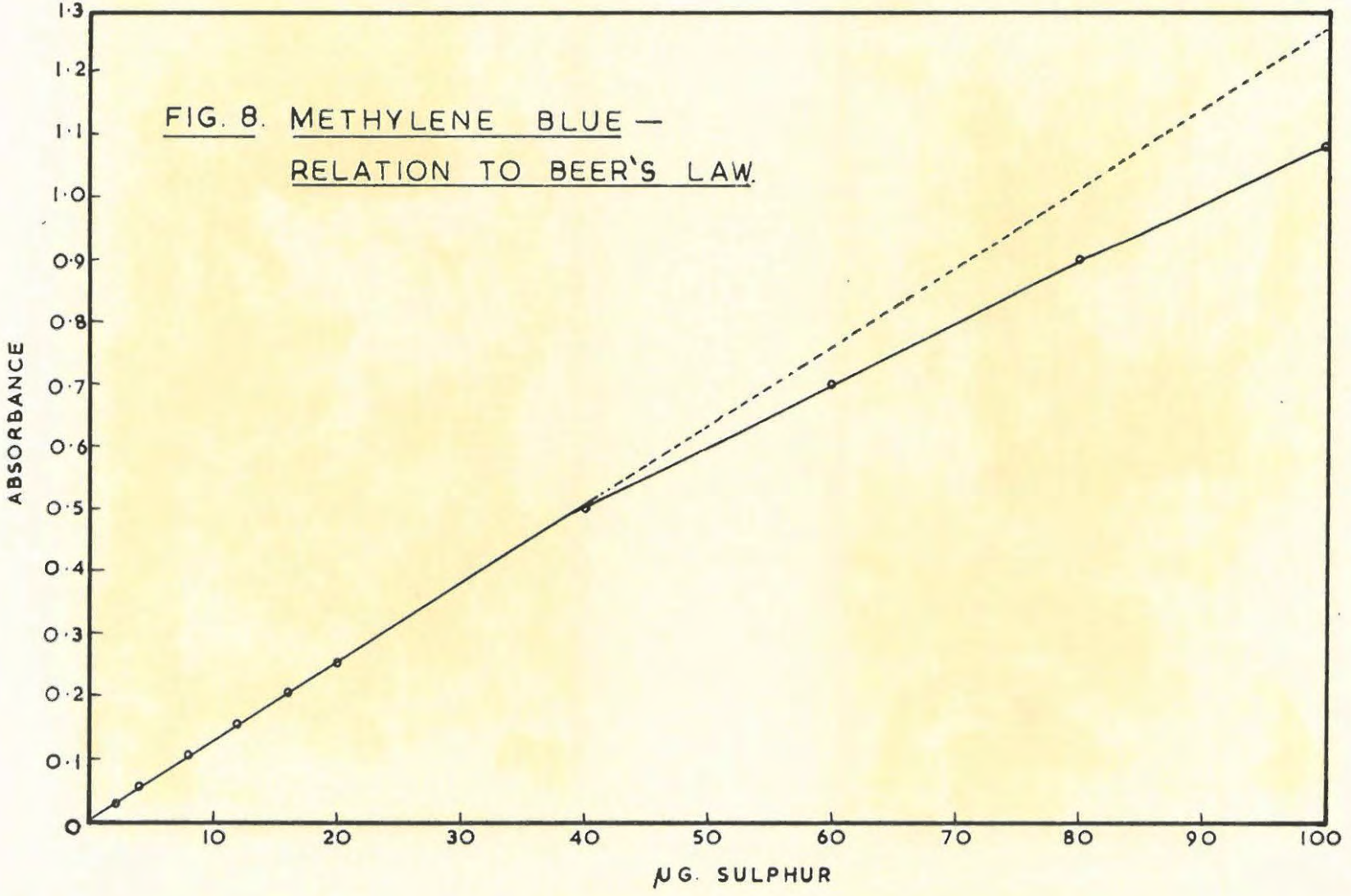


FIG. 8. METHYLENE BLUE —
RELATION TO BEER'S LAW.
ABSORBANCE
1·3
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
10
20
30
40
50
60
70
80
90
100
µG. SULPHUR

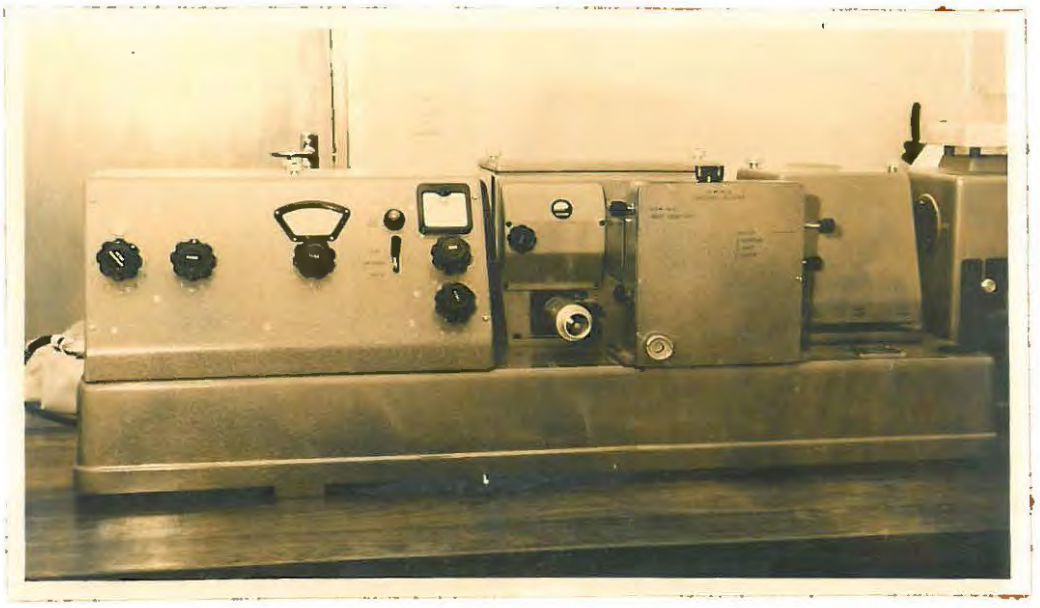


FIG. 9. HILGER UVISPEK SPECTROPHOTOMETER.

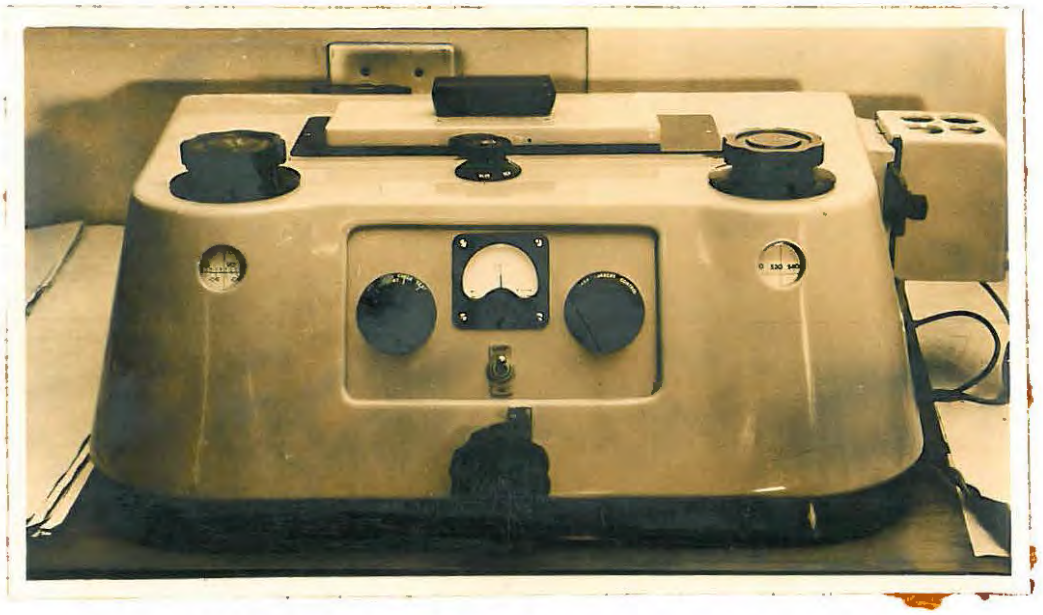


FIG. 10. UNICAM SP600 SPECTROPHOTOMETER.

suggested by Johnson and Nishita. For 1 cm. cells, the blanks ranged from 0·004 to 0·007 and, for 4 cm. cells, from 0·015 to 0·019. A reduction carried out using as sample an amount of lubricant approximately five times that normally used in lubricating the ground-glass joints gave an absorbance of 0·016 for 1 cm. cells. Assuming that a minimum of lubricant would normally be used, an absorbance of 0·004 appeared to be the normal blank value for 1 cm. cells.

3.59 Effect of the presence of traces of heavy metals in the absorber.

Since the commonest metallic ions found in distilled water are copper ions, an investigation was carried out into the effects of traces of copper and also manganese, mercury and lead ions in the absorption solution. The results are shown in Table X. The amounts of metals present are per 100 ml. absorption flask.

TABLE X

The Effect of Traces of Heavy Metals in the Absorber.

Sulphur content of sample = 10·28 µg.

Metal ions added	µg. metal added	µg. sulphur found	% Error
Copper	5	7·71	- 25·1
	10	5·14	- 50·1
	20	1·40	- 86·4
	30	0·00	- 100·0
Manganese	5	10·28	0·0
	10	9·89	- 3·8
Mercury	5	9·34	- 9·1
	10	7·78	- 24·3
Lead	20	10·28	0·0

The molar ratio (copper added : loss of sulphur) is approximately 1 : 1 which indicates the formation of cupric sulphide more or less quantitatively. The molar ratio (mercury added : loss of sulphur) appears to approach 1 : 2 indicating the probable formation of the complex $Hg(SH)_2$. Small quantities of manganese have little effect and lead appears to be without effect. Additions of these elements after colour development had no effect on the absorbance of the MB solutions. In view of the very serious interference of copper ions, all water used was deionised by passage through a resin column containing Amberlite MB 1 and MB 3.

3.6 GENERAL OBSERVATIONS.

3.61 Cleaning of Apparatus.

Lewin (78) has drawn the attention of research workers to the dangers involved in the use of chromic acid mixture for the cleaning of glassware. The sulphuric acid is very strongly adsorbed by the glass and, even after as many as twelve washings, sulphate is still detectable by tests such as the MB procedure. The present author had experience of these errors and accordingly avoided the use of chromic acid in the cleaning of glass apparatus.

After use, the reduction flasks were rinsed out twice with distilled water and twice with deionised distilled water, all lubricating grease being removed by means of a cotton wool pad on a glass rod. Finally each flask was rinsed out with acetone, redistilled from an all-glass apparatus and later reclaimed. This removed the last traces of grease and facilitated drying of the flasks. All other apparatus

was cleaned using dilute teepol solution, when necessary, and thoroughly washed with distilled water followed by deionised distilled water.

3.62 Preparing Samples for the Reduction Procedure.

The plant solutions used in the present investigations were prepared as described in Section 4 (page 73). A trial analysis showed that the volume of plant solution required was 0·4 - 0·5 ml. This volume was transferred to the reduction flask by means of a 1 ml. graduated pipette and the flask, contained in a suitable beaker, was heated in a drying oven at about 120°C for 45 minutes to 1 hour. The flask was then allowed to cool in the air. It proved convenient to have six such reduction flasks so that the drying of six sample aliquots might be carried out simultaneously, the drying stage being the most lengthy in the analysis, apart from the preparation of the leaf solutions.

3.63 Spectrophotometric Measurements.

Throughout the above investigations, two instruments were used viz. the Hilger Uvispek Spectrophotometer (fig. 9) and the Unicam SP 600 Spectrophotometer (fig. 10), using 1 cm. and 4 cm. cells. As the blank, deionised distilled water was used.

4. THE PREPARATION OF THE PLANT SOLUTION.

4.1 Preparation of the Leaf Sample.

Immediately after sampling and picking (79) the samples were transported to the laboratory in open-wove cloth bags with a minimum of delay, transferred to polythene bags and stored in a refrigerator. The leaves were then prepared for analysis as described by Steyn (80):-

Five polythene dishes were prepared as follows. The first contained about 750 ml. 0·1% teepol solution, the second and third each contained 750 ml. distilled water, the fourth an equal volume of deionised distilled water and the fifth dish was used to receive the wet leaves. In order to remove surface dirt and soil particles, each leaf was individually sponged in the teepol sclution with cotton wool and then rinsed consecutively in the three sets of water. The mid-ribs were not removed as originally described (80) since their presence was found to have little effect on the values obtained in the elemental analysis.

The leaves were folded into a sheet of Whatman No. 1 drying paper and placed in a forced-draught oven at 65°C for 48 hours. The dried leaves were finally ground finely in an agate ball mill and the intimately mixed samples stored in screw-capped bottles. Immediately before analysis, each sample was dried for a further 24 hours at 65°C, since the dried leaf powder was fairly hygroscopic.

4.2 Preparation of the Leaf Solutions.

To 1 g. leaf powder, weighed into a 100 ml. wide-necked Erlenmeyer flask (fig. 11), was added 12·5 ml. acid digestion mixture

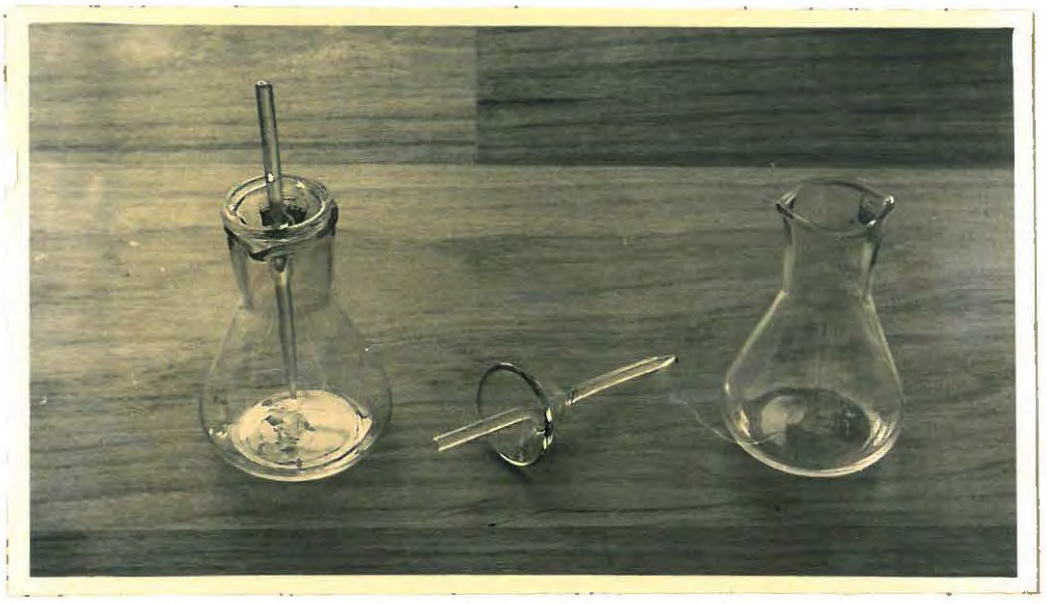


FIG. 11. LEAF SAMPLE DIGESTION FLASKS.

(800 ml. distilled C.P. Nitric Acid mixed with 200 ml. Perchloric Acid, pro analysi). Cover glasses to fit the conical flasks, shaped like small funnels, each with a tapered $1\frac{3}{4}$" glass rod fused to the apex, were placed over the flasks which were left overnight in the cold. The flasks were then heated on a Simmerstat electric hot-plate for $1\frac{1}{2}$ hours at about 75°C, $\frac{3}{4}$ hour at about 95°C, 1 hour at about 115°C and $\frac{1}{2}$ hour at about 140°C. The cover glasses were then rinsed and removed. When all the nitric acid had boiled off, the temperature was raised to about 180°C for a further $\frac{1}{2}$ hour or almost until dryness. The residue was taken up with boiling water and 1 drop of concentrated hydrochloric acid was added. The hot solution was filtered through Whatman No. 40 filter paper into a 100 ml. volumetric flask, the filter having previously been well washed with boiling water. The erlenmeyer flask and filter were rinsed six or more times with boiling water and the volumetric flask allowed to stand overnight. After standing for 1 hour in a thermostat at 20°C, the solution was made to the mark with deionised distilled water.

5. THE MODIFIED PROCEDURE FOR THE DETERMINATION OF SULPHUR IN PLANT MATERIAL AS METHYLENE BLUE.

REAGENTS:

(i) Carrier Gas.

Cylinder nitrogen gas, purified by passage through a strong solution of mercuric chloride in potassium permanganate solution.

(ii) Wash Solution.

For washing the hydrogen sulphide gas, liberated during the reduction procedure, deionised distilled water is used (see below).

(iii) Nitrogen Purification Solution.

Between 5 and 10 grams mercuric chloride (i.e. an excess) is added to 100 ml. 2% potassium permanganate solution.

(iv) Absorption Solution.

100 g. Zinc acetate dihydrate and 25 grams sodium acetate trihydrate are dissolved in deionised distilled water and made up to 2 litres. About 1 ml. glacial acetic acid (reagent grade) is added and the solution filtered after standing for 48 hours. Any turbidity appearing after a period of time may be disregarded.

(v) Amine Reagent.

5 Millimoles p-amino-dimethylaniline sulphate (i.e. 0·93 g. of the sulphate, $NH_2C_6H_4N(CH_3)_2 \cdot \frac{1}{2}H_2SO_4$, Eastman Kodak No. 1333, or 0·684 g. p-amino-dimethylaniline, Eastman Kodak P. 2147) is dissolved in 750 ml. deionised distilled water, 2·50 moles (187 ml.) concentrated reagent grade sulphuric acid is added and the cooled solution diluted to 1 litre with deionised distilled water. This reagent keeps for many months.

(vi) Ferric Reagent.

To 25 g. reagent grade ferric ammonium sulphate is added 5 ml. concentrated reagent grade sulphuric acid and the solution is diluted to 200 ml.

(vii) Sulphur-free Distilled Water.

Deionised distilled water should be used in the preparation of all reagents. The recommended resin-column contains a mixture of the monobed-resins, Amberlite MB 1 and MB 3.

(viii) Reduction Mixture.

Great care should be taken in the preparation of the reduction mixture. 100 ml. FRESH reagent grade hydriodic acid (S.G. 1·7, methoxyl grade), 50 ml. reagent grade 90% formic acid and 25 ml. reagent grade hypophosphorus acid are placed in a three-necked 250 ml. 'quickfit' flask. Nitrogen is bubbled through the solution and the flask is heated by means of a heating mantle. After boiling for five minutes, the flask is fitted with a reflux condenser and the solution is boiled for a further five minutes. The solution is then allowed to cool in the flask, the nitrogen flow is discontinued and the reduction mixture is transferred to a brown glass-stoppered bottle for storage. The 'quickfit' apparatus used in the preparation of the reduction mixture is shown in fig. 12. Provided that plant samples used in the reductions are evaporated to dryness and contain little perchloric acid, the reduction reagent may be used two or even three times. It should not be reheated to boiling point for regeneration owing to the danger of phosphine formation.

/ (ix) Standard Sulphate Solution.

(ix) Standard Sulphate Solution.

A standard potassium sulphate solution is prepared containing 1·087 g. of the reagent grade salt in 1 litre. This solution contains 200 p.p.m. sulphur and is further diluted as required. A solution containing 20 p.p.m. sulphur proves convenient since, in the preparation of the calibration curve, sample volumes less than 1 ml. may be evaporated to dryness fairly rapidly.

(x) Lubricant for Glass Joints.

Fisher 'Celloseal' burette grease is suitable provided that very small quantities are used.

STORAGE OF SOLUTIONS.

Solutions may be stored in pyrex flasks or bottles without deterioration. The reduction mixture should, however, be stored in a glass-stoppered brown-glass bottle.

CLEANING OF GLASSWARE.

The use of chromic acid should be avoided. Glassware should be thoroughly cleaned with 'teepol' solution and deionised distilled water as described in section 3.61.

APPARATUS.

The apparatus used in the preparation of the reduction mixture is shown if fig. 12 and the distillation apparatus used in the methylene blue determination is shown in fig. 13. The ground-glass joints above the condenser and gas-washing column may be omitted since the apparatus is washed in situ. In addition, a capillary flow-meter

FIG. 12. PREPARATION OF REDUCTION MIXTURE.

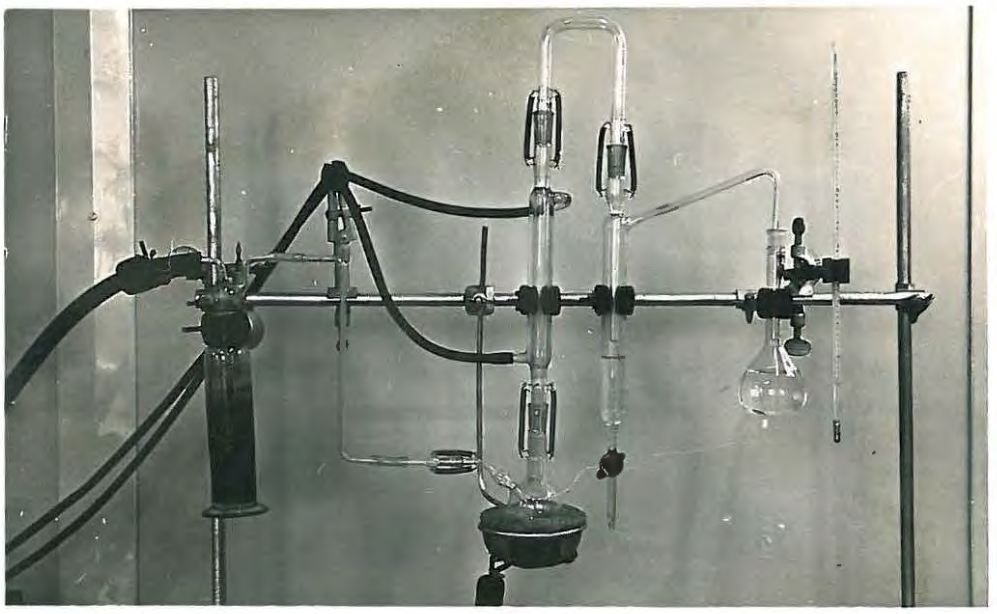


FIG. 13A MB REDUCTION & DISTILLATION APPARATUS.

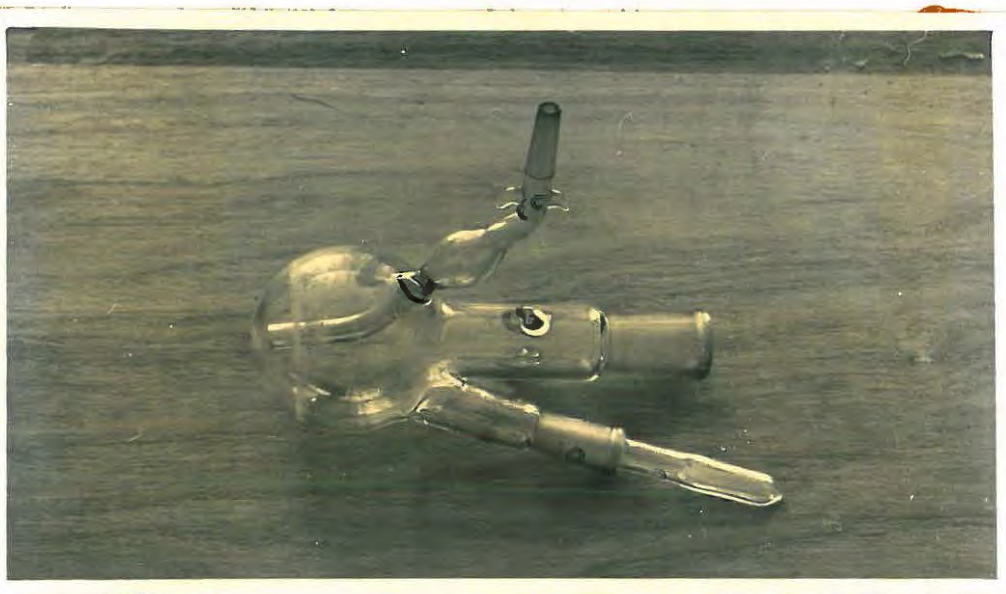


FIG. 13B REDUCTION FLASK.

may be inserted between the nitrogen-purification wash-bottle and the reduction flask. A 'glas-col' heating mantle is used for heating the reduction flask. To avoid overheating the walls of the flask, a small sheet of asbestos board, with a central hole 1¼ inches in diameter, may be placed between the mantle and the flask. Tygon tubing is used for all glass-to-glass connections.

PROCEDURE.

The sample aliquot, preferably not exceeding 2 ml. in volume, is carefully introduced into the reduction flask. The flask is supported in a suitable beaker and the sample evaporated to dryness in a drying oven at about 120° for 1 hour or until evaporation is complete. The flask is allowed to cool to room temperature. Since 10 - 15 ml. deionised distilled water is siphoned into the gas-washing column after the washing of the apparatus, the column will not require filling at this stage.

20 ml. Absorbing solution (iv) is pipetted into a 100 ml. volumetric flask and a further 60 ml. deionised distilled water added. A short length of glass tubing is attached to the exit tube of the gas-washing column by means of a short length of 'tygon' tubing and the volumetric flask is clamped in position so that the detachable glass tube almost reaches the bottom of the flask.

The reduction flask is attached to the condenser and nitrogen inlet tube by means of spring-fastenings and the nitrogen flow is adjusted to 150 - 200 ml. per minute. Through the smaller neck of the reduction flask (see fig. 13 b), 3 ml. reduction mixture (viii) is

rapidly introduced using a fast-delivery pipette and the lightly greased ground-glass stopper is immediately replaced.

The heating mantle (preferably already at the required temperature) is moved into position below the reduction flask. The temperature should be controlled so that the mixture comes to a gentle boil within about 1 minute. The reduction is continued for 15 minutes.

The glass absorption tube is carefully detached and allowed to drop into the absorption flask which is then stoppered and removed. The heating mantle is swung away from the reduction flask which is carefully loosened and removed. Another absorption tube is attached to the gas-washing column, another prepared absorption flask is placed in position, a further reduction flask is attached to the condenser and the reduction procedure repeated as described above.

By means of a very fast-delivery pipette, 10 ml. amine reagent (v) is introduced into the absorption flask by pipetting into the upper end of the detached absorption tube so that it forms a layer at the bottom of the flask. The stopper is immediately replaced and, after gently swirling two or three times to mix the contents, 2 ml. ferric reagent (vi) is added by means of another fast-delivery pipette. The flask is immediately stoppered and shaken vigorously for ½ to 1 minute.

After 10 minutes or longer, the detached absorption tube is carefully removed from the volumetric flask by means of tweezers, rinsed thoroughly and the solution is made to the mark with deionised distilled water. The extinction of the methylene blue solution is measured the same day on a suitable spectrophotometer at 667 mμ.

This wavelength is critical and the instrument should be checked regularly to ensure that readings are taken at the wavelength of maximum absorption. Until the absorbance is measured, the flasks containing the methylene blue solutions should be stored in subdued light.

Washing the Apparatus.

After six to ten determinations (depending on such factors as the nitrogen flow-rate, the efficiency of the condenser, the heating rate and the time of reduction), the water in the gas-washing column is run out, the stopcock closed and a beaker of deionised distilled water placed under the condenser in place of the reduction flask. The water is sucked through the condenser and wash-column by means of a length of glass tubing inserted at the point where the detachable tube to the absorption flask would normally be attached. This procedure is repeated four times, the rinsing solution being drained off after each washing except in the case of the final washing when about 15 ml. is allowed to remain in the column for the next group of determinations. The inner surface of the condenser is allowed to drain overnight until dry.

Calibration Curve.

The calibration curve is prepared exactly as described for plant samples but using aliquots of standard sulphate solution (ix) containing 0, 5, 10, 15 and 20 µg. sulphur respectively, and plotting the extinction of the methylene blue solutions against the sulphur concentrations.

/ Additional Notes.

Additional Notes.

1. A preliminary run, using an aliquot containing a small amount of sulphur, should be carried out before beginning a series of determinations, since the first determination frequently yields low results. The preliminary aliquot need not necessarily be evaporated to dryness and the colour need not be developed in the absorption flask.
2. There should be a minimum of delay between successive determinations in order to avoid the atmospheric oxidation of traces of hydriodic acid in the condenser.
3. The temperature of all the reagents and solutions should be maintained at a fixed temperature (within $\pm$ 0.5°C), preferably near room temperature, until the colour has been completely developed.
4. Where possible, all determinations should be performed in duplicate, since erratic results sometimes occur due to traces of phosphine in the reduction mixture, losses of hydrogen sulphide on addition of the colour reagents or other factors.

6. STATISTICAL STUDY OF THE ACCURACY AND PRECISION OF THE PROPOSED METHOD.

The modified methylene blue method was subjected to rigorous statistical tests to evaluate the accuracy and precision of the method under routine laboratory conditions.

6.1 Calibration Curve.

A calibration curve (fig. 14) was prepared following the procedure described in section 5. The results obtained are recorded in Table XI.

TABLE XI

Calibration Curve.

Unicam SP 600 Spectrophotometer - 4 cm. cells - 24°C.

µg. sulphur	% transmission at 667 mµ	µg. sulphur	% transmission
0	0·017	10	0·530
2	0·105	12	0·640
4	0·225	15	0·782
5	0·272	16	0·835
8	0·411	20	1·031

The calibration curve obtained was a straight line.

6.2 Accuracy of the Method.

The absolute accuracy of the method was examined by the addition of sulphur as sulphate to the citrus leaf samples, as follows. Twenty 0·5 g. aliquots of the homogeneous plant sample, prepared as detailed in section 4.1, were weighed separately. 1·250 mg. sulphur as standard potassium sulphate solution was added to each sample. Each of

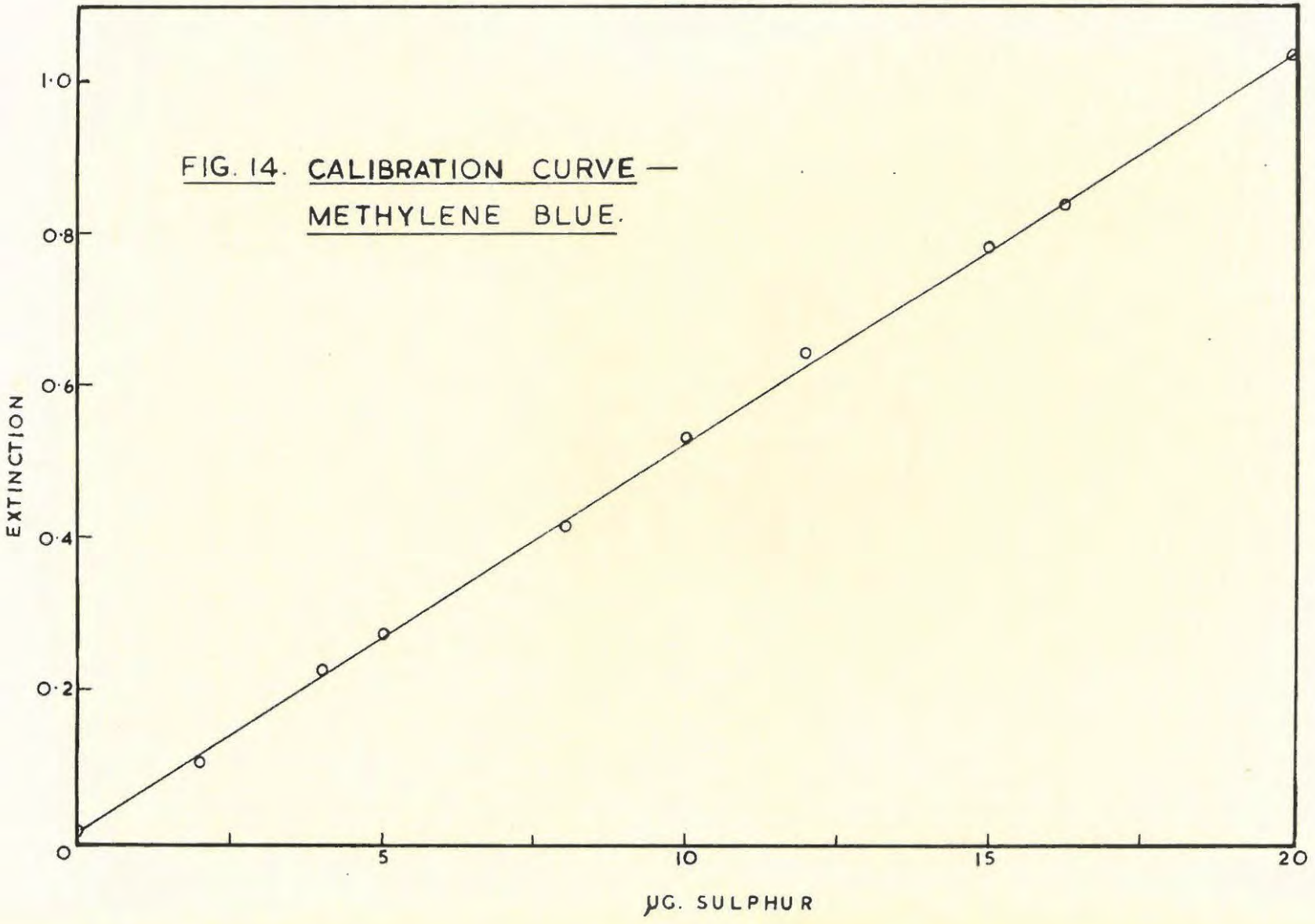


FIG. 14. CALIBRATION CURVE — METHYLENE BLUE.

the samples was brought into solution following the procedure detailed in section 4.2. The sulphur content of each sample was determined as described in section 5, in duplicate. The results obtained are reproduced in Table XII (page 84).

The sulphur content of the plant material in p.p.m. sulphur was calculated from the weight (mg.) of sulphur determined and the sample weight. The 1·250 mg. sulphur aliquot added was deducted in each case. The results are recorded in column 5. The mean of the thirty precision determinations (see section 6.3) was found to be 2680 p.p.m. sulphur. The ratio of each individual determination to this mean was calculated as a percentage. This value is given in column 6. From these data it was concluded that this procedure gave a true reflection of the sulphur content of the plant material.

6.3 Precision of the Method.

The precision of the method was evaluated as follows. A homogeneous plant sample was prepared. This was dried in an oven at 65°C. and fifteen 1 g. samples were weighed out. Each sample was individually dissolved in the acid digestion mixture and the solution prepared as detailed in section 4.2. The sulphur content of the resulting solutions was determined using the recommended procedure.

This series of tests was performed under conditions closely approximating those in a routine laboratory. The tests were performed over a period of several days. The temperature of the reagents was kept constant at about 24° ± 0·5°C. Fresh reagents were prepared as required. The results are recorded in Table XIII (page 85).

These results revealed that the procedure could determine down to 2·7 mg. sulphur in plant material with a coeff. of variation of 1·7%.

TABLE XIII

THE PRECISION OF THE MODIFIED PROCEDURE.

Determination (n)	Sample weight g.	Sulphur found p.p.m.	M - Y	$(M - Y)^2$
1	1·057	2634	46	2116
2		2634	46	2116
3	1·015	2625	55	3025
4		2644	36	1296
5	1·019	2752	72	5184
6		2655	25	625
7	1·023	2681	1	1
8		2604	76	5776
9	1·006	2686	6	36
10		2572	108	11664
11	1·025	2678	2	4
12		2696	16	256
13	1·027	2670	10	100
14		2708	28	784
15	1·010	2603	77	5929
16		2659	21	441
17	1·027	2689	9	81
18		2708	28	784
19	1·017	2735	55	3025
20		2735	55	3025
21	1·009	2662	18	324
22		2760	80	6400
23	1·014	2687	7	49
24		2706	26	676
25	1·008	2722	42	1764
26		2762	82	6724
27	1·015	2686	6	36
28		2686	6	36
29	1·022	2686	6	36
30		2668	12	144
n - 1 = 29		2680 (mean)		62457 $= \sum(M - Y)^2$

The Standard Deviation (σ) = $\sqrt{\frac{\sum(M - Y)^2}{n - 1}}$ = 46·40 p.p.m.

Coefficient of Variation (% Std. Dev.) = $\frac{46{\cdot}40}{2680} \times 100$ = 1·73 %.

7. A PRELIMINARY TEST ANALYSIS OF CITRUS LEAF SAMPLES FROM THE GAMTOOS VALLEY.

The methylene blue procedure was applied to the analysis of citrus samples from the Gamtoos River valley. These citrus trees had been undergoing treatment with various fertilizers. The results are given in Table XIV.

TABLE XIV. SULPHUR ANALYSIS IN GAMTOOS VALLEY CITRUS LEAF SAMPLES.

Sample number	Variety	Age (years)	Soil type	Fertilizers applied	Percentage sulphur
R_1Ia	Navels	7	Light sandy	Ammonium sulphate	0·384
S_2Ia	Navels	5	Heavy loam	Ammonium sulphate	0·245
K-I-V	Valencias	8	Sandy loam	Ammonium sulphate Potassium sulphate	0·262
M-I-V	Valencias	15	Sandy silt loam	Ammonium sulphate	0·319
P-I-N	Navels	22	Silt loam	Ammonium sulphate	0·320
A-3-N	Navels	25	Sandy	No sulphate	0·339
A 4	Navels	10	Sandy	Ammonium sulphate	0·261
B 4	Navels	10	Sandy	Ammonium sulphate Superphosphate	0·258
C 4	Navels	10	Sandy	Ammonium sulphate Potassium sulphate	0·267
D 4	Navels	10	Sandy	Ammonium sulphate Potassium sulphate Superphosphate	0·258

From the Table it would appear that there is no deficiency in sulphur in any of these citrus leaf samples, since 0·2 - 0·4 % is considered satisfactory for the percentage sulphur.

8. THE MOLYBDENUM BLUE METHOD.

8.1 The Molybdenum Blue Reduction.

This method, recommended by Bethge (81) in 1952, makes use of the coloured compound, usually referred to as molybdenum blue, whose composition is uncertain. This blue compound would appear to be formed by the reduction of molybdates by any strong reducing agent in the presence of the correct acid concentration.

Little if any relevant work appears to have been carried out, previously or since, on the application of this compound to the colorimetric determination of sulphur. However, Woods and Mellon (82) have mentioned the instability of the molybdenum blue colour, the difficulty of obtaining complete development of the colour, and the importance of the reagent quantities. They also found that the colour conformed to Beer's Law for only very low concentrations.

Basically, the method involves the absorption of hydrogen sulphide (formed by reduction of the sulphur) in zinc acetate solution and the reaction of the hydrogen sulphide with ammonium molybdate on the addition of a suitable amount of acid.

Bethge studied the respective efficiencies of sulphuric and phosphoric acids and found phosphoric acid more suitable since the colour intensity was not influenced so markedly by slight variations in the acidity and the absorbance was found to attain constancy 20 minutes after mixing. In addition, the calibration curve was found to be linear when phosphoric acid was used but not when sulphuric acid was used.

He also investigated what quantities of all the reagents

would yield optimum coloured solutions for spectrophotometry.

8.2 THE METHOD OF BETHGE.

REAGENTS.

Zinc acetate solution.

50 g. zinc acetate dihydrate and 10 g. sodium acetate trihydrate are dissolved in 500 ml. distilled water, set aside overnight, the solution filtered and diluted to 1 litre.

Ammonium molybdate solution.

100 g. ammonium molybdate tetrahydrate is dissolved in 1 litre of water by gentle warming and the solution filtered after 2 days.

Phosphoric acid.

342 ml. of 55% orthophosphoric acid are diluted to 1 litre.

All reagents are reagent grade.

PROCEDURE.

The hydrogen sulphide is absorbed in 10 ml. of the zinc acetate solution and the mixture transferred to a 50 ml. volumetric flask. 2.5 ml. ammonium molybdate solution is added, followed by 6 ml. phosphoric acid solution. The white precipitate, formed when the first ml. of phosphoric acid is added, dissolves easily on the addition of the remainder of the acid. The solution is diluted to the mark with distilled water and the absorbance of the blue solution is measured at 7,000 Å, not less than 20 minutes nor more than 50 minutes after mixing.

The calibration curve is obtained using a solution of sodium

sulphide, standardised iodometrically.

It is claimed that the above reaction, unlike the methylene blue reaction, is not at all sensitive to iodide ions.

8.3 EXPERIMENTAL STUDIES ON THE MOLYBDENUM BLUE METHOD.

8.31 Transmission Curve of Molybdenum Blue Solution.

A transmission curve (figure 15) was prepared using a molybdenum blue solution, prepared according to the recommended procedure of Bethge.

Although greater absorbance occurs in the infra-red range, and at 825 mμ, the absorbance maximum at 700 mμ was used for all further experimental studies, being more convenient than either of the other maxima.

8.32 Tests carried out.

Although the calibration curves prepared were invariably straight lines, it was found impossible to reproduce any one line. The same series of solutions, measured a short while later, but still within the limits set by Bethge, showed a general increase in absorbance despite very careful thermostatic control of temperature.

An investigation was carried out on three identical solutions made up as follows:-

To 50 ml. ZnAc - NaAc solution was added 7·5 ml. sodium sulphide solution, 12·5 ml. ammonium molybdate solution and 30 ml. phosphoric acid, and the solution was diluted to 250 ml. After 15 minutes, the absorbance of each solution was measured and the measurements repeated at 5 min. intervals. The results appear in Table XV.

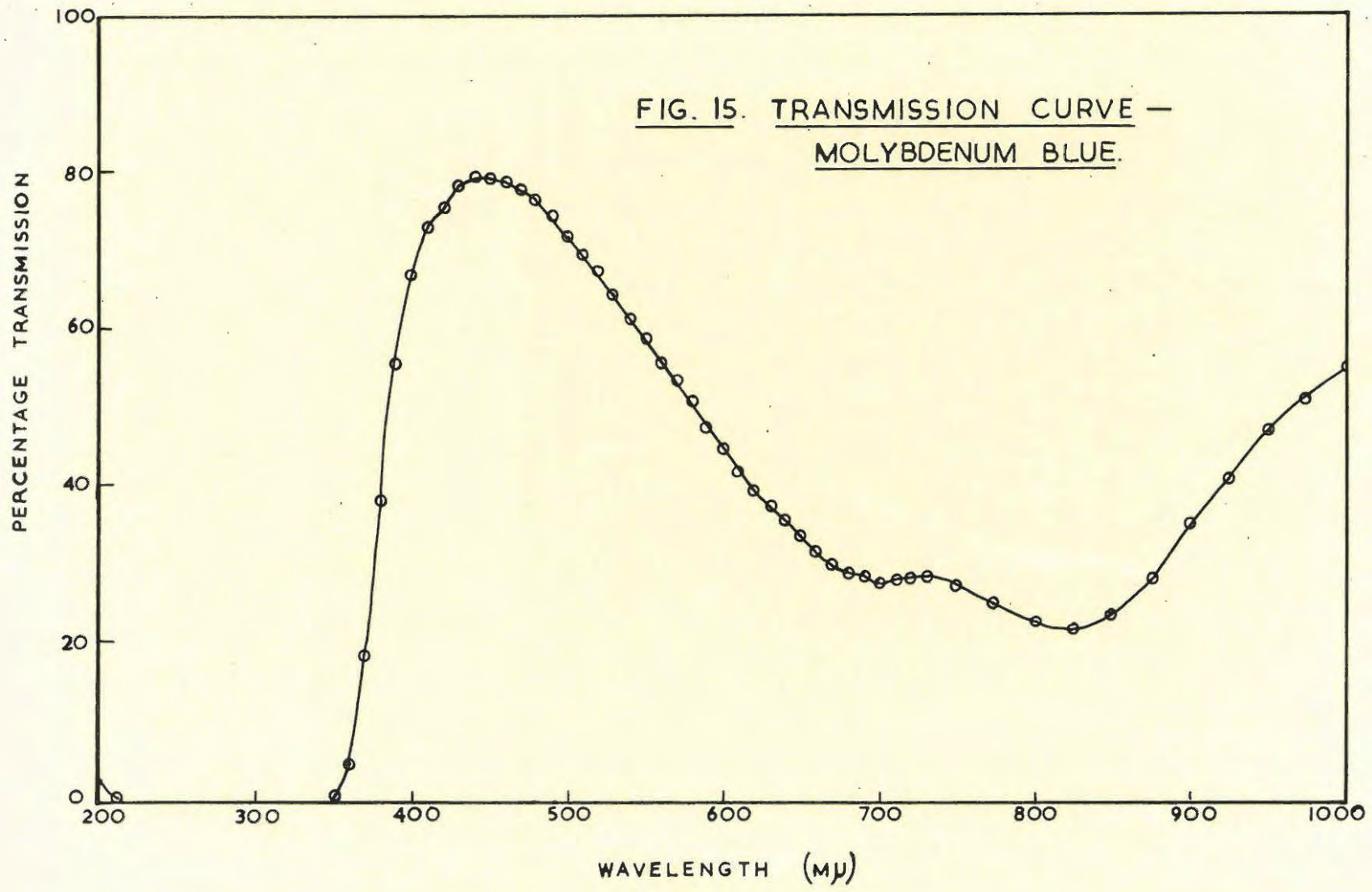


FIG. 15. TRANSMISSION CURVE — MOLYBDENUM BLUE.

TABLE XV

Variation in absorbance of three, identical, molybdate solutions.

Time after mixing. (min.)	Absorbance			Time after mixing. (min.)	Absorbance		
	Soln. No. 1	Soln. No. 2	Soln. No. 3		Soln. No. 1	Soln. No. 2	Soln. No. 3
15	0·197	0·230	0·223	50	0·207	0·247	0·232
20	0·200	0·231	0·223	55	0·207	0·241	0·233
25	0·200	0·234	0·224	60	0·204	0·243	0·236
30	0·200	0·237	0·226	65	0·207	0·247	0·234
35	0·201	0·238	0·221	70	0·208	0·245	0·240
40	0·204	0·243	0·230	75	0·208	0·248	0·237
45	0·212	0·243	0·231	80	0·210	0·251	0·245

From this table it was obvious that a serious, somewhat erratic increase in absorbance occurred, and at no stage was a definite maximum reached.

Varying the quantities of reagents, and taking every possible precaution against temperature effects, yielded no improvement in the results obtained.

Solutions, prepared by absorption of hydrogen sulphide formed according to the methylene blue reduction procedure, behaved in a similar fashion and the taking of readings at fixed times after mixing did not improve the constancy of readings.

In addition, it was found that the molybdenum blue solutions rapidly discoloured the spectrophotometer cells and these required thorough cleaning after each reading.

8.4 CONCLUSIONS.

These studies confirmed the findings of Woods and Mellon and contradicted the experimental findings of Bethge. Since the coloured solutions appeared to be unsuitable for the spectrophotometric determination of sulphur, this method was abandoned.

P A R T III.

THE BARIUM CHLORANILATE METHOD FOR THE SPECTROPHOTOMETRIC DETERMINATION OF SULPHUR.

1. REVIEW OF PUBLICATIONS ON THIS METHOD.

1.1 The Colour Reaction.

Chloranilic acid (2:5 - dichloro 3:6 - dihydroxyquinone) was first synthesized and named by Erdman (83) in 1843. It was found to be a strong dibasic organic acid, dissolving sparingly in water to give intensely coloured solutions, resembling in colour the purple-violet of permanganate solutions.

E.H. Tyner (84), in 1948, investigated the absorbance of chloranilic acid solutions, finding a maximum at 430 mμ and a minimum at 550 mμ. The graph of percentage transmission versus chloranilic acid concentration was found to conform to Beer's Law for all values from 0 to 20 mg. per 50 ml. at 430 mμ and from 4 to 14 mg. per 50 ml. at 550 mμ, the latter wavelength proving more sensitive. It was also found that the transmission decreased as the pH decreased, being more constant on the acid side of the chloranilic acid pH. Temperature also proved to be an important factor, the transmission decreasing if the precipitation was carried out at a higher temperature.

Tyner determined calcium in plant material, utilising the diminution in colour accompanying the precipitation of calcium chloranilate from the intensely coloured chloranilic acid solution.

In 1957, Bertolacini and Barney (8) made use of the highly coloured acid - chloranilate ion to determine sulphate. They used barium chloranilate, the sulphate being precipitated as barium sulphate while the chloranilate ion was liberated. The reaction was carried out

at pH 4 in a 50% ethanol medium, the precipitate being removed by centrifuging or filtration. Since the solubility product of barium sulphate was considerably less than that of barium chloranilate, the reaction was quantitative. The absorbance of the filtrate was measured at 530 mμ against a blank prepared in the same manner. (This absorbance maximum has also been reported at 535 and 550 mμ (85)).

In 1958, Bertolacini and Barney (86) described a procedure in which the absorbance measurements were made at 332 mμ, the absorbance being greater at this wavelength since the absorption of light by the chloranilate ion was 30 times greater than at 530 mμ. They stressed that glassware should be cleaned with 1:1 hydrochloric acid since detergents absorbed strongly in the 300 - 335 mμ range.

In 1959, Lysyj and Zarembo (87) confirmed the use of this wavelength, giving it accurately as 332 mμ and recommending it for use with samples containing 0·2 to 0·3 mg. sulphur. Lloyd (88), however, advocated measurements at 350 mμ while Spencer (89) carried out measurements at 327·5 mμ.

1.2 The Medium of Precipitation.

Bertolacini and Barney (8) found the optimum medium for the precipitation to be a 50% ethanol solution. They quoted the following solubility figures (20°C):-

Barium sulphate in water = $9{\cdot}6 \times 10^{-6}$ mole/litre.

Barium chloranilate in water = $2{\cdot}2 \times 10^{-4}$ mole/litre.

Barium sulphate in 50% ethanol = $2{\cdot}5 \times 10^{-7}$ mole/litre.

Barium chloranilate in 50% ethanol = $5{\cdot}2 \times 10^{-6}$ mole/litre.

The use of 50% ethanolic solution thus reduced appreciably

the solubility products of barium sulphate and barium chloranilate and the sensitivity of the method was correspondingly enhanced. Later research work confirmed these findings.

1.3 pH Control.

The careful control of pH has been stressed in all the publications on this method. The interference of phosphate ions by reaction with the barium chloranilate above pH 4 made it necessary to carry out the reaction at a pH value under this figure. Lower pH values, however, resulted in greater solubility of the barium chloranilate with corresponding loss in sensitivity.

Bertolacini and Barney (8) made use of a 0·05 M solution of potassium acid phthalate as a pH 4·0 buffer, the pH being approximately adjusted to this value beforehand by the addition of dilute ammonium hydroxide or hydrochloric acid and the use of pH paper.

In 1959, Klipp and Barney (90) used a sodium acetate - acetic acid buffer (0·1 M with respect to each), while Spencer (89) used a 0·5 M acetate buffer. In 1960, Scharrer and Deloch (91) used an acetate buffer according to Gottschalk (92). They mixed 1980 ml. 5N acetic acid with 20 ml. 5N ammonium acetate solution. This buffer solution, while having a pH 2·73 in aqueous solution, had an apparent pH 3·63 in 50% alcohol solution.

The acetate buffer systems appeared to have a greater buffering capacity than the phthallate buffer.

1.4 The Effect of other Ions in Solution.

It was found that, with the exception of the ammonium ion,

all cations interfered and hence cation exchange necessarily preceded each determination carried out. Dowex 50 was generally employed though Magar and Pollard (93) made use of Zeo-carb 225.

Bertolacini and Barney (8) found that phosphate, oxalate, bicarbonate, chlorido and nitrate did not interfere and as little as 2 p.p.m. sulphate could be determined in the presence of 100 p.p.m. other common anions. Scharrer and Deloch (91) found that the addition of 500 µg. N as nitrate, Cl as hydrochloric acid, CO_3^{2-} as ammonium carbonate, oxalic acid, citric and tartaric acids, to 500 µg. sulphur in solutions had no influence on the absorbance. The addition of 5000 µg. silicon as Na_2SiO_3 increased the absorbance by 1·9% while the addition of 5000 µg. phosphorus as phosphate caused an increase of 2·9%. However, such unsatisfactory S : P or S : Si ratios were scarcely likely to be encountered in biological samples.

1.5 Stability of the Colour.

Bertolacini and Barney (8) found that the colour stability was reached 15 minutes after adding the barium chloranilate and the absorbance increased by 5% in 24 hours, the temperature being kept constant. Klipp and Barney (90) instructed that the absorbance should be measured within 2 hours. Scharrer and Deloch (91) found the colour to be practically constant for 12 hours after development.

1.6 The Sensitivity and Accuracy of the Method.

Bertolacini and Barney (8) stated that the system obeyed Beer's Law at least up to 400 µg./ml. i.e. the original sample containing up to 40 mg. sulphate made up to 100 ml. sample solution.

They found the sensitivity to be 1 µg./ml. in the final solution, using 5 cm. cells at 530 mµ. The standard deviation and relative error of the method were 1%. Working at 332 mµ. and using 1 cm. cells, the standard deviation was found to be 0·2 p.p.m. for the analysis of ammonium sulphate solutions containing 0·96, 1·93 and 3·86 p.p.m. S.

Klipp and Barney (90) gave the lower limits of sensitivity as 1 µg. sulphur at 330 mµ using 1 cm. cells and 18 µg. sulphur at 530 mµ using 5 cm. cells. The standard deviation at 375 p.p.m. sulphur level was 5·5 p.p.m.

Scharrer and Deloch (91) claimed that the method was suitable for samples containing 75 - 2500 µg. sulphur at 530 mµ, it being possible to increase the sensitivity by using 1 cm. cells at 332 mµ. The standard deviation of determinations for 75 - 2500 µg. sulphur was 2% and the maximum deviation from the mean was 1.7%.

1.7 Preparation of the Barium Chloranilate Reagent.

The preparation of barium chloranilate has been described by Bertolacini and Barney (8) and Thomas (94).

In the former preparation, 1 litre of 5% barium chloride solution was mixed with 1 litre 0·1% aqueous chloranilic acid and allowed to stand overnight at room temperature. The aged precipitate was washed well with water until the supernatant liquid was free of chloride ions. Water was removed by centrifuging the precipitate three times with alcohol and once with diethyl ether. The crystals were then dried for 1 hour at 60°C in a vacuum oven.

In the preparation described by Thomas, about 700 ml. water

and 300 ml. acetone was added to 16 g. chloranilic acid in a 3 litre beaker. The mixture was heated until the acid had dissolved. While stirring vigorously, a hot, freshly-filtered solution of barium hydroxide, containing 20 g. of the hydroxide per litre, was added until the purplish-red colour of the solution diminished in intensity to a pink colour. Overshooting the end-point caused excess barium ions to be absorbed onto the precipitate. If the end-point was overshot, more chloranilic acid, dissolved in acetone, was added until a decidedly pink colour was again obtained. The tan, somewhat curdy precipitate, formed initially, changed into a dense purplish-brown crystalline material on ageing. After standing overnight, the supernatant liquid was decanted and the crystals washed free of excess chloranilic acid with several portions of 1:1 aqueous ethanol. The yield was claimed to be almost theoretical.

1.8 Application of the Method.

The acid-chloranilate ion was first used for spectrophotometric determination of calcium in plant material (84). Lysyj and Zarembo (87) applied the barium chloranilate method of Bertolacini and Barney (8, 86) to the determination of small amounts of sulphur in organic compounds. In the same year, Klipp and Barney (90) determined sulphur traces in naphthas by this method, while Lloyd (88) used barium chloranilate to determine enzymically liberated sulphate. Scharrer and Deloch (91) described a general method for the determination of small amounts of sulphur in biochemical substances, while Magar and Pollard (93) applied the method to sulphate determination in Morgan extracts of soils.

2. THE METHOD OF BERTOLACINI AND BARNEY.

In 1957, Bertolacini and Barney published their paper on the application of the coloured acid-chloranilate ion to the determination of sulphate. A description of the method follows:-

REAGENTS.

Barium Chloranilate.

This is prepared from reagent grade barium chloride and chloranilic acid as described on page 96.

Buffer, pH 4·0.

A 0·05 M solution of reagent grade potassium acid phthalate is prepared.

Ion exchange resin.

Dowex 50 × 8, 20 - 50 mesh, hydrogen form, is used.

Potassium sulphate.

Reagent grade potassium sulphate is used for the preparation of standards.

PROCEDURE.

An aqueous solution containing sulphate ions is passed through a column 15 cms. long containing Dowex resin. The effluent is adjusted to pH 4 with dilute hydrochloric acid or ammonium hydroxide and pH paper.

To an aliquot containing up to 40 mg. sulphate in less than 40 ml. in a 100 ml. volumetric flask are added 10 ml. of the buffer and 50 ml. of 95% ethanol. The mixture is diluted to volume with distilled water.

Approximately 0·3 g. barium chloranilate is added and the

flask shaken for 10 minutes. The excess barium chloranilate and barium sulphate precipitate are removed by centrifuging or filtering. The absorbance of the filtrate is measured with a colorimeter or spectrophotometer at 530 mμ against a blank prepared in the same manner. The sulphate concentration is then obtained from a calibration curve prepared from standard potassium sulphate solutions.

3. EXPERIMENTAL STUDIES ON THE BARIUM CHLORANILATE METHOD.

3.1 The Preparation of Standard Sulphate Solutions.

The 250 p.p.m. stock solution of potassium sulphate prepared for use in the EDTA method was also used in these studies.

In addition, a 100 p.p.m. solution was prepared by diluting 400 ml. of the stock solution to 1 litre.

All standard solutions were stored in polythene bottles.

3.2 Storage of Solutions.

The standard sulphate solutions, buffer solutions and deionised distilled water were stored in polythene bottles. The 95% ethanol was stored in a pyrex glass bottle.

3.3 Transmission Curve for the Acid-Chloranilate Ion.

An aliquot of standard sulphate solution containing about 0·5 mg. sulphur was passed through an exchange column containing Nalcite HCR, H^+ form (see page 36). The colour was developed as described in the procedure of Bertolacini and Barney, in section 2.

The transmission of the filtrate was measured using a Hilger Uvispek spectrophotometer with 1 cm. cells (fig. 9). A transmission curve (fig. 16) was plotted.

From fig. 16 it was deduced that maximum absorption occurred between 310 and 340 mμ in the ultraviolet region and at about 535 mμ in the visible region. This agreed with the findings of earlier research workers. For most of the following experimental studies, the absorption maximum at 535 mμ was used. This maximum is not so sharp as the MB maximum at 667 mμ.

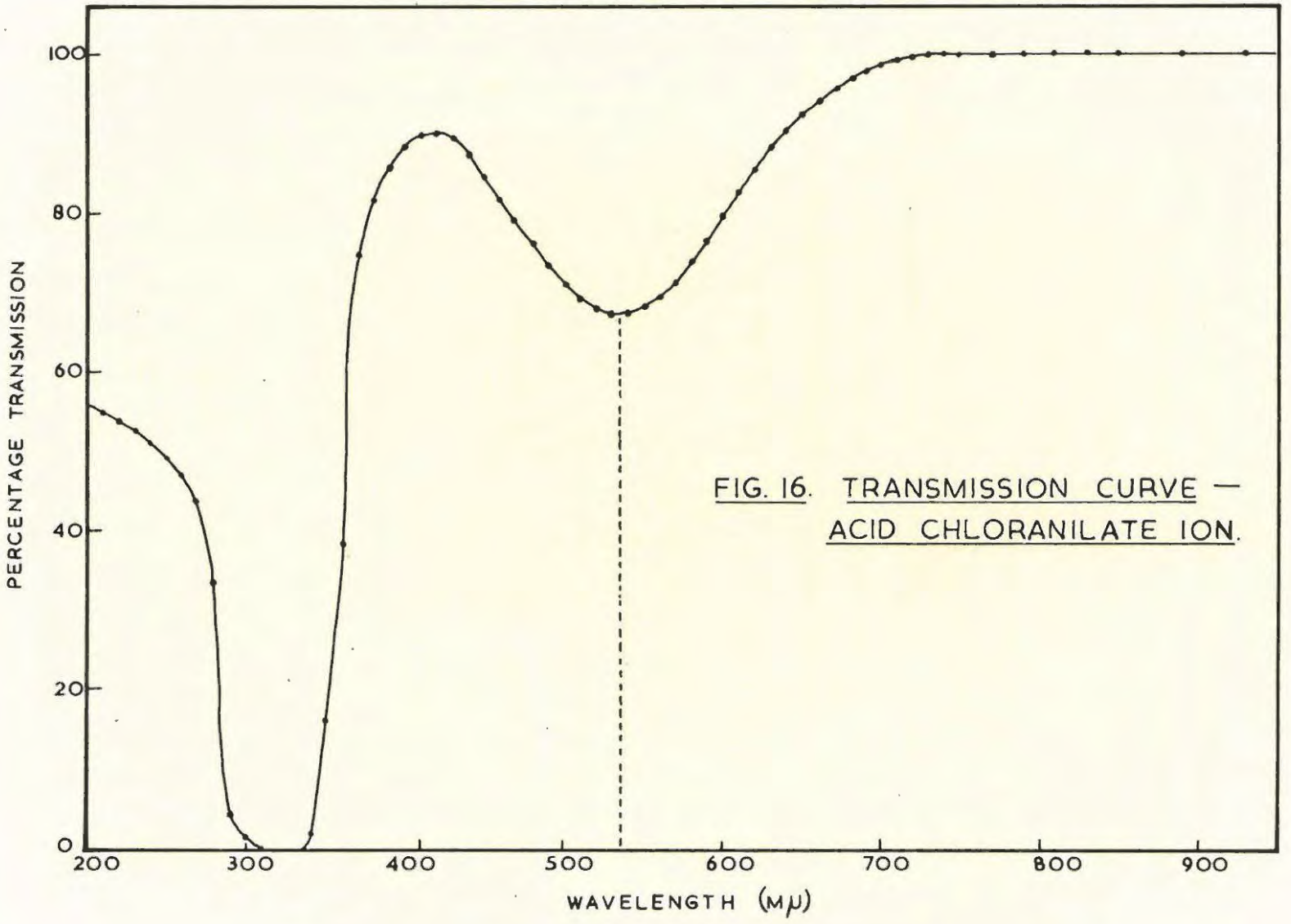


FIG. 16. TRANSMISSION CURVE — ACID CHLORANILATE ION.

3.4 Preparation of the Barium Chloranilate Reagent.

The commercially produced reagent was found to be rather unsatisfactory, varying considerably in quality. Large crystals were found, on crushing, to contain a large proportion of the unsatisfactory tan-coloured powder and, generally, it proved impossible to obtain reproducible absorbance values even from identical solutions.

Accordingly an attempt was made to prepare the reagent from chloranilic acid as described by Bertolacini and Barney (see page 96). However, even on ageing, a tan-coloured, somewhat colloidal product was obtained. The preparation described by Thomas (see page 96-7) was then applied. The first yield of the reagent closely resembled that obtained using the method of Bertolacini and Barney, the solution being allowed to stand overnight immediately sufficient barium hydroxide had been added. The method was repeated but the solution was boiled for 40 minutes after sufficient barium hydroxide had been added and only then was it allowed to age overnight. A deep purple, very fine, crystalline product was obtained and this reagent proved to be very satisfactory for all later work. Considerable care was excercised in the addition of the barium hydroxide solution since the end-point was easily overshot. This was due to the fact that the final mother liquor was orange in colour and difficulty was encountered in distinguishing between the pink solution of the chloranilic acid and the final orange coloured solution.

In view of the cost of the reagent and the fact that, in this procedure, it is used in considerable excess, much thus being wasted, the author found it convenient to reclaim part of the chloranilic

acid, after sulphate determinations, as follows. From the weight of used barium chloranilate (filtered off after use), the weight of sulphuric acid required to react with about two-thirds of this weight of barium chloranilate was added, the solution boiled, stirred well and filtered through Whatman No. 40 paper. It was important that the barium chloranilate should be in excess as excess sulphate ions would be troublesome in later determinations. The solution was then evaporated down considerably and the chloranilic acid (orange-red crystals) filtered off and dried for future use.

3.5 Buffer Solutions and pH Control.

Initially, the potassium acid phthalate buffer, recommended by Bertolacini and Barney, was employed but this was later discarded in favour of an acetate buffer as described by Scharrer and Deloch (91) and prepared by dissolving 3·85 g. ammonium acetate in 297·25 g. glacial acetic acid and diluting to 1 litre with deionised distilled water. This solution proved to have a greater buffering capacity and, for dilute solutions, it was hoped that preliminary approximate adjustment of the pH by means of dilute hydrochloric acid or ammonium hydroxide would be obviated. However, in the preliminary work, it appeared that, since 10 ml. plant solution was required in each determination, the buffer solution (5 ml. buffer per 50 ml. volumetric flask) was unable to control the acidity of the resin effluent. Each 10 ml. sample of plant solution was accordingly evaporated in a small beaker in a drying oven at about 150° and finally heated at 180° until perchloric acid fumes were no longer evolved. The residue was taken up in a little

water and the cations removed by passage through the resin column. Although the pH values of the resulting solutions were much closer to that of a blank solution, they remained slightly low and this caused serious errors. It proved necessary to measure the pH of each solution using a pH meter and to adjust the acidity by means of a dilute ammonium hydroxide solution.

The following table shows the errors introduced by slight variations in the pH of the final solutions:-

TABLE XVI

Effect of pH on Chloranilate Absorbance Values.

Solution	pH of Blank	pH of Solution	Absorbance	mg. S	Approx. % Error
1	3·80	2·72	0·338	8·468	216
2	3·80	2·85	0·289	7·307	173
3	3·80	3·25	0·186	4·863	81·5
4	3·80	3·40	0·134	3·629	35·4
5	3·80	3·80	0·094	2·680	0

Absorbance values for solutions 2 and 3 were obtained when the 10 ml. plant samples were not evaporated to dryness. Values for solutions 3 and 4 were obtained when the solutions were evaporated to dryness but the final pH was not adjusted with dilute NH_4OH solution. Solution 5 was evaporated to dryness and the pH of the final solution adjusted by means of dilute NH_4OH solution.

An error of as little as 0·05 pH unit introduced an error of

These results contradicted the findings of Bertolacini and Barney (see section 1.5). However, provided readings are taken as soon as possible after complete colour development, no great error should result from any instability of colour.

3.8 Relation to Beer's Law.

Solutions were prepared according to the recommended procedure (section 4) containing 100, 200, 300, 400, 500, 600, 1000, 2000, 3000, 4000 µg. sulphur per 50 ml. volumetric flask. Above about 2000 µg. the colours were too intense for the absorbance to be read accurately. The results are illustrated graphically in fig. 17. The Law is apparently only obeyed in the range 100 - 600 µg. sulphur.

3.9 Additional notes.

Time did not permit a thorough investigation to be made into the medium of precipitation and the effect of other ions in solution. However, these factors appear to have been thoroughly investigated by previous research workers.

In order to reduce the size of the required plant solution aliquot, an attempt was made to make measurements in the UV range at 327·5 mµ. Accordingly, a calibration curve was prepared using 0·20, 0·40, 0·60, 0·80 and 1·00 ml. standard sulphate solution (100 p.p.m.). However, at this wavelength, the only available spectrophotometer was unstable and each absorbance reading constantly diminished, altering by about 0·04 units or about 10% of the absorbance within a period of 3 minutes. Work at this wavelength had thus to be abandoned.

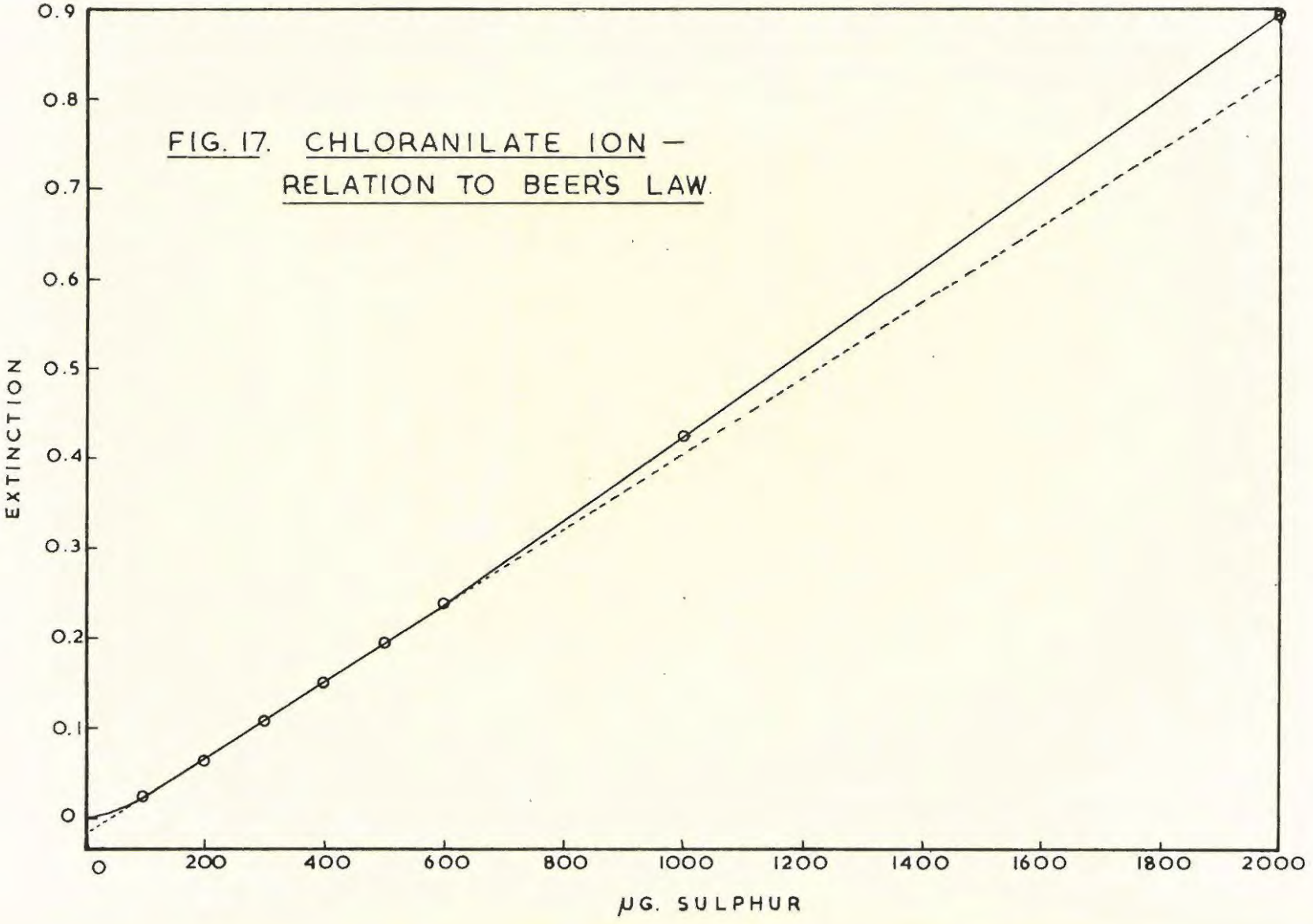


FIG. 17. CHLORANILATE ION — RELATION TO BEER'S LAW.
EXTINCTION
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
µG. SULPHUR

4. THE MODIFIED BARIUM CHLORANILATE METHOD FOR THE DETERMINATION OF SULPHATE IN PLANT MATERIAL.

REAGENTS.

(i) Water.

Deionised distilled water should be used in the preparation of all solutions. The recommended resin column contains a mixture of the monobed resins, Amberlite MB 1 and MB 3.

(ii) Ethyl Alcohol.

Reagent grade 95% ethyl alcohol is used.

(iii) Cation Exchange Resin.

The resin used is a column 12 × 100 mm. of Malcite HCR or Dowex 50, prepared as described on page 35.

(iv) Buffer Solution.

The buffer solution is prepared by dissolving 3·85 g. reagent grade ammonium acetate in 297·25 g. glacial acetic acid and diluting to 1 litre with deionised distilled water.

(v) Barium Chloranilate Reagent.

The use of the commercial product is not advocated.

About 700 ml. water and 300 ml. acetone is added to 16 g. chloranilic acid in a 3 litre beaker. The mixture is heated until the acid has dissolved. While stirring vigorously, a hot, freshly-filtered solution of barium hydroxide, containing 20 g. of the hydroxide per litre, is added until the purplish-red colour of the solution has diminished in intensity to a pink colour, not to be confused with the orange-red colour of the mother liquor. For convenience, the hot hydroxide solution may be filtered directly into

the chloranilic acid solution. If the end-point be overshot, further chloranilic acid dissolved in acetone should be added until the solution is decidedly pink again. The solution is maintained at boiling point for a further 40 minutes or longer and allowed to stand overnight. The supernatant liquid is decanted and the crystals filtered off and washed with several portions of 1:1 aqueous ethanol to remove excess chloranilic acid. The yield is almost theoretical.

APPARATUS.

(i) Six ion exchange columns (see page 35).

(ii) Six numbered 50 ml. volumetric flasks.

(iii) Seven 50 ml. test tubes (pyrex), fitted with stoppers and rubber 'collars' (on which the number is marked) to enable the seven tubes to be shaken simultaneously in the hand without slipping.

(iv) An accurate pH meter.

(v) Spectrophotometer with 1 cm. cells.

(vi) Seven small short-stemmed filter funnels to take 7 cm. circles of filter paper, and seven ordinary 5/8" diam. pyrex test tubes.

PROCEDURE.

The plant solutions are prepared as described in detail on pages 73 and 74.

An aliquot of 10 ml. of plant solution is evaporated for about 1 hour in a drying oven at 150°C and finally at 180° until perchloric acid fumes are no longer evolved. The residue is taken up in a little water and washed into the prepared resin column, the effluent being collected in a 50 ml. volumetric flask containing 25 ml. 95% ethanol

and 5 ml. buffer solution. The beaker is rinsed into the column with a number of small portions of deionised distilled water. After each addition, the water level is allowed to drop to the level of the glass-wool plug before the next addition is made. This procedure is repeated until each flask has been filled to the mark.

The flasks are well shaken and the levels again adjusted to the mark if necessary, since a contraction occurs on mixing. A stock blank solution is prepared, comprising 50% ethanol, 10% buffer solution and 40% distilled water. Using an accurate pH meter, the pH of the blank solution is measured and the pH of each ethanolic sample solution is adjusted to the same pH using a dilute ammonium hydroxide solution containing 10 ml. concentrated reagent grade ammonium hydroxide per 100 ml. solution.

Into each of the seven 50 ml. test tubes is weighed out, approximately, 0·03 g. barium chloranilate. Approximately 12·5 ml. of each ethanolic sample solution is transferred to each of six of the numbered test tubes and an equal volume of the stock blank solution to the seventh tube. The tubes are stoppered and, while held together in a bundle, the tubes are shaken vigorously for 2 minute periods at 2 minute intervals until they have been shaken for a total of 10 minutes. Using small funnels, the solutions are then filtered through 7 cm. Whatman No. 40 filter circles into ordinary pyrex test tubes and the absorbance is measured against the blank solution at 535 mμ on a suitable spectrophotometer with 1 cm. cells. A calibration curve is prepared in the same manner using 1, 2, 3, 4, 5, and 6 ml. 100 p.p.m. standard K_2SO_4

solution in place of the plant solutions, but without heating them to dryness.

The sulphur concentration of each plant solutions is then obtained from the calibration curve.

Duplicate or triplicate determinations may be carried out using further 12·5 ml. portions of the ethanolic sample solutions.

5. STATISTICAL STUDY OF THE ACCURACY AND PRECISION OF THE PROPOSED METHOD.

The modified barium chloranilate method was subjected to statistical tests to evaluate the accuracy and precision of the method under routine laboratory conditions.

5.1 CALIBRATION CURVE.

A calibration curve was prepared at 535 mμ according to the recommended procedure (see section 4) using 1, 2, 3, 4, 5, 6 ml. of 100 p.p.m. standard K_2SO_4 solution.

The absorbance values obtained are given in Table XVII and the curve obtained, using 1 cm. cells, is reproduced in fig. 18.

TABLE XVII

Absorbance of Chloranilate Solutions - Calibration Curve.

μg. Sulphur	100	200	300	400	500	600
Absorbance	0.024	0.066	0.108	0.153	0.190	0.235

The curve was a straight line which did not pass through the origin, thus confirming the findings of Scharrer and Deloch (91).

5.2 THE ACCURACY OF THE METHOD.

The plant solutions used in the accuracy determinations in section 6.2 (page 82) were also used in the determination of the accuracy of the barium chloranilate method. The sulphur content of each solution was determined as described in section 4 (page 106), although time did not permit duplicate determinations to be carried out. The results obtained are recorded in Table XVIII.

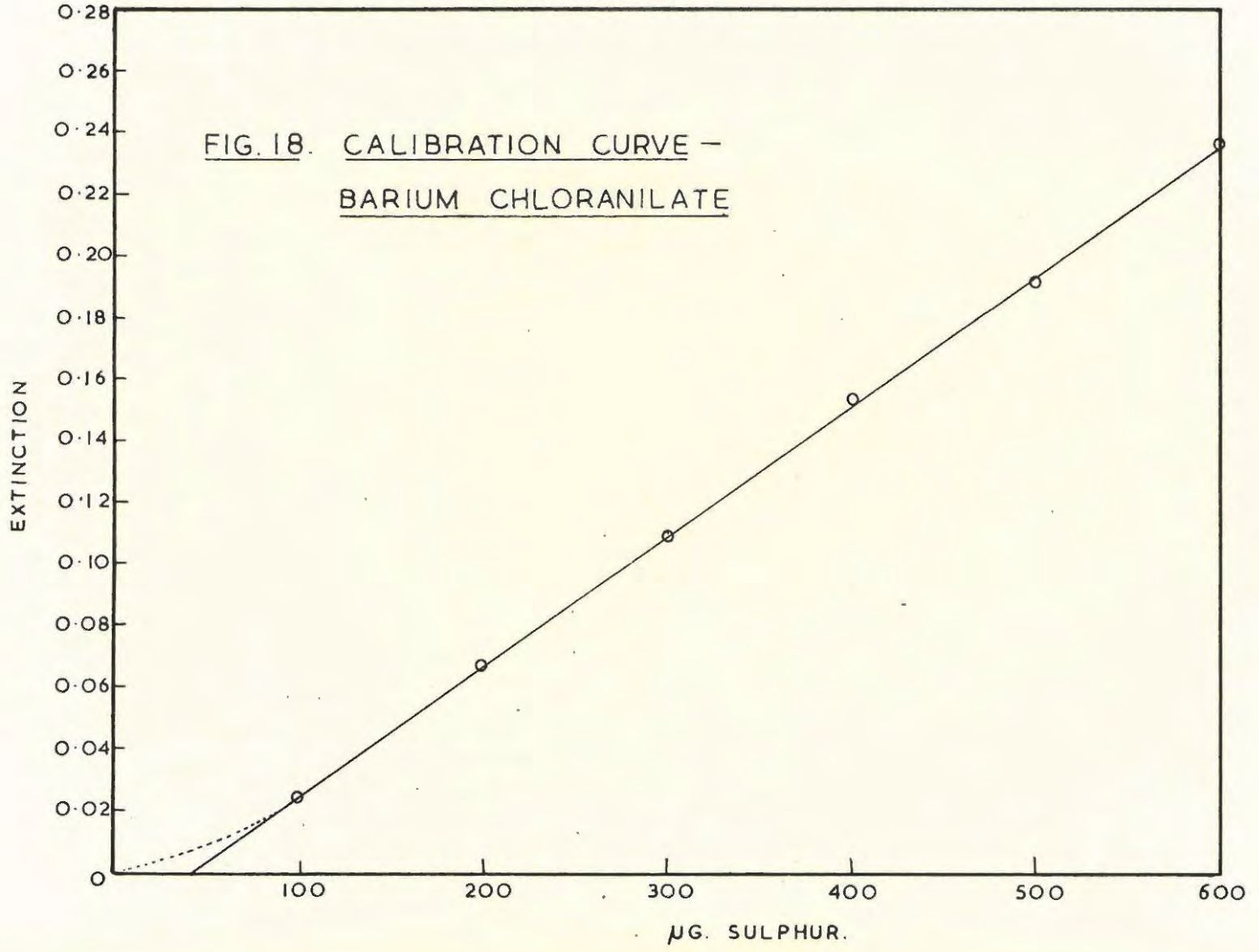


FIG. 18. CALIBRATION CURVE –
BARIUM CHLORANILATE
EXTINCTION
0·28
0·26
0·24
0·22
0·20
0·18
0·16
0·14
0·12
0·10
0·08
0·06
0·04
0·02
0
100
200
300
400
500
600
µG. SULPHUR.

TABLE XVIII

THE ACCURACY OF THE PROPOSED BARIUM CHLORANILATE PROCEDURE.

Determination	Weight of Plant sample (g.)	Weight of S added (mg.)	Weight of S found (mg.)	Sulphur found (p.p.m.)	Percentage Recovery
1	0·5113	1·250	2·704	2844	108·10
2	0·5041	1·250	2·609	2702	102·70
3	0·5074	1·250	2·704	2866	108·93
4	0·5023	1·250	2·538	2564	97·45
5	0·5042	1·250	2·538	2555	97·11
6	0·5199	1·250	2·728	2843	108·06
7	0·5042	1·250	2·562	2602	98·90
8	0·5134	1·250	2·704	2832	107·64
9	0·5036	1·250	2·538	2558	97·23
10	0·5119	1·250	2·609	2655	100·91
11	0·5129	1·250	2·585	2603	98·94
12	0·5212	1·250	2·728	2836	107·79
13	0·5331	1·250	2·562	2461	93·54
14	0·5272	1·250	2·680	2712	103·08
15	0·5177	1·250	2·538	2488	94·57
16	0·5346	1·250	2·728	2765	105·10
17	0·5037	1·250	2·538	2558	97·23
18	0·5288	1·250	2·752	2841	107·98
19	0·5218	1·250	2·585	2560	97·30
20	0·5045	1·250	2·680	2834	107·72
				MEAN:	102·01

From the weight of sulphur determined and the sample weight, the sulphur content of the plant material in p.p.m. was calculated, the 1·250 mg. sulphur aliquot added being deducted in each case. The results are recorded in column 5. The mean of 15 precision determinations (see section 5.3) was found to be 2631 p.p.m. sulphur. The ratio of each individual determination to this mean was calculated as a percentage. This value is given in column 6. (Had the mean value of 2680 p.p.m. sulphur (Methylene Blue determination) been used, the mean percentage would have been 100·2 %.) From these data it was concluded that this procedure gave a fair reflection of the sulphur content of the plant material.

5.3 THE PRECISION OF THE METHOD.

The precision of the method was evaluated as follows. The solutions used were those prepared for the precision determination in section 6.3 (page 83). The sulphur content of these solutions was determined by the recommended procedure detailed in section 4 (page 106).

This series of tests was performed under conditions closely approximating those in a routine laboratory and were performed over a period of several days. The temperature was not maintained constant and varied between 19° and 26°C. It is possible that improved precision would have been obtained by carrying out the determinations at a constant temperature but unfortunately this could not be tested.

The results of the precision determinations are recorded in Table XIX. These results revealed that the procedure could determine down to 2·7 mg. sulphur in plant material with a coefficient of variation of 4·6%.

TABLE XIX

THE PRECISION OF THE PROPOSED BARIUM CHLORANILATE PROCEDURE

Determination (n)	Sample weight (g.)	Sulphur found (p.p.m.)	M - Y	$(M - Y)^2$
1	1·057	2491	140	19600
2	1·015	2594	37	1369
3	1·019	2631	0	0
4	1·023	2571	60	3600
5	1·006	2500	131	17161
6	1·025	2662	31	961
7	1·027	2518	113	12769
8	1·010	2842	211	44521
9	1·027	2633	2	4
10	1·017	2450	181	32761
11	1·009	2775	144	20736
12	1·014	2784	153	23409
13	1·008	2707	76	5776
14	1·015	2548	83	6889
15	1·022	2762	131	17161
n - 1 = 14		2631 (mean)		206717 $= \sum(M - Y)^2$

The Standard Deviation (σ) = $\sqrt{\frac{\sum(M - Y)^2}{n - 1}}$ = 121·51 p.p.m.

Coefficient of Variation (% Std. Dev.) = $\frac{121·51}{2631} \times 100 = 4·62$ %.

SUMMARY AND CONCLUSION.

In an effort to find a more suitable routine method for determining sulphur in plant material than the $BaSO_4$ precipitation method, the following three methods were investigated; the indirect complexometric method involving the precipitation of the sulphate as $BaSO_4$ and titration of the excess barium ions with standard EDTA (disodium ethylenediaminetetraacetate), the reduction of the sulphur to hydrogen sulphide and subsequent determination of the sulphide colorimetrically as methylene blue, and the precipitation of the sulphate as $BaSO_4$ with barium chloranilate and spectrophotometric measurement of the liberated acid chloranilate ion.

In the complexometric method, phosphate was found to interfere seriously and the author was unable to remove this interference satisfactorily. The method is thus not applicable to plant material unless the phosphate concentration is very low and only an approximate figure for the sulphur content is required. When carried out in the absence of phosphate and in the presence of the $BaSO_4$ precipitate, the percentage standard deviation was found to be 1·02% and in the absence of the precipitate 1·60%.

The methylene blue procedure was found to be satisfactory and extremely sensitive. The reduction mixture was found to be the most sensitive factor and considerable care is required in its preparation. Hydrogen sulphide in the air must be avoided and the colour development must be carried out at a constant temperature. The standard deviation was found to be 46·40 p.p.m. for a sample containing 2680 p.p.m. sulphur, i.e. a percentage standard deviation of 1·73%.

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