THE ANALYST

# Determination of the Steam-volatile Phenols Present in Cigarette-smoke Condensate

## Part I.* Colorimetric Determination of the Total Steam-volatile Phenols

BY G. A. L. SMITH AND D. A. KING
(*Carreras Research Division, Bruce Grove, Wickford, Essex*)

The use of diazotised *p*-nitroaniline for the colorimetric determination of steam-volatile phenols in cigarette-smoke condensate is described. The variables involved in the reaction have been evaluated, and the optimum conditions determined. A procedure that is rapid and more accurate than existing procedures is suggested for the routine analytical determination of the steam-volatile phenols in cigarette-smoke condensate.

IT has become increasingly important of late that a rapid and accurate method of determining microgram amounts of total phenols in smoke should be obtained. Gas-chromatographic methods[1,2,3] are time-consuming, and it is desirable to improve upon their accuracy. Inaccuracies are caused, in part, by the multiplicity of the extractions used for obtaining the solution to be chromatographed,[4] and no corrections have been made by the authors quoted for differences between the behaviour of the markers and the phenols determined.

The time required to complete a single analysis with gas chromatography precludes its use as a routine analytical procedure. Attention, therefore, was directed towards an examination of other published methods on phenol determinations. The method of Rayburn, Harlan and Hamner[5] also includes several solvent extractions, and the time required to develop the paper chromatogram is 16 to 18 hours. Therefore, it was not considered suitable as a routine analytical procedure. A comparison of the analytical methods available for determining phenols has been made by Mohler and Jacob,[6] who showed that the best method available involves the use of aminophenazone for the colorimetric determination. This reagent was first proposed as a new colour test for phenols by Emerson,[7] and has since been used by Ettinger, Ruchhoft and Lishka,[8] Gottlieb and Marsh,[9] Martin[10] and Lacoste, Venable and Stone.[11] More recently it has been used by Lorentzen and Neurath[12] for determining the total phenol content of cigarette-smoke condensate. The method cannot be used for determining *p*-substituted phenols.

Since no suitable method was available for the routine analysis of the phenols present in cigarette-smoke condensate, an investigation of the colour reaction between *p*-nitrobenzenediazonium chloride and phenols in alkaline solution was undertaken. This investigation culminated in an analytical procedure that is both rapid and accurate being laid down. The reagents used are relatively stable, and the method is applicable over a wide range of phenol concentrations. *p*-Substituted phenols react with the reagent and are determined by this procedure, although the colours formed are not so intense.

### EXPERIMENTAL

ABSORPTION MAXIMA OF THE AZO DYES PRODUCED BY COUPLING PHENOLS WITH DIAZOTISED *p*-NITROANILINE IN ALKALINE SOLUTION—

A slight excess of the diazonium salt of *p*-nitroaniline at 5° C was added to an alkaline solution containing 500 μg of phenol, also at 5° C. The resulting solution was tested to

* For details of Part II of this series, see reference list, p. 311.

make sure it was alkaline. The absorption maximum for phenol was then determined on a Perkin-Elmer 137 UV Spectrophotometer, and was found to be at 480 mμ. In the same way, the absorption maxima were determined for *o*-, *m*- and *p*-cresols, and found to be 500, 492 and 510 mμ, respectively; see Fig. 1.

EFFECT OF VARYING THE pH OF THE SOLUTION ON THE INTENSITY OF THE COLOUR OF THE AZO DYE—

A series of solutions, each containing 500 μg of phenol, in which the volume of diazonium chloride solution added was kept constant, were prepared. By adding increasing volumes of 0·1 N sodium hydroxide to each flask, and diluting the solutions to 100 ml with water, a pH range of 7·0 to 13·0 was covered. The optical densities of the solutions were measured at 490 mμ with either a Unicam SP500 spectrophotometer or an Evans Electroselenium Ltd. Spectra.

The results of this work show that all the phenols possess an optical-density "plateau" over a small, but useful, pH range; see Fig. 2.

The same graphs were obtained when, instead of using different volumes of sodium hydroxide to produce a range of pH values, 50-ml volumes of boric acid - potassium chloride - sodium hydroxide buffer solutions were transferred with a pipette into the flasks.

A suitable pH working range ensuring stability and reproducibility of colour lay between 9·7 and 10·3. This range was strictly adhered to throughout the rest of the work reported here.

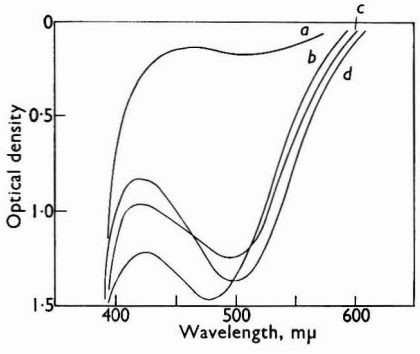



Fig. 1. Optical-density curves of phenols coupled with diazotised *p*-nitroaniline in alkaline solution: (*a*) *p*-cresol; (*b*) phenol; (*c*) *m*-cresol; (*d*) *o*-cresol

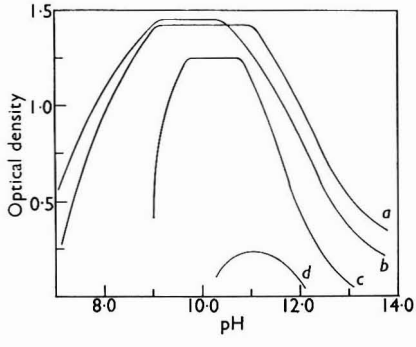



Fig. 2. The effect of pH on the optical density of the azo dyes: (*a*) *o*-cresol; (*b*) phenol; (*c*) *m*-cresol; (*d*) *p*-cresol

EFFECT OF THE CONCENTRATION OF DIAZONIUM CHLORIDE ON COLOUR FORMATION—

A boric acid - potassium chloride - sodium hydroxide buffer solution was used to keep the pH of the solution within the limits previously described. Excesses of *p*-nitrobenzene-diazonium chloride ranging from 0 to 3500 per cent. were used, and the results obtained are presented graphically in Fig. 3.

The graph shows that a large excess of reagent is required to produce the maximum colour, and that the colour produced by the reagent blank solution is proportional to the amount of diazonium solution used. Accordingly, the addition of diazonium chloride solution was kept constant and represented a 1000 per cent. excess on a 300-μg sample of phenol. The actual volume of reagent used was 5·5 ml. The preparation of the diazonium chloride solution is described on p. 308.

EFFECTS OF TIME, TEMPERATURE AND SODIUM CHLORIDE CONCENTRATION ON COLOUR FORMATION—

The optical density of the azo-dye solution was measured repeatedly, up to 24 hours after its preparation, at temperatures between 10° and 50° C. The optical density did not change within this 1-day period, and it was found that the addition of up to 5 g of sodium chloride in excess of that already present, has no effect upon the optical density of the solution.

EFFECT OF VARYING THE ORDER OF ADDITION OF THE REAGENTS ON COLOUR FORMATION—

The maximum colour formation is only obtained when the diazonium salt solution is added last. If it is added at any other stage the formation of the azo dye is repressed considerably. It does not matter in which order the other reagents are added.

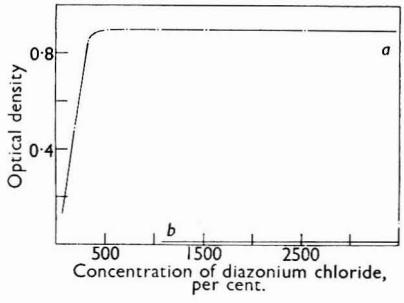



Fig. 3. The effect of varying the concentration of diazonium chloride on the colour produced: (*a*) phenol; (*b*) reagent blank solution

PREPARATION OF CALIBRATION GRAPH—

A series of test solutions of differing phenol concentrations was used to show that the colour formed does in fact obey Beer's law. The pH of these test solutions was adjusted to between 9·7 and 10·3 as described before, the only variant in these solutions being the amount of phenol added (0 to 250 $\mu$g). The blank value, under the conditions used, is so small as to be negligible.

The graphs obtained from the coloured reaction products of *p*-nitrobenzenediazonium chloride and phenol, *o*-, *m*- and *p*-cresols, and the two prepared mixtures all obey Beer's law; see Fig. 4. The optical densities were determined at 490 m$\mu$ in 1-cm cells; hence, if 4-cm cells were used, as little as 1 $\mu$g could be determined with great accuracy.

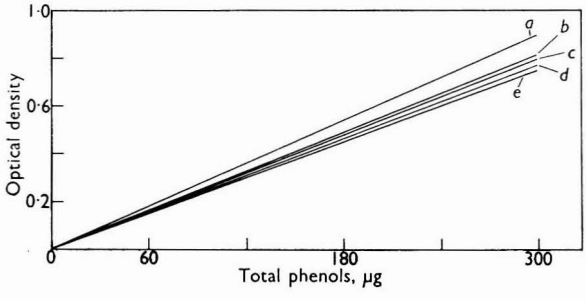



Fig. 4. Calibration graphs prepared from individual phenols and synthetic mixtures: (*a*) phenol; (*b*) *o*-cresol; (*c*) mixture of phenol, *o*-, *m*- and *p*-cresols, 8 + 1 + 1 + 1; (*d*) mixture of phenol, *o*-, *m*- and *p*-cresols, 4 + 1 + 1 + 1; (*e*) *m*-cresol

The two prepared mixtures were made up of phenol, *o*-, *m*- and *p*-cresol in the ratio of 6·5 + 1 + 1 + 1, and 4 + 1 + 1 + 1, for use with plain and filtered cigarettes, respectively. The ratios of phenols to the cresols in plain and filtered cigarette-smoke condensate were determined in Part II[13] of this work.

EFFICIENCY OF STEAM-DISTILLATION—

Known amounts of individual phenols were placed in a steam-distillation apparatus and the first 100 ml of distillate collected. The phenols in the distillate were then determined colorimetrically, and the results obtained showed that up to a total of 1000 $\mu$g could be recovered in 100 ml of distillate without any losses occurring. It is unlikely that there will ever be more than 400 $\mu$g of mixed phenols to distil when this method is used for cigarette-smoke condensate.

## METHOD

REAGENTS—

All reagents should be of analytical-reagent grade unless otherwise specified.

*Methanol*—General-purpose reagent.

*Sodium hydroxide, 0·2 and 0·1* N.

*Sulphuric acid, 2* N.

p-*Nitroaniline hydrochloride solution*—Weigh out accurately 1 g of general-purpose reagent *p*-nitroaniline, transfer it to a 100-ml calibrated flask and dissolve it in 20 ml of hydrochloric acid, sp.gr. 1·18. Dilute to the mark with water.

*Sodium nitrite solution, 2·2 per cent. w/v, aqueous.*

*Diazonium salt of* p-*nitroaniline, aqueous solution*—Solution A: transfer with a pipette 10 ml of *p*-nitroaniline hydrochloride solution to a 100-ml calibrated flask and dilute to the mark with water. Cool the solution to below 5° C before use. Solution B: transfer with a pipette 10 ml of the sodium nitrite solution to a 100-ml calibrated flask and dilute to the mark with water. Cool the solution to below 5° C before use.

Transfer with a pipette a known volume of solution A into a clean, dry beaker immersed in an ice-bath. From a burette add solution B, with gentle agitation, until a blue colour is obtained when the solution in the beaker is tested with starch - iodide paper. (The theoretically calculated ratio of the volumes of solution A to solution B is 4·5 to 1, and this is confirmed by practical experiment.) Store the diazonium salt solution in ice and place it in a dark cupboard till it is required for use. If kept in this manner it will remain usable for several hours. The diazonium salt solution should have little colour and should be crystal clear.

*Stock solution of phenols*—The individual phenols must be of the purest grade obtainable commercially, and solutions of phenol and the three cresols are prepared by accurate weighing and dilution to the mark with water. They are diluted just before use to prepare standard 0·001 per cent. w/v solutions of phenol, *o*-, *m*- and *p*-cresol.

*Buffer solution*—Weigh accurately 12·37 g of boric acid and 14·90 g of potassium chloride and dissolve them in 1 litre of cold distilled water. Transfer 125 ml of this solution and 110 ml of 0·2 N sodium hydroxide to a 500-ml calibrated flask and dilute to the mark with water. If the pH is lower than 9·8, adjust it to this value by adding more sodium hydroxide solution.

APPARATUS—

*Direct-reading electronic weigher*—Obtainable from A.M.F. Ltd.

*Smoking unit, C.S.M.* 10—Obtainable from Cigarette Components Ltd.

Either *Cambridge filter* or *electrostatic precipitating tubes*—Fitted to the smoking unit.

*Steam-distillation unit*(*s*).

*Spectrophotometer—e.g.*, either a Unicam SP500 or an E.E.L. Spectra.

PREPARATION OF CALIBRATION GRAPH—

A standard volume of 5·5 ml of diazonium salt solution must be added to the flask in every determination. The pH is adjusted as follows.

With a burette run 11 ml of the diazonium salt solution into a clean 100-ml beaker. Place the electrodes of the pH meter into the beaker and add sufficient water to cover them to a depth of at least 1 inch. Run in the 0·1 N sodium hydroxide slowly and with agitation until the pH of the solution reaches 7·0 to 7·3. Note the volume added. Now run the sodium hydroxide solution in, drop by drop, till the pH has risen to 10. Again, note the volume added.

Let $x$ ml of 0·1 N sodium hydroxide be the volume required to adjust the pH of the solution to between 7·0 and 7·3 and $y$ ml be the volume required to raise the pH from between 7·0 and 7·3 to 10·0.

Then $x$ ml of 0·1 N sodium hydroxide $\equiv$ 11 ml of diazonium salt solution,

hence $\frac{x}{2}$ ml of 0·1 N sodium hydroxide $\equiv$ 5·5 ml of diazonium salt solution.

The volume of 0·1 N sodium hydroxide required to adjust the pH of the solution to 10·0 is then given by—

$$\left(\frac{x}{2} + y\right) \text{ml.}$$

The value of $\left(\frac{x}{2} + y\right)$ was usually found to be between 10·0 and 12·0 ml but the value must be determined for each and every batch of diazonium salt solution prepared.

With a burette or a pipette, transfer the volumes of solution listed below into five separate 100-ml calibrated flasks. For plain cigarettes, into the first flask put—

> 3·25 ml of standard phenol solution and 0·5 ml of each of the standard *o*-, *m*- and *p*-cresol solutions.

Place twice these volumes into the second flask, three times the volumes into the third, and so on up to the fifth flask. For filter-tipped cigarettes, into the first flask put—

> 2·0 ml of standard phenol solution and 0·5 ml of each of the standard *o*-, *m*- and *p*-cresol solutions.

Place twice these volumes into the second, three times into the third and so on up to the fifth flask.

Then add 25·0 ml of buffer solution and $\left(\frac{x}{2} + y\right)$ ml of 0·1 N sodium hydroxide to each of the flasks. When all the solutions except the diazonium salt solution have been added, immerse the flasks in an ice-bath for 15 minutes. Finally add 5·5 ml of the diazonium salt solution to each flask. Dilute to the marks with water.

Set the solutions aside for 15 minutes and then determine the optical densities of the solutions at 490 m$\mu$ in 1-cm cells by using either a Unicam SP500 or an E.E.L. Spectra. Since the blank value, under the conditions used, has been found to be so small, the need for a reagent blank solution is obviated and water can be used instead. Plot a graph of optical density *versus* micrograms of total phenols. A straight-line relationship should be obtained.

### Conditioning and selection of cigarettes—

Condition the cigarettes to be used in the determination for 48 hours in a humidifying cabinet, or a constant-humidity room with the relative humidity at 58 per cent. Remove the cigarettes from the cabinet or the constant-humidity room and weigh 100 of them. Determine the average weight of a single cigarette in milligrams. Select 10 cigarettes whose weights are within $\pm$20 mg of the average weight of one cigarette by using the direct-reading electronic weigher.

### Smoking procedure—

Set up the C.S.M. 10 smoking unit so that each of the four channels operates on a 2-second puff of appropriate volume every minute. The volume of the puff must be calibrated by using a bubble meter before each smoking operation. Mark the weight-selected cigarettes at a butt length of 23 mm. Smoke five cigarettes on each channel, and collect the smoke on either a Cambridge filter or in electrostatic precipitating tubes.

Note—

The Tobacco Manufacturers Standing Committee (now the Tobacco Research Council)[14] advocates the use of a 2-second puff of 25-ml volume every minute, but the 2-second puff of 35-ml volume every minute, has wider usage in America[15] and Europe.

EXTRACTION OF TAR FROM THE CAMBRIDGE FILTER—

Remove the filter holders and uncouple them. Take out the filter and place it in a sintered-glass funnel that is attached to a Buchner flask. First wash the holder with methanol to remove all the tar adhering to it, and then wash the filter until it is clean. Carefully transfer the solution of tar in methanol to a 100-ml calibrated flask and dilute to the mark with methanol.

STEAM-DISTILLATION—

Transfer with a pipette 20 ml of the methanolic tar solution and 10 ml of 2 N sulphuric acid into a 250-ml long-necked round-bottomed flask. Attach the flask to a steam-distillation unit. Steam-distil the mixture, and collect 100 ml of distillate in a 100-ml calibrated flask. Transfer the steam-distillate to a stoppered bottle, and store it in the refrigerator until required.

DETERMINATION OF STEAM-VOLATILE PHENOLS—

Transfer with a pipette or a burette, 25 ml of buffer solution, 50 ml of the steam-distillate and $\left(\frac{x}{2} + y\right)$ ml of 0·1 N sodium hydroxide into a clean, dry 100-ml calibrated flask. Stopper the flask, shake it vigorously and cool it to 5° C in an ice-bath. Run in 5·5 ml of diazonium chloride solution from a burette, stopper the flask and shake the solution vigorously. Set it aside for 5 minutes and dilute to the mark with cold water. Place the solution in a 1-cm glass cell, and read its optical density at 490 mμ, either on a Unicam SP500, or on an E.E.L. Spectra. By using the optical density of the sample solution, read off from the calibration graph its equivalent in μg of total phenols; see Fig. 4. Let this equal $a$ μg. Let $b$ equal the number of cigarettes smoked in the preparation of the original tar solution.

$$\text{Then total phenols per cigarette} = \frac{a}{b} \times \frac{100}{20} \times \frac{100}{50}\ \mu g$$

$$= \frac{10a}{b}\ \mu g.$$

In this instance $b = 5$.

Then the total steam-volatile phenol content per cigarette $= 2a$ μg.

## RESULTS AND PRECISION

Cambridge filters were used throughout, but the electrostatic precipitating tubes were used as a check and a good agreement obtained. Also a cold trap (solid carbon dioxide - ethanol) was placed between the filter and the machine to determine the amount of the phenols that had passed through the filter. Under the smoking conditions prescribed, only a negligible amount of phenols was found to have passed through the filter.

A series of replicate determinations of total phenol concentration in tobacco smoke was performed on a single brand of plain cigarettes, and the results are shown in Table I.

TABLE I

STATISTICAL ANALYSIS OF A SERIES OF REPLICATE DETERMINATIONS OF TOTAL PHENOLS ON A SINGLE BRAND OF PLAIN CIGARETTES

| Laboratory | Number of determinations | Mean, μg per cigarette | Range, μg per cigarette | Standard deviation, μg per cigarette | Coefficient of variation, % |
|---|---|---|---|---|---|
| 1 | 17 | 324 | 312 to 338 | 7·7 | 2·4 |
| 2 | 20 | 327 | 388 to 352 | 19 | 5·8 |

A comparison was then made between the total phenol content of the condensate obtained from plain and filter tipped cigarettes of the same brand. The results are shown in Table II.

From the results in Table II it can be seen that the total phenol content of the smoke obtained from a filter-tipped cigarette is considerably less than that obtained from the corresponding plain cigarette.

## Discussion

Provided that the ratio of the phenols produced by smoking cigarettes of a certain brand is first determined, the procedure described will give an accurate and precise determination of the total phenol content of the smoke condensate.

## Table II

Comparison of total phenols produced by plain and filtered cigarettes

| Brand | A | B | C | D | E |
|---|---|---|---|---|---|
| Plain, μg of total phenol per cigarette | 324 | 300 | 225 | 260 | 280 |
| Filter-tipped, μg of total phenol per cigarette | 160 | 150 | 150 | 150 | 205 |

For routine analyses of both plain and filter-tipped British cigarettes, it is suggested that a calibration graph having a ratio of phenol to the cresols of 5 + 1 + 1 + 1, respectively, should be used. The error incurred is at the most about ±2 per cent. (see Fig. 4) and for routine analyses of this type this is sufficiently small to be ignored.

There is good agreement between results for the total phenol determination quoted in Table I, of this Paper and those obtained by adding the means of phenol, the cresol isomers and guaiacol, quoted in Table III in Part II.[13] Comparison can be made since these were the same batch of a single brand of plain cigarettes. Though only phenol, the cresols and guaiacol were determined in Part II, they total approximately 96 per cent. of the figure obtained by using the method described in this Paper. If all of the minor phenolic components had been determined, the figure would undoubtedly have been much nearer to 100 per cent.

The total phenol content can be determined in duplicate on 12 samples in an 8-hour day. This is considerably more than could be determined in a comparable working day by the gas-chromatographic methods available at present. There is also a large saving in the number of cigarettes smoked and consequently the time taken in smoking them is much less. The colorimetric method described requires only five plain or filter-tipped cigarettes, whereas the gas-chromatographic method requires one hundred plain or two hundred filter-tipped cigarettes.

Complete resolution of phenol, the cresols, the xylenols and the ethylphenols, has been achieved by gas - liquid chromatography of their acetates.[16] The method has been put on a quantitative basis, and it is hoped to publish the results of this investigation, along with a comparison of the results obtained by the thin-layer chromatographic method of Smith and Sullivan.[13]

We thank the Directors of Carreras Limited for permission to publish this work and Mr. A. A. Watson for his co-operation in providing us with one of the series of results (see Table I) used to ascertain the precision of the method.

## References

1. Hoffmann, D., and Wynder, E. L., *Beitr. TabakForsch.*, 1961, **1**, 101.
2. Spears, A. W., *Anal. Chem.*, 1963, **35**, 320.
3. Crouse, R. H., Garner, J. W., and O'Neill, H. J., *J. Gas Chromatography*, 1963, **1**, No. 2, 18.
4. Stedman, R. L., and Miller, R. L., *J. Chromatography*, 1963, **11**, 409.
5. Rayburn, C. H., Harlan, W. R., and Hamner, H. R., *Anal. Chem.*, 1953, **25**, 1419.
6. Mohler, E. F., jun., and Jacob, L. N., *Ibid.*, 1957, **29**, 1369.
7. Emerson, E., *J. Org. Chem.*, 1943, **8**, 417.
8. Ettinger, M. B., Ruchhoft, C. C., and Lishka, R. J., *Anal. Chem.*, 1951, **23**, 1783.
9. Gottlieb, S., and Marsh, P. B., *Ind. Eng. Chem., Anal. Ed.*, 1946, **18**, 16.
10. Martin, R. W., *Anal. Chem.*, 1949, **21**, 1419.
11. Lacoste, R. J., Venable, S. H., and Stone, J. C., *Ibid.*, 1959, **31**, 1246.
12. Lorentzen, G., and Neurath, G., *Beitr. TabakForsch.*, 1963, **2**, 73.
13. Smith, G. A. L., and Sullivan, P. J., *Analyst*, 1964, **89**, 312.
14. Bentley, H. R., and Burgan, J. G., "Cigarette Smoke Condensate: Preparation and Laboratory Estimation. *Research Paper No.* 4," Second Edition, Tobacco Manufacturers' Standing Committee, London, 1961, p. 2.
15. Bradford, J. A., Harlan, W. R., and Hamner, H. R., *Ind. Eng. Chem.*, 1936, **28**, 836.
16. Smith, G. A. L., and King, D. A. K., *Chem. & Ind.*, 1964, 540.

Note—Reference 13 is to Part II of this series.

Received *October 7th*, 1963

# Determination of the Steam-volatile Phenols Present in Cigarette-smoke Condensate

## Part II.* Determination of Phenol, the Cresols and Guaiacol by Thin-layer Chromatography

By G. A. L. SMITH and P. J. SULLIVAN
(*Carreras Research Division, Bruce Grove, Wickford, Essex*)

A method is described for determining phenol, *o*-, *m*- and *p*-cresols and guaiacol present in tobacco-smoke condensate. The phenols are coupled with diazotised *p*-nitroaniline under specified conditions and extracted into ether. The ethereal solution is streaked on to a plate of kieselguhr G impregnated with formamide and developed with either a benzene - cyclohexane - dipropylene glycol (30 + 70 + 3 by volume) or a benzene - cyclohexane - diethylamine (5 + 5 + 1 by volume) solvent mixture. A good separation is obtained, and the individual bands are scraped off and dissolved in ammonia solution, sp.gr. 0·88. The individual phenols are then determined colorimetrically by using a Unicam SP500 spectrophotometer.

As little as 0·5 μg of each phenol can be determined quantitatively by this method.

Paper chromatography was used by Rayburn, Harlan and Hamner[1] for separating, identifying and determining the amounts of phenol and cresols present in tobacco-smoke condensate. We were unable to obtain as good a separation as claimed by them. In general, the separations of phenols of low molecular weight are frequently poor, *e.g.*, the cresol isomers were rarely resolved.[2,3,4] Recently, however, Crump[5] achieved an excellent separation of phenol and the cresol isomers. We have modified the method of Crump and have used thin-layer instead of paper chromatography to separate the phenols. The time required for the separation by using thin-layer chromatography was 30 minutes as compared with 2½ hours by using the method of Crump.

A correlation has been found between the colorimetric method reported in Part I of this series[6] and the method described below (see also under "Discussion" in Part I). Finally, cigarettes made from various types of cured tobacco were used in an investigation to determine whether or not the ratio of the phenols produced by them differed significantly.

### Experimental

#### Absorption maxima of the azo dyes produced by coupling phenols with diazotised *p*-nitroaniline—

Specimens of the pure dyestuffs were made by coupling the phenols in alkaline solution with the diazonium hydrochloride of *p*-nitroaniline, filtering off the crystals, and recrystallising them three times from glacial acetic acid. The crystals were washed free of acid with de-ionised water and dried in a desiccator. The absorption maxima were determined by dissolving a little of each of the dyestuffs in ammonia solution, sp.gr. 0·88, and recording their spectra on a Perkin-Elmer model 137 UV spectrophotometer. The results are shown in Table I.

#### Effect of varying the concentration of the ammonia solution on the absorption frequency of the dyestuffs—

The absorption frequency, and the optical density measured at the absorption maximum corresponding to its concentration of ammonia solution, increases with increase in the concentration of the ammonia solution (see Fig. 1).

* For details of Part I of this series, see reference list, p. 318.

Since the optical density was at a maximum when the dyes were dissolved in ammonia solution, sp.gr. 0·88, all measurements of optical density should be made in this medium.

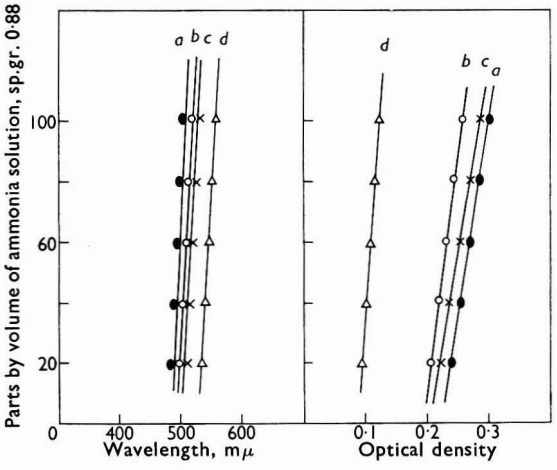



Fig. 1. The effect of varying the concentration of ammonia solution, sp.gr. 0·88, on the wavelength of the absorption maxima of the dyes and on the optical density of the solution measured at the maximum corresponding to its concentration of ammonia solution. The concentration of the solution measured was 0·2 μg per ml. Graphs: (*a*) phenol; (*b*) *m*-cresol; (*c*) *o*-cresol; (*d*) *p*-cresol

Five Winchesters of ammonia solution, sp.gr. 0·88, were analysed, and were all found to contain 34·5 ± 0·5 per cent. w/v of ammonia. After about two months it was found that the ammonia concentration in the Winchesters had dropped by less than 1 per cent.

In view of these analyses it was considered that any error introduced by the variation of ammonia concentration between Winchesters was negligible.

TABLE I

ABSORPTION MAXIMA OF DYESTUFFS IN AMMONIA SOLUTION, SP.GR. 0·88

| Compound | Absorption maxima, mμ | Compound | Absorption maxima, mμ |
|---|---|---|---|
| Phenol .. .. .. | 506 | 2,6-Xylenol .. .. | **550**, 410 |
| *o*-Cresol .. .. .. | 530 | 3,4-Xylenol .. .. | 544, **385** |
| *m*-Cresol .. .. .. | 521 | 3,5-Xylenol .. .. | 512, **410** |
| *p*-Cresol .. .. .. | **560**, 360 | *o*-Ethylphenol .. .. | 530, **402** |
| 2,3-Xylenol .. .. | **550**, 405 | *m*-Ethylphenol .. .. | **527**, 410 |
| 2,4-Xylenol .. .. | 546, **395** | *p*-Ethylphenol .. .. | 545, **395** |
| 2,5-Xylenol .. .. | **545**, 415 | Guaiacol .. .. .. | 540, 404 |

Where more than one peak was recorded in the spectrum of the dye, the wavelength of the peak having the higher optical density has been set in bold type. The two peaks of guaiacol are of equal intensity.

PREPARATION OF CALIBRATION GRAPHS—

A series of test solutions of known concentration was prepared, covering the range 0 to 10 μg of phenol, guaiacol and the cresol isomers, by dissolving known weights of the pure dyes in ammonia solution, sp.gr. 0·88, and diluting each to 500 ml in calibrated flasks with ammonia solution. Portions were then transferred by pipette to 10-ml calibrated flasks, and diluted to the mark with ammonia solution. The optical densities of the test solutions in 4-cm cells were then read on a Unicam SP500 spectrophotometer, at 506, 530, 521, 560 and 540 mμ for phenol, *o*-, *m*- and *p*-cresol and guaiacol, respectively. The solutions

obeyed Beer's law, the relationship between optical density and concentration of dyestuff being linear in each instance. The equations for the lines, all of which went through the origin were—

Phenol, $y = 6{\cdot}8x$; *o*-cresol, $y = 7{\cdot}8x$; *m*-cresol, $y = 8{\cdot}3x$;
*p*-cresol, $y = 11{\cdot}3x$; guaiacol, $y = 7{\cdot}7x$.

The graphs obtained from the pure dyes were checked by starting from known weights of the phenols themselves, coupling them in alkaline solution with diazotised *p*-nitroaniline as described in Part I,[6] and then acidifying the alkaline solution to pH 4 with dilute hydrochloric acid and extracting the dye with ether. The ethereal solution was dried and then evaporated to approximately 10 ml, and transferred quantitatively to a 25-ml calibrated flask and diluted to the mark with ether.

Portions of the ethereal solution were then streaked on to thin layers of kieselguhr G, and the ether evaporated by gently blowing on the plate. The bands were scraped off and transferred with great care to 10-ml calibrated flasks, which were then filled to the mark with ammonia solution, sp.gr. 0·88. After being shaken for 10 minutes, the contents of the flasks were poured into clean, dry centrifuge tubes and spun in a centrifuge for 10 minutes to ensure complete removal of all the kieselguhr. The clear, supernatant solutions were transferred to clean, dry, 4-cm cells and the optical densities determined at the particular absorption maximum of each solution with a Unicam SP500 spectrophotometer.

Before the removal of the bands from the plates, the $R_F$ value of each phenol was determined and recorded, and the results are given in Table II.

TABLE II

$R_F$ VALUES OF THE DYES PRODUCED BY COUPLING PHENOLS WITH DIAZOTISED *p*-NITROANILINE

| Azo compound | $R_F$ value in benzene - cyclohexane - dipropylene glycol solvent | $R_F$ value in benzene - cyclohexane - diethylamine solvent | Azo compound | $R_F$ value in benzene - cyclohexane - dipropylene glycol solvent | $R_F$ value in benzene - cyclohexane - diethylamine solvent |
|---|---|---|---|---|---|
| Phenol .. .. | 0·30 | 0·23 | 2,5-Xylenol .. | 0·74 | 0·54 |
| *m*-Cresol .. .. | 0·53 | 0·39 | 2,6-Xylenol .. | 0·85 | 0·30 |
| *o*-Cresol .. .. | 0·61 | 0·44 | 3,4-Xylenol .. | 1·00 | 0·90 to 1·00 |
| *p*-Cresol .. .. | 1·00 | 0·90 to 1·00 | 3,5-Xylenol .. | 0·56 | 0·75 |
| Guaiacol .. .. | 0·90 | 0·10 | *o*-Ethylphenol .. | 0·64 | 0·64 |
| 2,3-Xylenol .. | 0·71 | 0·70 | *m*-Ethylphenol .. | 0·68 | 0·54 |
| 2,4-Xylenol .. | 1·00 | 0·90 to 1·00 | *p*-Ethylphenol .. | 1·00 | 0·90 to 1·00 |

The linear relationships derived by starting from the pure dyes were identical with those from the pure phenols.

If the acidity is allowed to exceed pH 4 when the sodium salts of the dyes are acidified, the dyes are not completely recovered during the ether extraction.

PREPARATION OF THE THIN-LAYER CHROMATOGRAM

REAGENTS—

*Kieselguhr G*—Obtainable from Camlab (Glass) Ltd.
*Formamide*—A 5 per cent. v/v solution in acetone.

PROCEDURE—

Prepare a slurry of 40 g of kieselguhr G and 100 ml of water by grinding these materials together in a mortar. Pour the slurry on to 20-cm × 20-cm flat, glass plates in a layer 250$\mu$ thick. Dry the plates for half an hour in an oven at 100° C. Impregnate the plates one at a time with formamide, by immersing them in the formamide solution contained in a dip-tank. Remove each plate from the dip-tank, allow it to drip for half a minute, and then leave to dry for half an hour at room temperature.

Remove 1 inch of absorbent from the sides of the plates, and 1 cm from the bottom; this prevents the solvent from bowing. Allow the chromatographic tank that contains the developing solvent mixture to stand for 1 hour with the sides hung with filter-paper soaked in the solvent; this procedure allows the atmosphere to reach equilibrium before the plates are used.

### Recovery of dyes from thin-layer plates

Known weights of pure dyes were dissolved in ether and diluted to a known volume. Portions were streaked on to the plates, the streaks being 5 to 7 cm in length. Great care was taken to avoid puncturing the thin layer. The plates were placed in the tank and developed for three quarters of an hour. The bands were then scraped off the plates and each transferred to a 10-ml calibrated flask, and diluted to the mark with ammonia solution, sp.gr. 0·88. After removing the absorbent by centrifugation, the optical densities of the solutions were measured at their respective wavelengths. In all instances, recoveries were greater than 98 per cent.

The same recoveries were also recorded when synthetic mixtures of dyes were prepared and subjected to the whole procedure.

### Method

The apparatus for this procedure is the same as that described previously in Part I of this series,[6] as is the conditioning and selection of cigarettes and the smoking procedure.

#### Reagents—

All reagents should be of analytical-reagent grade unless otherwise specified.

*Methanol*—General-purpose reagent grade.

*Sodium hydroxide*, 0·1 N.

*Sulphuric acid*, 2 N.

*Sodium nitrite solution*, 2·2 *per cent. w/v, aqueous.*

p-*Nitroaniline hydrochloride solution*—Weigh accurately 1 g of general-purpose reagent grade *p*-nitroaniline, transfer it to a 100-ml calibrated flask and dissolve it in 20 ml of hydrochloric acid, sp.gr. 1·18. Dilute to the mark with water.

*Diazonium salt of* p-*nitroaniline, aqueous solution*—Prepare the solution as described in Part I of this series.[6]

#### Extraction of tar from the Cambridge filter—

Remove the filter holders and uncouple them. Take out the filter and place it in a sintered-glass funnel that is attached to a Buchner flask. First wash the holder with methanol to remove all the tar adhering to it, and then wash the filter until it is clean. Transfer the tar solution to a 250-ml round-bottomed flask, taking care to wash the Buchner flask clean with more methanol. The solution and washings should have a total volume of between 50 and 60 ml.

#### Steam-distillation—

Transfer with a pipette 20 ml of 2 N sulphuric acid into the flask containing the methanolic tar solution, mix well and add 100 ml of water. Attach the flask to a steam-distillation unit, steam-distil the solution and collect 250 ml of distillate.

#### Determination of phenols—

Transfer the 250 ml of steam-distillate to a 600-ml beaker. Wash out the flask with de-ionised water and add the washings to the solution in the beaker. Place the electrodes of a pH meter into the beaker, making sure that they are about 2 inches below the surface of the liquid. Run in 0·1 N sodium hydroxide slowly, agitating the solution with a mechanical stirrer, until it reaches a pH of 10. Then run in that volume of 0·1 N sodium hydroxide required to bring 22·0 ml of diazonium chloride solution to pH 10 (see under "Preparation of Calibration Graph," Part I, p. 308[6]). Then add 22·0 ml of the diazonium chloride solution with rapid stirring. Allow the stirring to continue for 5 minutes to ensure complete reaction. Run in 0·1 N hydrochloric acid slowly with agitation until the pH of the solution is 4.

Transfer the solution of dye completely to a 500-ml separating funnel. Extract the aqueous solution with 4 successive 50-ml portions of ether. Dry the ethereal solution of dye by adding 1 g of magnesium sulphate, and shake the mixture periodically for half an hour. Filter off the drying agent on a Buchner funnel. Wash the solid twice with 30-ml of ether to remove the last traces of dye. Combine the filtrate and washings in a 250-ml flask and evaporate them to a volume of about 10 to 15 ml. Transfer the liquid quantitatively to a 25-ml calibrated flask and dilute to the mark with more ether.

The preparation of the thin-layer plates and the development technique are the same as described under "Preparation of the Thin-layer Chromatogram," p. 314.

Apply 100 $\mu$l of the ethereal solution in a streak about 5 to 7 cm in length to the thin layer. Great care must be taken to avoid puncturing the thin layer. Place the plate in the developing chamber containing the solvent and develop it for 30 minutes. Remove the plate and allow it to dry in air for 1 to 2 minutes. Scrape off the individual bands, transfer them to 10-ml calibrated flasks and dilute to the mark with more ammonia solution. After shaking the flasks for 10 minutes, transfer the ammoniacal dye solutions to clean, dry centrifuge tubes and spin them in a centrifuge for 10 minutes to ensure complete removal of all the kieselguhr. Transfer the clear supernatant liquids to clean, dry 4-cm cells and measure the optical densities at the absorption maxima of each solution on a Unicam SP500 spectrophotometer. By using the optical densities obtained, read off the amount of each individual phenol present in the streak from the appropriate calibration graph, and calculate the amount of each present in a single cigarette.

NOTE—

The solvent system, benzene - cyclohexane - dipropylene glycol, was used for determining phenol and the cresol isomers. The band of $R_F$ value 1·0, contained all the *p*-substituted phenols, *e.g.*, *p*-cresol, *p*-ethylphenol, 2,4-xylenol and 3,4-xylenol, and since *p*-cresol was the major component, the band, when scraped off, was determined as *p*-cresol.

The solvent system, benzene - cyclohexane - diethylamine, was used for determining guaiacol as well as phenol and the cresols. However, the separation achieved between *o*- and *m*-cresols with this developing system is not as good as that obtained by using the first-mentioned system.

## RESULTS AND PRECISION

A series of replicate determinations, 15 in number, was performed on a single batch of an unfiltered brand of cigarette, and the results obtained are shown in Table III.

TABLE III

REPRODUCIBILITY AND PRECISION OF METHOD

| Compound | Range, $\mu$g per cigarette | Mean, $\mu$g per cigarette | Standard deviation, $\mu$g | Ratio of phenols produced |
|---|---|---|---|---|
| (*a*) *Benzene - cyclohexane - dipropylene glycol solvent*— | | | | |
| Phenol | 195 to 215 | 202 | 5·9 | 6·5 |
| *o*-Cresol | 25 to 35 | 31 | 3·3 | 1·0 |
| *m*-Cresol | 20 to 30 | 25 | 4·8 | 0·8 |
| *p*-Substituted phenols as *p*-cresol | 30 to 40 | 35 | 2·9 | 1·1 |
| (*b*) *Benzene - cyclohexane - diethylamine solvent*— | | | | |
| Phenol | 193 to 200 | 197 | 2·6 | 6·0 |
| *o*-Cresol | 30 to 35 | 33 | 2·2 | 1·0 |
| *m*-Cresol | 25 to 30 | 28 | 1·5 | 0·9 |
| *p*-Substituted phenols as *p*-cresol | 33 to 40 | 33 | 2·5 | 1·1 |
| Guaiacol | 12 to 15 | 14 | 1·2 | 0·4 |

It was of great importance that the ratio of phenols produced by smoking cigarettes made from different types of tobacco should be determined, since the accuracy of the colorimetric determination described in Part I of this series[6] depended on this being known.

IDENTIFICATION OF THE OTHER PHENOLS PRESENT—

The phenols listed below were identified by their $R_F$ values and spectra, but not determined since they were all very minor constituents—

2,3-xylenol; 2,6-xylenol; 2,5-xylenol or *m*-ethylphenol or both; 3,5-xylenol; *o*-ethylphenol;

and a band at or very near to the solvent front which contained the *p*-substituted phenols.

SEPARATION AND IDENTIFICATION OF THE *p*-SUBSTITUTED PHENOLS—

By using a thin layer of kieselguhr G impregnated with formamide and the cyclohexane - diethylamine (9 + 1 by volume) solvent system, a synthetic mixture of the four *p*-substituted phenols was resolved into three spots. These compounds could be identified, since the $R_F$ values of the individual compounds had previously been determined.

Removal of the band of $R_F$ value 0·9 to 1·00 obtained from the cigarette-smoke condensate and examined as described above, also produced three spots that were identified as *p*-cresol or *p*-ethylphenol or both, 3,4-xylenol, and 2,4-xylenol; see Table IV.

TABLE IV

COMPARISON OF $R_F$ VALUES

| Azo compound | $R_F$ of known compound | $R_F$ of compound in cigarette smoke |
|---|---|---|
| *p*-Cresol .. .. .. | 0·15 | 0·15 * |
| *p*-Ethylphenol .. .. | 0·15 | 0·15 * |
| 3,4-Xylenol .. .. .. | 0·23 | 0·23 |
| 2,4-Xylenol .. .. .. | 0·53 | 0·53 |

* One spot.

By using a thin layer of kieselguhr and the cyclohexane - benzene - diethylamine (7 + 3 + 1 by volume) solvent mixture, the mixture of *p*-substituted phenols present in cigarette smoke could be separated into three spots. These were identified by measuring their $R_F$ values and comparing them with those obtained under the same conditions with known compounds. The results are shown in Table V.

TABLE V

COMPARISON OF $R_F$ VALUES

| Azo compound | $R_F$ of known compound | $R_F$ of compound in cigarette smoke |
|---|---|---|
| *p*-Cresol .. .. .. | 0·30 | 0·30 |
| *p*-Ethylphenol .. .. | 0·50 | 0·50 * |
| 3,4-Xylenol .. .. .. | 0·50 | 0·50 * |
| 2,4-Xylenol .. .. .. | 0·85 | 0·85 |

* One spot.

TABLE VI

RATIO OF PHENOLS PRODUCED BY SMOKING CIGARETTES MADE FROM DIFFERENT TYPES OF TOBACCO

| Type of tobacco | Type of curing | Phenol, μg per cigarette | *o*-Cresol, μg per cigarette | *m*-Cresol, μg per cigarette | *p*-Substituted phenols, as *p*-cresol, μg per cigarette | Ratio of phenols produced |
|---|---|---|---|---|---|---|
| Virginia blend 1, filter cigarette | Flue-cured | 100 | 15 | 20 | 25 | 5·0 + 1·0 + 0·8 + 1·2 |
| Virginia blend 2, plain cigarette | Flue-cured | 202 | 31 | 25 | 35 | 6·5 + 1·0 + 0·8 + 1·1 |
| Virginia blend 2, filter cigarette | Flue-cured | 103 | 20 | 18 | 22 | 5·2 + 1·0 + 0·9 + 1·1 |
| Virginia blend 3, plain cigarette | Flue-cured | 185 | 31 | 28 | 34 | 6·0 + 1·0 + 0·9 + 1·1 |
| Virginia blend 3, filter cigarette | Flue-cured | 50 | 15 | 15 | 19 | 3·3 + 1·0 + 1·0 + 1·3 |
| Virginia blend 4, filter cigarette | Flue-cured and small amount air-cured | 60 | 20 | 15 | 22 | 3·0 + 1·0 + 0·8 + 1·1 |
| Virginia blend 5, filter cigarette | Flue-cured | 75 | 23 | 18 | 25 | 3·3 + 1·0 + 0·8 + 1·1 |
| Turkish blend 1, plain cigarette | Air-cured | 180 | 29 | 25 | 44 | 6·2 + 1·0 + 0·9 + 1·5 |
| Turkish blend 2, plain cigarette | Air-cured | 160 | 30 | 21 | 38 | 5·3 + 1·0 + 0·7 + 1·3 |
| Continental blend, plain cigarette | Air-cured and fermented | 185 | 31 | 26 | 39 | 6·0 + 1·0 + 0·8 + 1·3 |
| American blend, plain cigarette without casing | Flue-cured and air-cured | 150 | 33 | 24 | 86 | 4·5 + 1·0 + 0·7 + 2·6 |
| Cigar | Air-cured and fermented | 145 | 17 | 17 | 155 | 8·5 + 1·0 + 1·0 + 9·1 |

The presence of *p*-cresol, 2,4-xylenol and 3,4-xylenol in cigarette-smoke condensate has clearly been demonstrated by using these two systems. Undoubtedly, *p*-ethylphenol is there as well, but as yet we have not been able to isolate it.

By far the largest component of the four in the mixture is *p*-cresol, accounting for approximately 75 per cent. of the total.

## Discussion

The ratio of phenols produced by the four types of tobacco used, does not differ significantly, as can be seen from results shown in Table VI. The only noticeable difference is shown by the American blended tobacco, which produced between 2 and 3 times as much *p*-substituted phenols as did the other three types of tobacco used.

There was a significant difference between the cigar tobacco and all types of the cigarette tobacco. The *p*-substituted phenols had now become the major component of the mixture, being slightly larger than phenol.

The introduction of a filter (see Table VI, Virginia blends 2 and 3) greatly reduced the total phenols and also altered the ratio of them in the smoke. Phenol appeared to be selectively removed by the filter, and an appropriate ratio of phenols and cresols retained by the filter was 10 + 1 + 1 + 1.

We thank the Directors of Carreras Limited for permission to publish this work.

## References

1. Rayburn, C. H., Harlan, W. R., and Hamner, H. R., *Anal. Chem.*, 1953, **25**, 1419.
2. Reio, L., *J. Chromatography*, 1958, **1**, 338.
3. Macak, J., and Kubes, J., *Coll. Czech. Chem. Commun.*, 1960, **25**, 301.
4. Leibnitz, E., Behrens, U., Riedel, L., and Gabert, A., *J. prakt. Chem.*, 1960, **11**, 125.
5. Crump, G. B., *J. Chromatography*, 1963, **10**, 21.
6. Smith, G. A. L., and King, D. A., *Analyst*, 1964, **89**, 305.

Note—Reference 6 is to Part I of this series.

Received *October 7th*, 1963

# The Use of Infrared Spectroscopy in the Analysis of Pesticide Residues

By N. T. CROSBY and E. Q. LAWS

*(Department of Scientific and Industrial Research, Laboratory of the Government Chemist, Cornwall House, Stamford Street, London, S.E.1)*

A method is described for identifying residues of organo-phosphorus pesticides in vegetables by infrared spectroscopy. A general procedure is described, in which column chromatography, for isolating unknown residues, and then gas chromatography of the separated extracts is used before identifying and measuring them by the infrared method. The procedure is discussed in relation to other possible methods.

When the pesticide treatment a sample of foodstuff has received is unknown, it may be as important to ascertain the nature of the residue as it is to determine the amount present; if both are known, then the hazard to the consumer of the food can be properly assessed.

Methods that have been used for analysing organo-phosphorus compounds include polarography, anti-cholinesterase measurement, chromatography and phosphorus determination.

Polarography is only applicable to a limited number of pesticides, and it is less sensitive than the other methods. Cholinesterase measurement is extremely sensitive and yields evidence of physiological activity, but it does not distinguish between different pesticides.

Paper or thin-layer chromatographic methods yield considerable information on the identity of the phosphorus pesticides, but they are difficult to apply in the presence of impurities co-extracted with the pesticide from the sample. Methods that rely on a phosphorus determination for assessing the amount of pesticide present give little information about the identity of the separated compounds. The "General Method" of Laws and Webley,[1] in which a colorimetric phosphorus measurement is used as the end-method for the determination of many phosphorus pesticides, has been usefully applied to many vegetables and has been the subject of collaborative study.[2] One valuable feature of the method is that it divides the pesticides into three groups: water-soluble pesticides, which are eluted from a carbon column by chloroform; petroleum-soluble compounds, which are eluted from alumina with light petroleum; and those that are subsequently eluted from the alumina with 15 per cent. v/v diethyl ether - light petroleum mixture.

The gas-chromatographic method, so successfully applied to chlorinated compounds by Goodwin, Goulden and Reynolds,[3] and later to the phosphorus pesticides by Egan, Hammond and Thomson,[4] gives partial evidence of the identity of the compound determined, and this type of evidence has been reinforced more recently by the application of the simultaneous multiple-column gas-chromatographic technique of Goulden, Goodwin and Davies,[5] which may be interpreted to give a "spectrum" or pattern that is stated to be characteristic of a particular pesticide.

The most satisfactory single method for the absolute identification of an organic compound is to record its absorption spectrum. This is particularly true of the infrared spectrum, in which many of the absorption bands may be related to structural groups forming part of the molecular arrangement of the compound studied. With pure compounds, the identification is fairly straightforward, but when a substance has been isolated from a complicated mixture derived from a vegetable or animal extract, the presence of even microgram amounts of impurities may prevent the interpretation of the spectrogram.

The problem has been studied by McCaully and Cook[6] who were able to detect 2 p.p.m. of certain organo-phosphorus pesticides in 1 kg of vegetables.

There are many extraction and purification systems that can be used, but, because the working of Laws and Webley's general method[1] was familiar to us, and since it is so versatile, both with regard to the number of pesticides extracted and the number of plant species with which it will function, it was selected for further studies by the infrared method. It

was found that the nature and amounts of the phosphorus-free residues obtained by the method, when it was applied to vegetables that had not been treated with pesticides, varied greatly; even, in some instances, with the same kind of plant. The results given in Table I illustrate this point.

TABLE I

WEIGHTS OF RESIDUES OBTAINED FROM 50-g SAMPLES OF VARIOUS CROPS

Samples were subjected to extraction and partition procedures only

All weights are expressed in milligrams

| Crop | Water-soluble portion, obtained from a carbon column and eluted with chloroform | Petroleum-soluble portion, obtained from an alumina column and eluted with— | |
|---|---|---|---|
| | | light petroleum | light petroleum - 15 per cent. diethyl ether mixture |
| Apple .. .. .. | 2 | 3, 6 | 4, 6, 9 |
| Cabbage .. .. | 13 | 36 | — |
| Carrot .. .. .. | Nil, Nil | — | — |
| Cauliflower .. .. | 2, 3 | — | — |
| Cherry .. .. | 2, 2 | 6, 7 | — |
| Cucumber .. .. | Nil | 2 | — |
| Lettuce .. .. | 2, 2 | 3 | 9, 11, 12* |
| Mushroom .. .. | — | — | 20 |
| Onion .. .. | 14, 6 | 6 | — |
| Potato .. .. | Nil, Nil, Nil, Nil | 2 | — |
| Swede .. .. .. | 1 | 4 | — |
| Sugar beet .. .. | Nil | — | — |
| Tomato .. .. | — | 3, 6 | — |

* After the sample had been subjected to Jones and Riddick's partition,[7] this weight was 4 mg.

The infrared spectra of such residues were recorded, and those that could possibly be used directly for infrared examination were noted. Table II lists suitable vegetable extracts.

TABLE II

VEGETABLE EXTRACTS SUITABLE FOR DIRECT INFRARED EXAMINATION

The general method[1] was used alone or in combination with Jones and Riddick's partition[7]

| Carbon column | Alumina column | Alumina column *plus* Jones and Riddick's partition |
|---|---|---|
| Apple | — | — |
| — | — | Carrot |
| Cucumber | Cucumber | — |
| Potato | — | Potato |
| Sprout | — | Sprout |
| Sugar beet | Sugar beet | — |

In those instances in which the column-chromatographic method did not give a satisfactory clean-up for the infrared examination, removal of interfering substances was attempted by using the techniques enumerated below—

(*i*) *Liquid - liquid partition* (*Jones and Riddick's method*[7])—This effected a considerable improvement with extracts from the light-petroleum fraction.

(*ii*) *Partition and adsorption chromatography*—Magnesia columns were satisfactory for morphothion in green vegetables; this was probably an example of displacement chromatography. Carbon columns gave little further improvement with chloroform extracts derived from the aqueous extracts. Fuller's earth retained the pesticides too strongly.

(*iii*) *Freezing-out of wax-like substances*—Freezing-out from acetone solution removed some impurities, but also removed pesticides.

(*iv*) *Molecular-sieve chromatography*—Molecular sieves that had been de-activated by prior water treatment showed some promise with oxydemeton-methyl.

(*v*) *Vacuum distillation of the pesticide*—This was successful with oxydemeton-methyl and phorate, but considerable plant material was distilled with the pesticides.

(*vi*) *Infrared compensation methods*—These were satisfactory when the plant extracts were already reasonably clean.

(*vii*) *Gas chromatography*—This method was satisfactory for all but the least volatile compounds or those which decompose with heat.

As a consequence of these experiments, the method chosen was a combination of the general method and a final gas-chromatographic clean-up stage, and then collection of the effluent from the gas chromatograph and the identification and measurement of the pesticide by means of infrared spectroscopy.

Pesticides that have been recovered satisfactorily are listed below—

Chlorthion, dimethoate, fenchlorphos, fenthion, malathion, mevinphos, oxydemeton-methyl, parathion, parathion-methyl, phenkapton, phorate, the sulphone of the oxygen analogue of phorate, phosphamidon, schradan and thiometon.

## Instrumentation

### Gas chromatograph—

The gas chromatograph is used here solely for separating pesticide residues from co-extracted plant materials. These sample weights, up to 50 mg (see Table I), require large volumes of injection solvent. As much as 150 $\mu$l of the solvent, applied in three injections, may be needed for each analysis. These factors combine to reduce the efficiency of the analytical columns. A semi-preparative instrument was therefore designed that incorporated a device for collecting pesticide residues for direct examination by infrared spectroscopy.

The chromatographic parameters selected represent a compromise between optimum column performance and efficacy of removal of interfering materials.

The use of some liquid phases, such as silicone oils, is precluded by their interference in infrared analysis. A stationary phase comprising glass beads coated with Apiezon L grease and Epikote resin proved to be a satisfactory column packing and was used throughout this work. Further investigation will be necessary to establish whether it is the best available packing for this type of analysis. In the early stages of the development of this method, some pesticides were not completely recovered, and others were not recovered at all. The losses appear to be due to the use of copper columns, which cause decomposition of some of the organo-phosphorus pesticides, since substitution of stainless steel as the column material results in greatly improved recoveries of many of these compounds. This finding is similar to that of Beckman and Bevenue,[8] obtained for chlorinated pesticides with copper and stainless-steel columns.

### Infrared spectrophotometer—

The limit of detection of the instrument is determined by the signal-to-noise ratio of the photometer, which in turn depends on the sensitivity of the thermopile and the number of absorbing molecules in unit cross-sectional area of the sample beam.

The standard micro cells of 1-mm path-length supplied with most infrared spectrophotometers, including the Infracord 137, have a volume of about 100 $\mu$l, of which only one third lies in the optical path of the instrument. Recognisable spectra require at least 60 $\mu$g in this system. Increasing the path-length of the cell has little effect on the limit of detection, since the cell volume is increased at the same time, and, correspondingly, a greater weight of solute is required to produce a solution of the same concentration.

The use of cavity cells in conjunction with beam-condenser optics increases the sensitivity of the system so that a recognisable trace is obtained with as little as 5 $\mu$g of most organo-phosphorus pesticides. Cavity cells of 1-mm path-length have a volume of about 5 $\mu$l, all of which lies in the optical path.

Electronic expansion devices offer an alternative method of lowering the limit of detection, but it must be borne in mind that background interference also becomes more serious and considerable distortion of the spectrum may occur. Recent developments in this Laboratory include the use of cavity cells, without a beam condenser, in conjunction with the Grubb Parsons Spectromaster instrument, which incorporated a scale-expansion device. By using

this technique, a good spectrum is obtained with as little as 1 $\mu$g of the organo-phosphorus pesticides, and in some instances the spectrum can be readily distinguished at the 0·5 $\mu$g level. An example of a spectrum obtained from a 1-$\mu$g sample is given in Fig. 1. This was obtained by using the Grubb Parsons Spectromaster with a scale expansion factor of 5.

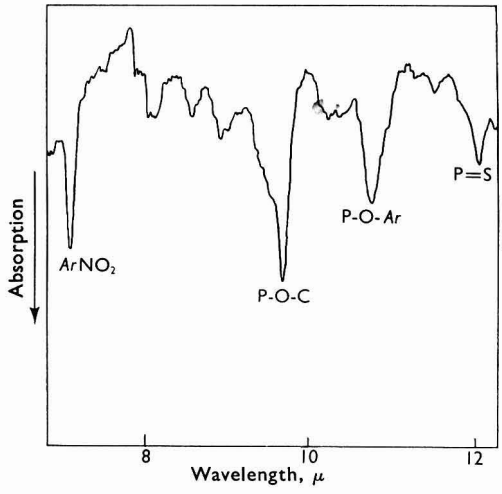



Fig. 1. Spectrogram obtained from a 1-$\mu$g sample of parathion with carbon disulphide as the solvent. Path-length, 1 mm. Scale expansion factor, × 5

## Method

The extraction of residues of organo-phosphorus pesticides from plant material, their separation into water-soluble and petroleum-soluble groups, and the chromatography of both groups by using activated carbon and alumina have been fully described by Laws and Webley.[1] Only modifications of that procedure incorporated into the present work will be described here, together with a detailed account of the gas-chromatographic stage and the determination of the final pesticide residues by infrared spectroscopy. Phosphorus determinations were performed by the method of Caverly and Hall.[9]

### Apparatus—

*Spectrophotometers*—Perkin-Elmer 137 Infracord and a Grubb Parsons Spectromaster instruments were used.

*Beam condensers*—Obtainable from Research and Industrial Instruments Ltd.

*Sodium chloride cavity cells*—Having a path-length of 1 mm, and a capacity of about 5 $\mu$l.

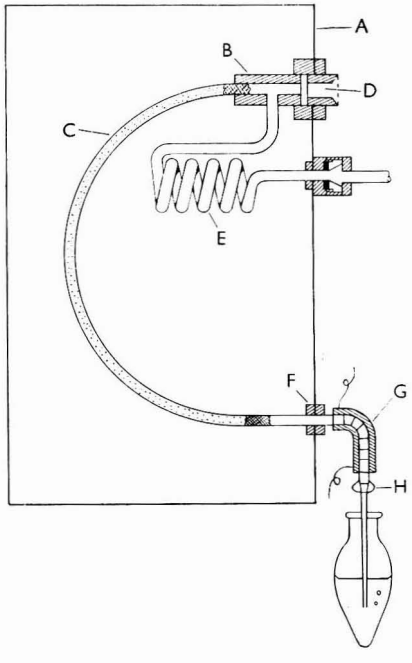
*Hamilton syringes*—Of capacity 50 $\mu$l and 10 $\mu$l.

*Semi-preparative gas chromatograph*—This was constructed in these laboratories (see Fig. 2).

A temperature-controlled oven, A, is fitted with a horizontal injection chamber, B, located inside the oven, with a septum, D, made of silicone rubber, permitting access to the outside. The exit tube, F, is L-shaped and passes through the oven wall, and is bound externally with a heating tape, G, to reduce the thermal gradient. Without this precaution, pesticides tend to collect around the right-angle bend. The lower end of the exit tube is connected to a Luer joint, H, to which a hypodermic needle may be attached. The air temperature of the oven is thermostatically controlled to within $\pm$0·1° C.

Columns, C, are C-shaped and are made from stainless-steel tubing, either having a 0·25-inch or a 0·375-inch outside diameter, and are 2 feet long.

The mobile phase is oxygen-free nitrogen passing at the rate of 100 ml per minute through a copper pre-heating spiral, E, inside the oven before entering the injection chamber perpendicular to the direction of injection. Injection is by means of a 50-$\mu$l capacity Hamilton sheath syringe operating through the silicone-rubber septum, which is made from Esco silicone rubber, $\frac{7}{16}$ inch in diameter and $\frac{1}{16}$ inch thick.




A = Temperature-controlled oven
B = Injection chamber
C = Column
D = Silicone-rubber septum
E = Copper pre-heating spiral
F = Exit tube
G = Heating tape
H = Luer joint

Fig. 2. Semi-preparative gas chromatograph

The column is operated isothermally within the range 150° to 185° C, and is left for at least one hour at any given temperature to reach equilibrium.

PREPARATION OF STATIONARY PHASE AND PACKING OF COLUMNS—

Columns are freshly prepared from glass beads (ballotini) of 0·177-mm diameter, coated with 0·1 per cent. w/w Epikote resin and 0·2 per cent. w/w Apiezon L grease. Before the stationary phase is applied, the beads are given two treatments with hot concentrated hydrochloric acid to remove iron and other impurities. They are then washed with water until free from acid.

Dissolve the stationary phase in warm benzene and pour the solution over the clean, dry beads in a shallow dish. Allow the solvent to evaporate while the beads are continuously stirred. Dry the beads for one hour at 105° C.

Pack the columns with the help of a mechanical vibrator. The narrow-bore tube takes 17 g of prepared beads and the wide-bore tube takes 25 g. Insert a pledget of cotton wool at the injection end of the column and close the exit end with a stainless-steel gauze to prevent the column packing from shifting.

REAGENTS—

All reagents should be of analytical-reagent grade. Further purification of chloroform and dichloromethane is necessary before they are used in infrared work.

*Apiezon L grease.*
*Carbon disulphide.*
*Chloroform, distilled.*
*Epikote resin* 1001.
*Dichloromethane, doubly distilled.*
*Hydrochloric acid, sp.gr.* 1·18.
*Diluted hydrochloric acid,* 0·01 N.
*Diluted ammonia solution* (1 + 20), *v/v.*
*Alumina, acid washed*—Prepare the alumina as specified by Laws and Webley.[1] Wash each batch with diluted hydrochloric acid. Remove the excess of acid by means of diluted ammonia solution, and heat the alumina to remove excess of ammonia and water. Heat the product at 500° C for 2 hours and, after cooling, adjust the activity to Brockmann grade V by adding 15 g of water to 100 g of alumina.
*Glass beads, A.S.T.M. grade* 80.
*Silicone-rubber sheet*—Quality TCL 56, obtainable from Esco (Rubber) Ltd.

PROCEDURE—

Evaporate the eluates from the carbon or alumina columns to a small volume, and transfer it to a 5-ml pear-shaped flask; use small amounts of fresh solvent to wash out the larger flask. Evaporate the solution carefully to dryness by warming the small flask in a beaker suspended in a steam-bath. Remove the last traces of solvent by using a gentle stream of air. Particular care must be exercised with oxydemeton-methyl residues to avoid over-heating, and with all pesticides to minimise mechanical losses during evaporation. Record the approximate weight of the residue obtained. When this is less than 1 mg, direct examination by infrared spectroscopy may be possible but, generally, further purification, by using the semi-preparative gas chromatograph, will be required.

Dissolve the residue in 40 $\mu$l of chloroform, and inject the solution on the column. Repeat this procedure until the whole residue has been transferred to the gas chromatograph.

Collect the column effluent by bubbling the gas stream obtained from the chromatograph for 20 minutes through dichloromethane contained in a 5-ml pear-shaped flask. Evaporate the solution carefully to dryness.

To the dry residue, add an exact 20-$\mu$l portion of the solvent in which the infrared measurements will be made (usually carbon disulphide), taking care that there are no losses by evaporation. By means of a microsyringe, transfer a portion of this solution, sufficient to fill the cavity cell, and examine it in the infrared spectrophotometer; use a beam condenser in the sample beam and a variable-path cell, filled with the solvent, in the reference beam.

Examine the sample in the range 5 to 15 $\mu$, measure the absorption of the major peaks and identify the compound by reference to a standard spectrum.

If an analysis for phosphorus is required, transfer the contents of the flask and cell, with suitable washing, to the wet-combustion flask and continue with the phosphorus determination.

## DISCUSSION OF RESULTS

GAS CHROMATOGRAPHY—

The performance of the semi-preparative gas-chromatographic column was first assessed by using the purest available sample of each pesticide. The percentage recovery varied with the column temperature. Table III shows the highest recovery and the optimum temperature for each pesticide.

CLEAN-UP—

The efficacy of the clean-up was next investigated. Plant extracts were prepared by the general method.[1] The weights of the extracts obtained are recorded in Table I. Pesticides were added to the eluates from the chromatographic columns and, after evaporation of the

solvent, were dissolved in chloroform and injected into the gas chromatograph. The pesticide residues showed little contamination with plant material at temperatures below 185° C.

TABLE III

GAS CHROMATOGRAPHY OF PESTICIDES ON APIEZON - EPIKOTE COLUMNS

| Pesticide | Column temperature, °C | Recovery, per cent. |
|---|---|---|
| Chlorthion | 165 | 90 |
| Dimethoate | 160 | 85 |
| Fenchlorphos | 165 | 85 |
| Fenthion | 160 | 80 |
| Malathion | 165 | 85 |
| Mevinphos | 160 | 85 |
| Oxydemeton-methyl | 175 | 75 |
| Parathion | 157 | 100 |
| Parathion-methyl | 165 | 90 |
| Phenkapton | 170 | 90 |
| Phorate | 165 | 90 |
| Sulphone of the oxygen analogue of phorate | 160 | 70 |
| Phosphamidon | 165 | 100 |
| Schradan | 165 | 95 |
| Thiometon | 165 | 100 |

RECOVERIES—

Recoveries are shown in Table IV. These are similar to the results obtained with pure pesticides; see Table III. The variable results given by oxydemeton-methyl were later found to be attributable to losses occurring at the solvent-evaporation stage.

TABLE IV

GAS-CHROMATOGRAPHIC SEPARATION OF PESTICIDES FROM CROP EXTRACTS

50-g samples of vegetables were extracted in each experiment

| Pesticide | Crop | Amount of pesticides found, μg | Recovery, per cent. |
|---|---|---|---|
| Dimethoate | Apple | 37 | 80 |
| | Lettuce | 26, 28 | 78, 85 |
| | Potato | 57 | 75 |
| Malathion | Apple | 23 | 62 |
| | Carrot | 33 | 65 |
| | Cucumber | 91 | 67 |
| | Lettuce | 24 | 90 |
| Oxydemeton-methy | Apple | 35 | 65 |
| | Carrot | 21 | 30 |
| | Potato | 21 | 75 |
| | Lettuce | 57 | 30 |
| | Swede | 50 | 50 |
| Parathion | Apple | 52 | 70 |
| | Potato | 24 | 92 |

MIXTURES OF PESTICIDES—

Finally, the entire procedure was used. Mixtures of pesticides were added to various fresh crops, which were extracted, and the pesticides separated into the three groups of the general method,[1] before final clean-up on the gas chromatograph. Table V shows the recoveries obtained for each experiment. The figures lie in the range 50 to 80 per cent. We consider this to be satisfactory, taking into account the large number of manipulations involved in the complete procedure.

EXTENSION OF THE METHOD TO LOWER AMOUNTS OF RESIDUE—

At levels of pesticide concentrations below 20 μg (0·4 parts per million on a 50-g sample), even qualitative identification becomes uncertain owing to difficulties inherent in the handling and transference of such small amounts. Losses occur at each stage of the method and are particularly high at the elution stage. Some improvement has been made by using 0·8-cm internal diameter columns in place of the 1·5-cm columns used in the general method. Alternatively, the limit of detection may be lowered by taking larger samples of crops. The results given in Table VI were obtained by adding 20 μg of pesticide to 200-g samples of vegetables. This represents 0·1 p.p.m. of pesticide. For these experiments, the amount of column material in the general method was increased by 50 per cent. and larger volumes

of solvents were used in the extractions; the method was otherwise unchanged. From the results, it can be seen that the residues are free from extraneous compounds. The identification of the pesticides is satisfactory. The quantitative recoveries at the 0·1 p.p.m. level are in the range 40 to 50 per cent., compared with some instances of nil recovery obtained previously on 50-g samples at the 0·2 p.p.m. level by using the standard columns.

TABLE V

RECOVERY OF MIXED PESTICIDES FROM CROP SAMPLES: COMPARISON OF INFRARED AND PHOSPHORUS DETERMINATIONS

50-g samples were used: concentrations were in the range 0·2 to 4·0 p.p.m.

| Experiment number and crop | Pesticide | Amount of added pesticide, μg | Weight of pesticide recovered, μg— by infrared measurement | Weight of pesticide recovered, μg— by phosphorus determination |
|---|---|---|---|---|
| 1. Apple .. .. | Dimethoate | 48, 24 | 25, 18 | 25, 21 |
| | Malathion | 101, — | 80, — | 67, — |
| | Parathion | 81, 41 | 58, 25 | 61, 17 |
| 2. Apple .. .. | Malathion | 50, 10 | 36, 6 | 24, 6 |
| | Oxydemeton-methyl | 63, 13 | 36, Nil | 34, 6 |
| | Phenkapton | 198, 99, 40 | 140, 60, 25 | 145, 60, 30 |
| 3. Apple .. .. | Phosphamidon | 53, 26 | 38, 19 | — |
| 4. Carrot .. .. | Dimethoate | 48, 24 | 44, 22 | 39, 19 |
| | Malathion | 51, 26 | 26, 15 | 24, 19 |
| | Parathion | 49, 25 | 40, 19 | 24, 28 |
| 5. Cauliflower .. | Oxydemeton-methyl | 56, 28 | 31, 15 | — |
| 6. Cherry .. .. | Malathion | 51, 26 | 37, 16 | — |
| 7. Cucumber .. | Dimethoate | 49, 24 | 43, 18 | 29, 14 |
| | Malathion | 51, 26 | 26, 15 | 24, 19 |
| | Parathion | 49, 24 | 47, 20 | 38, 38 |
| 8. Gooseberry .. | Dimethoate | 49, 25 | 22, 12 | 27, 15 |
| | Malathion | 51, 26 | 38, 21 | 35, 17 |
| 9. Lettuce .. .. | Oxydemeton-methyl | 50, 25 | 31, 12 | 36, 18 |
| 10. Lettuce .. .. | Dimethoate | 49, 25 | 27, 21 | 22, 23 |
| | Malathion | 51, 26 | 25, 16 | 30, 17 |
| | Parathion | 49, 25 | 35, 18 | — |
| 11. Mushroom .. | Malathion | 51, 26 | 30, 17 | — |
| 12. Potato .. .. | Oxydemeton-methyl | 50, 25 | 36, 18 | 14, 11 |
| 13. Potato .. .. | Dimethoate | 49, 25 | 42, 14 | 39, 15 |
| 14. Tomato .. .. | Dimethoate | 49, 25 | 33, 17 | 37, 22 |
| | Parathion | 48, 24 | 28, 15 | — |

TABLE VI

RECOVERY OF MIXED PESTICIDES FROM CROP SAMPLES AT THE 0·1 P.P.M. LEVEL

Comparison of infrared and phosphorus determinations on 200-g samples

| Crop | Pesticide | Amount of added pesticide, μg | Weight of pesticide recovered, μg— by infrared measurement | Weight of pesticide recovered, μg— by phosphorus determination |
|---|---|---|---|---|
| Cabbage .. .. | Dimethoate *plus* parathion | 15<br>17 | 8<br>7 | 8<br>9 |
| Cabbage .. .. | — | — | — | 0·3 |
| Carrot .. .. .. | Oxydemeton-methyl *plus* phenkapton | 18<br>20 | 10<br>18 | 8<br>11 |

QUALITATIVE IDENTIFICATION AND QUANTITATIVE DETERMINATION BY THE INFRARED METHOD—

The principal infrared structural correlations that have been used in these experiments are given in Table VII. Twenty organo-phosphorus compounds have been examined, and the wave-number assignments fall within the ranges given by Bellamy.[10] Not all of these absorption bands are suitable for quantitative measurements; some are too weak at low solute concentrations, but they may nevertheless be of value in the identification of certain groups. A factor that must always be borne in mind is that some spectral-absorption bands may be unreliable, owing to the presence of impurities.

The Infracord spectrophotometer gives transmittance values that are reproducible to within $\pm 1$ per cent., but, in practice, difficulties in the manipulation of small amounts limit the reproducibility to $\pm 5$ per cent. Throughout the range 5 to 50 $\mu$g, the absorptions follow Beer - Lambert chracteristics.

### Comparison of infrared and phosphorus methods—

The aim of the present work was the qualitative identication of pesticide residues, but, in many instances, quantitative infrared measurements have been tolerably close to parallel phosphorus determinations. Pesticide recoveries calculated from the phosphorus figures are generally lower than those found by infrared measurement, because of losses in transference from the infrared cell to the wet-combustion flask. On the other hand, some higher results were obtained by the chemical method, and these were attributed to the accumulation of decomposition products from previous analyses on the gas-chromatographic column. In consequence, eluates obtained directly from the columns used in the general method are preferred to fractions collected from the gas chromatograph for the phosphorus determinations.

Table VII

Infrared wavelengths and structural correlations for organo-phosphorus pesticides

| Structural group | Wavelength of measurement, $\mu$ | Strength of absorption |
|---|---|---|
| * P=O (free) | 7·9 | Moderate |
| * P—O—C (aromatic) | 8·1 | Moderate |
| * P—O—C (aromatic) | 10·7 | Strong |
| * P—O—C (aliphatic) | 9·7 to 9·8 | Strong |
| P—O—Ethyl | 8·6 | Weak |
| P—O—Methyl | 8·4 | Weak |
| * P=S | 12 to 13 | Moderate |
| * C—$NO_2$ (aromatic) | 7·4 | Strong |
| C=O | 5·7 to 5·9 | Strong |

* Suitable for quantitative measurements.

## Conclusions

The method has been tested with a selection of the most commonly used organo-phosphorus pesticides on a wide range of fruits and vegetables. The residual pesticides have been satisfactorily identified and the amount present evaluated down to the 0·1 p.p.m. level. This is below the useful detection limit required in residue analysis. In principle, the method should be applicable to other pesticides, including the chlorinated hydrocarbons, and also to animal products. Some organo-phosphorus pesticides are not amenable to the gas-chromatographic treatment before infrared examination. This may be because of their non-volatile character or because they are subject to thermal decomposition in the chromatograph. Examples of such pesticides are azinphos, diazinon, morphothion and trichlorphon. The use of gas chromatography under reduced pressure might solve this problem.

It seems to us that future research in the field of the toxic phosphorus esters is likely to centre around the isolation and identification of pesticide metabolites. The infrared method appears to offer a valuable approach to the solution of this problem.

In thanking the Government Chemist for permission to publish this Paper, we also thank our present and former colleagues who have taken part in these researches.

## References

1. Laws, E. Q., and Webley, D. J., *Analyst*, 1961, **86**, 249.
2. Joint Demeton-methyl Residues Panel, *Ibid.*, 1962, **87**, 485.
3. Goodwin, E. S., Goulden, R., and Reynolds, J. G., *Ibid.*, 1961, **86**, 697.
4. Egan, H., Hammond, E. W., and Thomson, J., *Ibid.*, 1964, **89**, 175.
5. Goulden, R., Goodwin, E. S., and Davies, L., *Ibid.*, 1963, **88**, 941.
6. McCaulley, D. F., and Cook, J. W., *J. Ass. Off. Agric. Chem.*, 1960, **43**, 3.
7. Jones, L. R., and Riddick, J. A., *Anal. Chem.*, 1952, **24**, 569.
8. Beckman, H., and Bevenue, A., *J. Chromatography*, 1963, **10**, 231.
9. Caverly, D., and Hall, P. S., *Analyst*, 1961, **86**, 478.
10. Bellamy, L. J., "The Infra-red Spectra of Complex Molecules," Methuen & Co. Ltd., London, 1958, p. 311.

Received *October 23rd*, 1963

# The Application of Paper and Thin-layer Chromatography to the Identification of Thyroxine in a Feeding-stuffs Additive

BY STELLA J. PATTERSON AND R. L. CLEMENTS

(*Department of Scientific and Industrial Research, Laboratory of the Government Chemist, Cornwall House, Stamford Street, London, S.E.*1)

A method is described for identifying and estimating thyroxine derived from an iodinated protein component of a feeding-stuffs additive. Procedures under investigation for the chemical assay of the pharmacologically active constituents of thyroid are applied and extended to include chromatography on starch-bound cellulose thin-layer plates. A confirmatory test for iodo compounds is also described.

IN the course of the analysis of a feeding-stuffs additive, recommended for the stimulation of milk production in cows, consisting mainly of protein, carbohydrate and fat, some free and organically bound iodine was found, indicating the possible presence of an iodinated protein. It was therefore decided to examine the sample by using the techniques for detecting thyroidal iodo compounds that are at present under investigation in this Laboratory in connection with our membership of Panel 7 of the Joint Committee of the Pharmaceutical Society and the Society for Analytical Chemistry on Methods of Assay of Crude Drugs.[1] There are also many references in the literature to the presence of biologically active material, identified as thyroxine, in proteins that have been iodinated under carefully controlled conditions.[2,3,4,5]

## EXPERIMENTAL

Both alkaline and enzymic hydrolyses were used to liberate the thyroxine from the protein material; the former method is described in detail because it is rapid and requires no special reagents.

The chromatographic separation of thyroxine from other iodo compounds in the t-amyl alcohol - ammonia solution solvent system described by Barker[6] has been improved by additional precautions that ensure an ammonia-saturated atmosphere. (Thus Barker gives $R_F$ of iodide = 0·20 and $R_F$ of thyroxine = 0·23; typical values obtained by the proposed procedure are: $R_F$ of iodide = 0·25 and $R_F$ of thyroxine = 0·45.)

In our modification of Gmelin and Virtanen's[7] FFCA (ferric chloride, potassium ferricyanide and arsenous acid mixture) spraying procedure for detecting iodo compounds on paper chromatograms, the paper chromatogram was impregnated with silver nitrate before colour development with the FFCA reagent. By this means a thyroxine spot derived from the sample was clearly visible, and reliable estimation against standards was possible.

Chromatography on starch-bound cellulose thin-layer plates is also described. A tough film, formed on the plates by using starch as a binder, withstands the water-washing that is essential after FFCA has been used for developing the spots, and uniform spraying of the plates is readily achieved. The spots are compact, alignment good and the time required for applying the spots and running the chromatogram is considerably reduced. Chromatography on thin-layer plates in alternative solvents of widely differing pH values was used to confirm that thyroxine had been correctly identified. These solvents are not ideal for the separation of thyroxine, and the thin-layer plates were brought into use after attempts to run similar chromatograms on paper had been unsuccessful.

Thyroxine was also detected in a sample of commercial iodinated casein when the sample was examined by the same procedure as that used for the feeding stuff.

## METHOD

### Hydrolysis and extraction with butanol

Special apparatus—

*Tubes*—Thick-walled test-tubes, *e.g.*, Pyrex extra-heavy-walled tubing, about 35 cm long × 11 mm internal diameter should be used.

Reagents—

*Sodium hydroxide*, 2 N and N.

*n-Butanol*—Use analytical-reagent grade material saturated with water.

Procedure—

Add 10 ml of 2 N sodium hydroxide to 0·25 g of the sample contained in a glass tube. De-gas the solution and seal off the tube under reduced pressure to a length of about 28 cm. Heat the tube in an oven at 105° to 110° C for 18 hours, shaking the tube gently every half hour for the first two hours to obtain a uniform mixture, and then allow the tube to cool to room temperature.

Open the tube, warm it to 50° C and filter the contents through a Whatman No. 541 filter-paper. Wash the tube and filter-paper with 20 ml of warm water. Adjust the pH of the filtrate to 3·0 with dilute sulphuric acid, by means of a pH meter, and extract it with 30-, 25-, 25- and 20-ml portions of n-butanol. Wash the combined butanolic extracts with 25 ml of 2 N sodium hydroxide and then 20 ml of N sodium hydroxide. Evaporate the washed butanolic extracts to dryness at 40° C under reduced pressure.

### Chromatography

Reagents—

*Methanol - ammonia solution mixture*—Take 5 ml of ammonia solution, sp.gr. 0·88, and dilute it to 100 ml with methanol.

*t-Amyl alcohol - ammonia solution solvent system*—Shake thoroughly a mixture of 100 ml of t-amyl alcohol, 80 ml of water and 20 ml of ammonia solution, sp.gr. 0·88, and set the mixture aside until the two phases separate.

*Ferric chloride solution*—Prepare a 2·7 per cent. solution of analytical-reagent grade ferric chloride, $FeCl_3.6H_2O$, in 2 N hydrochloric acid.

*Potassium ferricyanide solution*—Prepare a 3·5 per cent. w/v aqueous solution of analytical-reagent grade potassium ferricyanide, $K_3[Fe(CN)_6]$.

*Arsenous acid solution*—Warm 3·8 g of analytical-reagent grade arsenous oxide with 25 ml of 2 N sodium hydroxide until it dissolves. Cool the solution to approximately 5° C and add 50 ml of 2 N sulphuric acid that has been previously cooled to approximately 5° C. Dilute the mixture to 100 ml with water.

*FFCA reagent*—Mix portions of the above solutions of ferric chloride, potassium ferricyanide and arsenous acid in the ratio 5 + 5 + 1, immediately before use.

*Silver nitrate, solution in acetone*—Dissolve 0·2 g of silver nitrate in 5 ml of water and dilute the solution to 100 ml with analytical-reagent grade acetone. This solution must be freshly prepared before use.

*Standard thyroxine solution*—Prepare a solution that contains 0·1 mg of thyroxine (sodium salt) per ml of methanol - ammonia solution mixture.

*Standard solution of iodo compounds*—Prepare a solution containing 0·1 mg of each of potassium iodide, and of the sodium salts of 3,5-di-iodotyrosine, 3,5-di-iodothyronine, 3,5,3′-tri-iodothyronine and thyroxine per ml of methanol - ammonia solution mixture.

*Cellulose powder*—Cellulosepulver 300. (Obtainable from Macherey Nagel and Co.)

*Starch solution*—Prepare a 0·8 per cent. w/v aqueous solution of soluble starch.

Paper chromatography—

*Apparatus*—A chromatographic tank, just large enough to accommodate the chromatograms when they are suspended from a central trough in a stabilised atmosphere of ammonia, should be used. The atmosphere may be obtained by suspending two sheets of Whatman No. 3 chromatographic paper of the same width as the chromatograms (15 × 7½ inches) on either side of the central trough. These sheets should be saturated with the aqueous phase of the solvent system and should dip into an excess of the solution in the bottom of the tank.

*Procedure*—Dissolve the residue obtained from the evaporated butanolic extracts in 10 ml of methanol - ammonia solution mixture. Transfer suitable amounts of this solution to a Whatman No. 1 chromatographic paper by using a graduated micro-pipette. Place the solutions alongside the standards and allow each spot to dry before subsequent overspotting. (To obtain a suitable range for visual comparison, 32 $\mu$l of the sample solution and between 4 and 16 $\mu$l of the standard solution should be applied in 4-$\mu$l portions.) Spot the paper along a line drawn 2 inches from one end.

Position the prepared chromatogram in the central trough and allow it to hang in the ammonia atmosphere for 1 hour before adding the organic phase of the solvent to the trough, and then run the chromatogram by the descending-solvent technique for 16 to 17 hours.

*Impregnation with silver nitrate and colour development*—After the run is complete, dry the chromatogram at room temperature. Rapidly pass it through the silver nitrate solution and when it is dry, pass it through the FFCA reagent, remove it and hold it in the horizontal plane for 1 to 2 minutes until the standards achieve a suitable gradation, and then wash it thoroughly with water to remove any excess of reagent.

THIN-LAYER CHROMATOGRAPHY—

*Preparation of plates*—Stir 7·5 g of cellulose powder and 20 ml of freshly prepared 0·8 per cent. w/v starch solution together in a beaker until all the powder is wetted. Slowly add a further 30 ml of starch solution, stirring continuously, and then transfer it to a mortar and mix it well for 1 minute. Immediately spread the mixture on plates to a thickness of 0·25 mm, allow them to dry and heat them in an oven at 100° to 105° C for 1 hour. Before spotting takes place, wash a plate by elution in the solvent tank until the solvent has risen about 6 inches, and allow the plate to dry.

*Procedure*—Apply the sample and standard solutions to the washed portion of the plate. (The most suitable amounts for convenient visual examination were found to be 1 and 2 $\mu$l of standard and 4 $\mu$l of sample solution.) Allow the chromatogram to run in the ammonia-saturated tank (as described under "Paper Chromatography," p. 329) until the solvent front has risen about 4 inches above the starting line (3 to 4 hours) and then dry it. Spray the plate with FFCA reagent and, after suitable development of the spots, wash it well with water.

## ADDITIONAL CHROMATOGRAPHIC EVIDENCE FOR THE PRESENCE OF THYROXINE

To confirm that the material considered to be thyroxine had been correctly identified, thin-layer chromatograms were obtained by running a fraction previously eluted from a paper chromatogram that contained the supposed thyroxine, alongside, and in admixture with, a thyroxine marker, with the t-amyl alcohol - ammonia solution solvent, and also the n-butanol - acetic acid and methanol - ammonium acetate solvent systems described by Barker.[6]

In another experiment, a known amount of thyroxine was initially added to some iodinated casein and chromatographic estimation of the thyroxine by the proposed method gave a satisfactory recovery.

Confirmation that the material responsible for the formation of Prussian blue with the FFCA reagent was an iodinated compound, acting as a cyclic chemical catalyst, of the type indicated by Gmelin and Virtanen,[7] was demonstrated by the considerable reduction in intensity of the spots when the reagent was used without the arsenous acid.

## DISCUSSION AND RESULTS

On paper chromatograms the $R_F$ values for thyroxine derived from hydrolysed samples are lower than those of the thyroxine standards; the retardation is greater after alkaline hydrolysis. This unwanted effect is much reduced on thin-layer chromatograms, in which instances a smaller amount of sample is required. Similarities between the chromatographic patterns obtained for the feeding stuff and the iodinated casein after hydrolysis and extraction by the same procedure are discernible in Fig. 1. To illustrate the efficiency of the chromatographic separation, a standard solution of iodo compounds at different levels is shown in Fig. 1, but only the standard thyroxine solution is essential for the estimation of thyroxine in the sample.

The thyroxine content of the feeding stuff, assessed by visual comparison of the sample spots with standards, is of the order of 0·1 per cent. Similar results were obtained after both alkali hydrolysis as described above and enzymic hydrolysis at pH 8·5 as described by Devlin and Stevenson.[8]

We thank the Government Chemist for permission to publish this Paper, and Mr. R. E. A. Drey, a member of Panel 7,[1] whose combination of published conditions for the alkaline hydrolysis and extraction of thyroid tissue with n-butanol were used in this work.

## References

1. "Report of the Analytical Methods Committee," *Analyst*, 1963, **88**, 659.
2. Ludwig, W., and von Mutzenbecher, P., *Z. physiol. Chem.*, 1939, **258**, 195.
3. Harington, C. R., and Pitt-Rivers, R. V., *Nature*, 1939, **144**, 205.
4. Reineke, E. P., and Turner, C. W., *J. Biol. Chem.*, 1943, **149**, 555.
5. Roche, J., Michel, R., and Lafon, M., *Ann. Pharm. Franç.*, 1947, **5**, 337.
6. Barker, S. B., "Methods in Hormone Research," Academic Press Inc., New York and London, 1962, Volume I, p. 366.
7. Gmelin, R., and Virtanen, A. I., *Acta Chem. Scand.*, 1959, **13**, 1469.
8. Devlin, W. F., and Stevenson, N. R., *J. Pharm. Pharmacol.*, 1962, **14**, 597.

Received *November 20th*, 1963

# The Identification of Offals in Sausages

By N. H. HOLE,

(*Central Veterinary Laboratory, Ministry of Agriculture, Fisheries and Food*)

MERIEL PARRY ROBERTS* AND K. BRYCE JONES

(*Food Science and Plant Health Division, Ministry of Agriculture, Fisheries and Food, Great Westminster House, Horseferry Road, London, S.W.*1)

Histological methods have been applied to the detection of comminuted offals in sausages. It has been shown that udder, raw tripe and stomach are identifiable in fresh material. Photomicrographs of characteristic fragments of tripe, udder and stomach are given.

THE Offals in Meat Products Order, S.I. No. 246, 1953, prohibits the use of certain offals in uncooked open meat products. The availability of colloid-mills for reducing animal tissues to a smooth paste, a process sometimes referred to as emulsification, has given rise to the problem of detecting the illegal inclusion of certain offals that might be treated in this manner before incorporation into meat goods such as sausages. The suggestion was made that histological examination might prove to be a practical means of testing sausage meat for the presence of prohibited offals, and the assistance of the Central Veterinary Laboratory, Animal Health Division, was sought for the supervision and organisation of the technical aspects of such an investigation.

Even when it is reduced to a paste, the animal tissue in sausage meat is readily differentiated by histological means from the vegetable matter, but the identification of specific tissues may be affected by the degree of comminution. Skeletal muscle, the normal "meat" of ordinary sausages, is almost wholly made up of one type of cell that is recognisable in minute fragments, but other tissues or organs may be identifiable by some structural arrangement or by the association of certain cell types. While it is unlikely that any degree of commercial comminution will destroy individual cells, it is possible that colloid-mill processing may disintegrate composite tissues sufficiently to make histological recognition difficult.

Besides mechanical disintegration, other treatments, such as prolonged pre-cooking, might be expected to affect histological structure. It would appear, however, that the degree of heating applied to pre-cooked German sausages does not prevent the histological recognition of the meat constituents used. Kelch[1] has demonstrated the value of histological examination for determining the nature of the meat content of several types of German sausage, and the numerous illustrations in the series of plates show readily identifiable rind, paunch, lung, kidney, pancreas, stomach, lymph node, intestine, thymus and parotid gland in specimens prepared from sausages that in the course of preparation, had been "made tender" by steam-cooking, later heated to 70° C for 1 hour and finally smoked. The materials examined by Kelch had either been put through the 2-mm disc of a Wolf cutter several times (*i.e.*, fairly fine mincing) or treated in a Puck colloid-mill. The latter is a steel-bladed type of mill, whereas a stone-cone type was used in the work now reported.

Linke[2] has also used histological methods in his examinations of the meat content of canned sausages and luncheon meats; he found 20 per cent. of the samples examined to contain heart, lung and gastro-intestinal tract tissue.

This report is confined to observations made on raw sausage meat covered by the Offals in Meat Products Order, the primary object being to determine whether simple histological and staining methods could have a practical application to the routine examination of sausage meat for the presence of prohibited offals. The effect on structure of different degree of comminution of different tissues and the influence of decomposition have been assessed; the findings with selected tissues have been applied to sausage-meat preparations to which known amounts of offals have been added and mixed.

* Seconded to the Central Veterinary Laboratory, Weybridge.

## Experimental

### Techniques used—

Materials were examined after they had been minced in a bowl-cutter, and also after processing in a colloid-mill to varying degrees of comminution.

Trials were made both with frozen sections and with sections cut after the tissue had been embedded in paraffin wax. Although more laborious, the latter method was found to give more consistent and satisfactory results than the former for material of the fragmented nature of sausage meat. Embedding the sample in paraffin wax was therefore adopted as the standard procedure. Specimens were fixed for at least 48 hours in 10 per cent. formalin (4 per cent. formaldehyde) in normal saline solution, and then dehydrated in the usual alcohols, cleared in chloroform, and embedded in wax. Sections, cut 5 to 10 microns thick, were stained with haematoxylin and eosin, or by a picro-Masson trichrome method. Several different staining techniques were tried, but, of the simpler methods, haematoxylin and eosin staining was found to demonstrate good histological detail, whereas Masson trichrome methods applied to some tissues give a greater optical contrast, in that connective tissues are selectively stained blue. The conjunction of connective tissue and of epithelium, which may be demonstrated in this way, assists in the identification of tripe. (Connective tissue is also present in the supporting structure in normal muscle and in the walls of blood vessels.)

Details of these embedding and staining methods are given in any standard book on histological techniques.

### Materials examined—

Specimens of uterus, stomach, tripe, intestine, udder, spleen, lungs and thymus were obtained fresh, and sections prepared for histological examination after mincing and colloid-mill treatments.

Fifty-four samples of experimental pork sausage meat were prepared having a nominal meat content of 65 per cent. The meat comprised lean muscle, or in some instances lean muscle *plus* from 5 to 25 per cent. of some offal type of tissue, to simulate the illegal use of offals in sausage manufacture. These experimental materials were either minced in a bowl-cutter or processed in a colloid-mill in the course of preparation. Details of content and preparation are given in Table II, p. 336. Examinations were carried out under a code number that did not reveal the nature of the meat content. Two or three samples about the size of a hazel nut were cut at random from each specimen for embedding, and several sections taken at different levels were stained for examination.

## Results

### Illustrations—

The illustrations to this Paper have been deliberately chosen at random from photographs of sections prepared in the normal routine manner described; they have not been specially selected for clarity or perfection, the intention being to demonstrate the results that might be expected in routine examination, rather than those that would be obtained by specialised techniques and search.

The investigation has demonstrated that the histological recognition of several raw offals is not seriously affected by modern commercial methods of comminution of sausage meats, including treatment in colloid-mills, and that it is possible to detect the presence of 5 per cent. of certain raw offals added as an adulterant to sausage-meat even after treatment in a colloid-mill at number 10 setting.

These findings relate to raw sausage meats examined in a fresh state or after preservation by deep-freezing.

### Effect of the degree of comminution—

It was found that the effect of colloid-mill treatment varied considerably with the nature of the tissue. No difficulty has been experienced in identifying normal "meat" (skeletal muscle) submitted to the more severe processing, because of its simplicity of structure, and firm tissues such as uterus, udder and tripe, which all tend to be tough and resistant to fragmentation, have also been found to be scarcely affected. With such tissues, we formed the impression that comminution tends to be 2-dimensional only, and that strands of tissues

are liable to appear in the final product. Softer tissues, such as normal lungs, tend to become compressed into a solid cellular mass, and extremely soft and pulpy tissues like spleen and thymus suffer a loss of structure sufficient to interfere with their specific identification, although the presence of cells of a general lymphoid nature is readily detected. Comminution does not necessarily affect all the components of a complex tissue equally. For example, in the instance of stomach or intestine, the epithelial cells of the mucous membrane, the type and arrangement of which are one key to identification, are fairly easily stripped from the supporting basal tissues and are liable to be broken up in processing. Such types of tissue are therefore less easily identified after the samples have been more finely comminuted.

The histological structure of some tissues, particularly those concerned with reproduction, varies considerably at the different stages of physical development; for example, pre-lactating, lactating and post-lactating udder tissues have a markedly different microscopical appearance. Another point of importance is that the histology of a minute fragment of an organ such as the stomach will depend on the site of the origin of the fragment; the identification of stomach depends largely on the recognition of the type of epithelium, and this varies in different regions of the stomach. The oesophageal zone is lined with stratified squamous epithelium, not easily differentiated from tripe, whereas in the cardiac and fundic areas the epithelium is glandular and has a completely different appearance.

Table I shows the results obtained with a series of comminuted offal tissues.

TABLE I

THE EFFECT OF COMMINUTION ON RECOGNISABLE HISTOLOGICAL STRUCTURE

| | Treatment | | | | | | |
|---|---|---|---|---|---|---|---|
| | | Colloid-mill grading | | | | | |
| Tissue | Minced | 50 | 40 | 30 | 20 | 10 | 5 |
| Uterus .. .. | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| Stomach .. .. | +++ | +++ | +++ | +++ | +++ | ++ | ++ |
| Tripe .. .. .. | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| Intestine .. .. | +++ | +++ | +++ | +++ | ++ | ++ | — |
| Udder .. .. .. | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| Spleen .. .. .. | ++ | ++ | ++ | ++ | + | + | + |
| Lungs .. .. .. | +++ | +++ | +++ | +++ | ++ | ++ | ++ |
| Thymus .. .. | ++ | + | + | + | + | + | + |

+++ = recognisable. ++ = barely recognisable. + = not definitely recognisable.

IMPORTANCE OF THE STATE OF PRESERVATION—

Freshly taken non-decomposed animal tissue is obviously the material of choice for histological study. Cellular change and gradual breakdown set in immediately after death, and although the early changes are not sufficient to prevent the histological recognition of a tissue, the longer the interval between death and the immobilisation of these changes by low temperature or some fixation procedure the less recognisable fragments of tissue become. Freshly prepared minced or milled material is quite satisfactory, provided that the tissues

Fig. 1. Fragment of tripe epithelium on the left, and fragments of normal meat (muscle) amongst vegetable material. Haematoxylin and eosin. Magnification, × 110

Fig. 2. Two large fragments of tripe epithelium mid-right, with normal muscle on the left. Masson trichrome. Magnification, × 110

Fig. 3. Fragment of tripe epithelium still attached to basal connective tissue (blue). Masson trichrome. Magnification, × 110

Fig. 4. Enlargement of a fragment of tripe epithelium showing the typical squamous-type cells and chitinous outer surface. Haematoxylin and eosin. Magnification, × 480

Fig. 5. Fragment of bleached tripe in the centre amongst normal muscle and vegetable matter. Haematoxylin and eosin. Magnification, × 110

Fig. 6. Enlargement of Fig. 5, showing the dislocation of normal structure after bleaching; compare with Fig. 4. Haematoxylin and eosin. Magnification, × 480

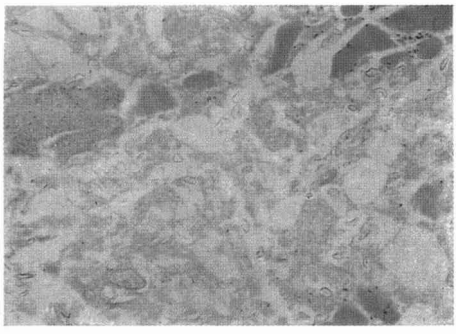


Fig. 1

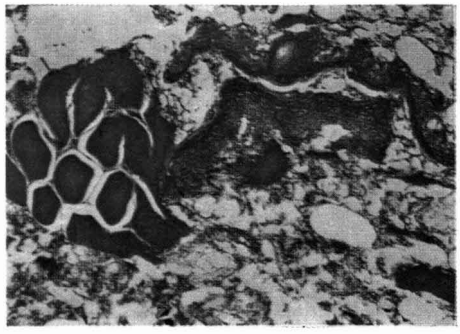


Fig. 2

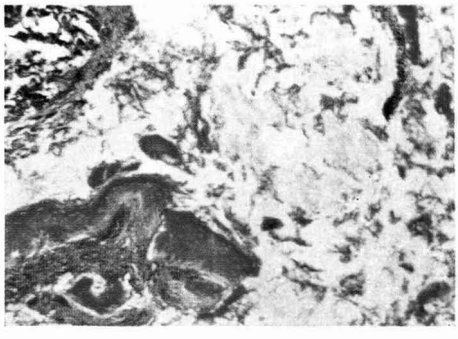


Fig. 3

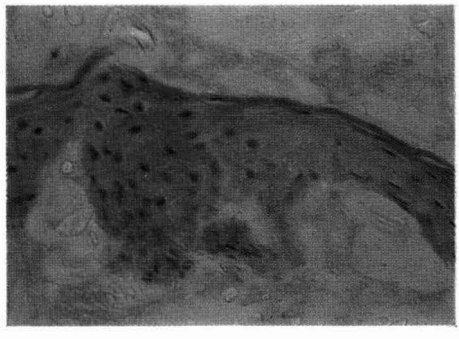


Fig. 4

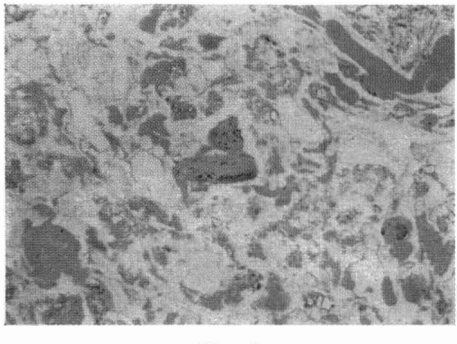


Fig. 5

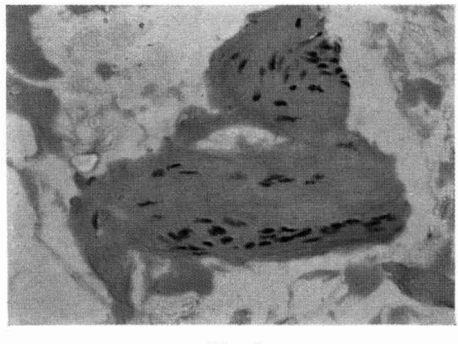


Fig. 6

[To face page 334

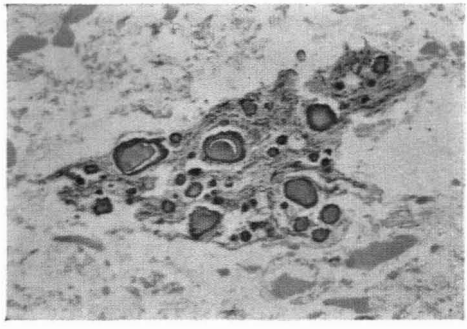


Fig. 7

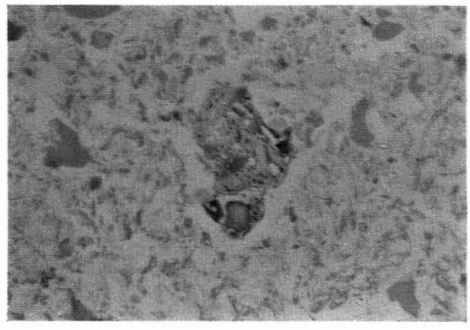


Fig. 8

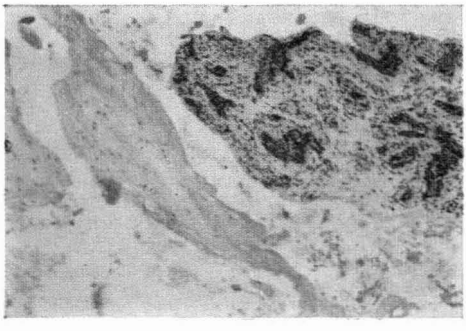


Fig. 9

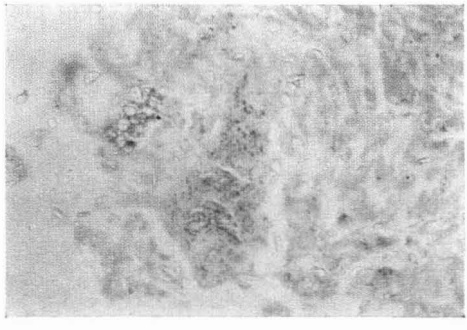


Fig. 10

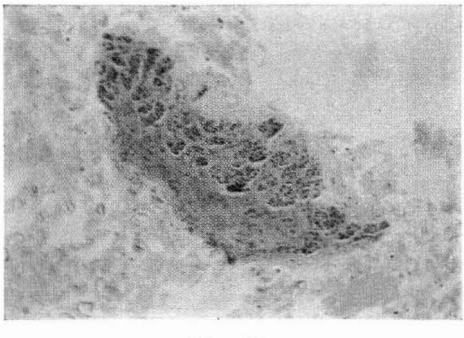


Fig. 11

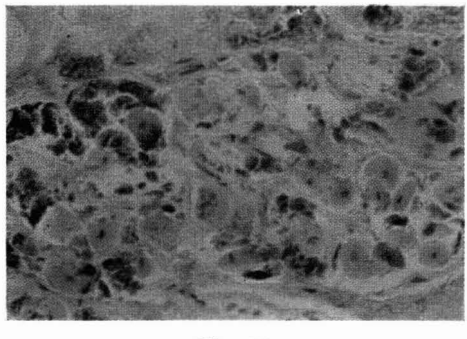


Fig. 12

used were themselves reasonably fresh or kept in the cold, but comminution tends to speed up autolytic change, and once prepared the material must immediately be put in cold storage unless it is wanted for examination on the spot. Decomposition results in a loss of staining affinities and recognisable structure, and the softer the tissue the quicker the process. Experimentally minced lung kept at room temperature became unrecognisable in two or three days, whereas samples of the same material kept in deep-freeze for 16 weeks were still readily identifiable.

EXAMINATION OF EXPERIMENTAL SAUSAGE MEATS—

The control and the adulterated specimens listed in Table II were examined by histological methods without knowledge of the content or the type of offal likely to be present. The histological findings are shown in the last column. Correlation of the factual additions and the histological reports can be summarised as under—

(*i*) All but one of the 16 samples that contained an addition of udder tissue were identified.

(*ii*) Ten of the samples contained an addition of raw tripe, and all 10 were recognised.

(*iii*) Ten of the samples contained pig's stomach; this was recognised in 6 of the samples and suspected in another 2. The failures proved to be the samples subjected to the finest colloid-mill treatment used.

(*iv*) Six of the samples contained an addition of bleached tripe. This was inadvertent, and in fact contrary to the conditions of the investigation, which emphasised the use of raw materials. The presence of some adulterant was recognised in all, but not identified as tripe.

(*v*) The remaining 12 samples were submitted as normal unadulterated sausage meat. No animal tissue other than muscle was detected in 10 samples, one was reported as suspected of containing some adulterant, and one was found to contain udder, a finding subsequently confirmed.

The failure to identify bleached tripe is attributable in part to the fact that search was being made for raw tissues and the bleaching process had sufficiently affected the histological structure to make its positive recognition as a raw tissue uncertain. The knowledge obtained of the histological appearance of bleached tripe would permit its ready identification in a further series of similar samples.

Udder was identified in sample 14, which had been designated as a control. Subsequent enquiries showed that sow's flank had been used in making up the test material. Either all the mammary tissue was not trimmed from the flank before use or there was some confusion in coding.

It must not be imagined that in positive material numerous fragments of adulterant are present in every microscope field or even in every section prepared. Usually only one or two recognisable pieces are seen per section, and in some instances more than one section has to be examined before definite evidence is obtained. The more finely the offal is divided the more widely it is likely to be dispersed, and the more frequently will fragments occur in any sections examined.

Fig. 7. Fragment of lactating udder with inspissated milk in the alveoli. Haematoxylin and eosin. Magnification, × 110

Fig. 8. Another fragment of udder tissue at a different stage of lactation. Haematoxylin and eosin. Magnification, × 110

Fig. 9. Fragment of non-lactating udder tissue. Haematoxylin and eosin. Magnification, × 110

Fig. 10. Fragment of the glandular mucous membrane of the stomach in the centre. Haematoxylin and eosin. Magnification, × 110

Fig. 11. Another fragment of glandular stomach tissue showing a different type of gland cell. Haematoxylin and eosin. Magnification, × 110

Fig. 12. Enlargement of Fig. 11, showing the characteristic acidophile parietal cells. Haematoxylin and eosin. Magnification, × 480

TABLE II

EXPERIMENTAL SAUSAGE-MEATS EXAMINED

| Sample number | Offal included as percentage of total meat | Comminuting method and colloid-mill plate setting | Histological findings |
|---|---|---|---|
| 1 | None | Bowl-cutter | No offal |
| 2 | None | Colloid-mill 50 | No offal |
| 3 | None | Colloid-mill 12 | No offal |
| 4 | Bleached tripe (5%) | Bowl-cutter | Unspecified adulterant |
| 5 | Bleached tripe (5%) | Colloid-mill 50 | |
| 6 | Bleached tripe (5%) | Colloid-mill 12 | |
| 7 | Bleached tripe (25%) | Bowl-cutter | |
| 8 | Bleached tripe (25%) | Colloid-mill 50 | |
| 9 | Bleached tripe (25%) | Colloid-mill 12 | |
| 10 | Udder (5%) | Colloid-mill 50 | Udder |
| 11 | Udder (5%) | Colloid-mill 12 | Udder |
| 12 | Udder (25%) | Colloid-mill 12 | Udder |
| 13 | Udder (5%) | Bowl-cutter | Udder |
| 14 | None | Bowl-cutter | Udder |
| 15 | None | Colloid-mill 50 | No offal |
| 16 | Udder (25%) | Bowl-cutter | Udder |
| 17 | Udder (25%) | Colloid-mill 50 | Udder |
| 18 | None | Colloid-mill 12 | No offal |
| 19 | Raw tripe (7%) | Colloid-mill 50 | Tripe |
| 20 | Raw tripe (7%) | Colloid-mill 50 | Tripe |
| 21 | Udder (7%) | Colloid-mill 50 | Udder |
| 22 | Stomach (7%) | Colloid-mill 50 | Stomach |
| 23 | Udder (7%) | Colloid-mill 10 | Udder |
| 24 | Stomach (7%) | Colloid-mill 10 | Suspected stomach |
| 25 | None | — | No offal |
| 26 | Tripe (7%) | Colloid-mill 10 | Tripe |
| 27 | None | — | No offal |
| 28 | Udder (7%) | Colloid-mill 10 | Udder |
| 29 | Stomach (7%) | Colloid-mill 50 | Stomach |
| 30 | Udder (7%) | Colloid-mill 50 | Udder |
| 31 | Udder (7%) | Colloid-mill 10 | Udder |
| 32 | Stomach (7%) | Colloid-mill 10 | Stomach |
| 33 | Tripe (7%) | Colloid-mill 50 | Tripe |
| 34 | None | — | No offal |
| 35 | Tripe (7%) | Colloid-mill 10 | Tripe |
| 36 | Stomach (7%) | Colloid-mill 10 | Suspected stomach |
| 37 | Udder (7%) | Colloid-mill 10 | Udder |
| 38 | Stomach (7%) | Colloid-mill 50 | Stomach |
| 39 | None | — | Suspected adulterant |
| 40 | Stomach (7%) | Colloid-mill 10 | No offal |
| 41 | Tripe (7%) | Colloid-mill 50 | Tripe |
| 42 | Udder (7%) | Colloid-mill 50 | Udder |
| 43 | Udder (7%) | Colloid-mill 50 | Udder |
| 44 | Udder (7%) | Colloid-mill 10 | Suspected stomach |
| 45 | Tripe (7%) | Colloid-mill 50 | Tripe |
| 46 | Stomach (7%) | Colloid-mill 50 | Stomach |
| 47 | None | — | No offal |
| 48 | Tripe (7%) | Colloid-mill 10 | Tripe |
| 49 | Udder (7%) | Colloid-mill 50 | Udder |
| 50 | Tripe (7%) | Colloid-mill 10 | Tripe |
| 51 | Stomach (7%) | Colloid-mill 50 | Stomach |
| 52 | Stomach (7%) | Colloid-mill 10 | Suspected adulterant |
| 53 | None | — | No offal |
| 54 | Tripe (7%) | Colloid-mill 10 | Tripe |

## CONCLUSIONS

Histological methods of examining raw non-decomposed sausage-meat for the purity and the nature of animal tissues present can be successfully applied to the detection of some common offals even after the materials have been "emulsified" in a colloid-mill. Some tissues, after being finely comminuted, may prove more difficult to identify specifically than others, although the presence of animal cells other than those of normal "meat" or muscle is readily recognised.

The actual amount of material it is practical to examine by histological means is minute, and therefore, while a positive finding is definite evidence of adulteration, a negative finding is not a certificate of purity for the bulk sample.

The execution of tests of this type necessitates suitable equipment, some experience of section preparation and some knowledge of basic animal histology.

We thank Mr. Gerrard, Mr. Wertheim and the National College of Food Technology at Weybridge for making apparatus available, and for advice and assistance in its use for preparing different grades of minced or milled animal tissues.

## References

1. Kelch, F., *Fleischwirtschaft*, 1957, **9**, 473, 604 and 757; 1958, **10**, 85, 211, 389, 537, 665 and 835; 1959, **11**, 107, 257, 581, 817 and 995; 1960, **12**, 87, 281, 544, 807 and 1019; 1961, **13**, 301 and 473.
2. Linke, H., *Ibid.*, 1960, **12**, 86 and 89.

Received *January 17th*, 1962

# A Critical Examination of the Aldridge Method and Some Modifications for Determining Small Amounts of Cyanide in Effluents

By H. G. HIGSON* and L. S. BARK

(*The Department of Chemistry and Applied Chemistry, The Royal College of Advanced Technology, Salford, Lancashire*)

The Aldridge method, which involves the action of cyanogen bromide on benzidine - pyridine solution, is critically examined. The effects of varying the concentration of benzidine is discussed, and it is shown that a more dilute solution, 2 per cent. as compared with 5 per cent., suitably extends the range of the method from 0·2 to 2·0 μg to 0·2 to 20 μg of cyanide in the sample.

The method recommended in the United Kingdom[1,2,3] for determining the cyanide contents of effluents that contain up to 2 mg per litre of sample or up to 20 mg per litre of sample after dilution, is based on the method developed by Aldridge.[4,5] The method is also recommended[6] for the analysis of waters.

Aldridge claimed that 0·2 μg of hydrogen cyanide, at a concentration of 0·1 μg per ml, could be determined with an accuracy of ±2 per cent., and stated the following points concerning the concentration of the reagents he used.

(*i*) *Pyridine*—An increase in the concentration of pyridine increases the time required for colour development and stability of the colour. The optimum concentration in the final solutions should be 25 to 30 per cent.

(*ii*) *Hydrochloric acid*—Up to 5 per cent. v/v of concentrated acid decreases the time required for colour development. The optimum concentration in the final solutions should be 4 to 5 per cent.

(*iii*) *Benzidine*—An increase in the concentration of benzidine decreases the time required for colour development. The optimum concentration in the final solutions should be 0·4 to 0·5 per cent.

Various workers have disagreed with Aldridge about the stability of the colour, the wavelength to be used for measurement of the colour and the concentration of benzidine to be used.

It was therefore decided to investigate the Aldridge method with special reference to each of these points.

## Stability of Colour and Wavelength for Measurement

Aldridge claimed that the colour is stable for 30 minutes at 20° C and for longer times at lower temperatures. Different workers[7,8] have reported that the colour fades in aqueous media at a steady rate after maximum absorption is reached.

Saltzman[8] used the original Aldridge method and reported that the colour was unstable, changing in both tint and optical density. The instability of the colour increased with temperature; measurement of the optical density of the colour was made at a wavelength of 510 mμ. Saltzman stated that he obtained good, reproducible results, by controlling the temperature and by allowing for the age of the sample colour by means of a set of readings made at various ages of the standards used.

Although Aldridge used filters that transmit light over a relatively broad spectrum, others have used a definite wavelength for colour measurement. These wavelengths have differed widely: 480,[8] 510,[7] 520[3,9] and 532[10] mμ have been suggested.

In order to discover the reason for these variations, and to find if such variations in wavelength could affect the results obtained, an examination of the Aldridge method was started.

* Present address: Lankro Chemicals Limited, Eccles, nr. Manchester.

The method as described by Aldridge was used, except that the optical density of each solution was not measured on a Spekker spectrophotometer, but was measured between 500 and 560 mμ by using a ratio-recording grating spectrophotometer, at different time intervals. The solutions examined contained 0·0, 0·1, 0·2, 0·5, 1·0, 1·5 and 2·0 μg of cyanide per ml, and were obtained from a standard cyanide solution containing 500 μg of cyanide per ml that had been standardised by potentiometric titration and by Liebig's titration method with *p*-dimethylaminobenzylidene rhodamine as indicator.

Colours were allowed to develop at 20° ± 1° C for 15 minutes after the reagents had been mixed with solutions. Stoppered glass cells of 1-cm optical path-length were filled with each solution, and each optical density was compared to that of water in a similar 1-cm cell, between 500 and 560 mμ at 5-minute intervals. Water was used as a reference solution throughout since blank solutions as well as sample solutions may vary with time. Further, the optical densities of the solutions were also measured by using a manually operated spectrophotometer at different time intervals at selected wavelengths (determined from the recorded spectra). The results obtained with the recording spectrophotometer are shown graphically in Fig. 1. The solution containing 2·0 p.p.m. of cyanide was too optically dense to be measured on the instruments used. The results of optical-density measurements made by using the manually operated instrument are given in Table I.

TABLE I

OPTICAL DENSITIES OF CYANIDE-CONTAINING SOLUTIONS

Results are corrected for the blank value

| Minutes after mixing | Wavelength, mμ | Optical density: Concentration of cyanide, μg per ml | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 0·0 | 0·1 | 0·2 | 0·5 | 1·0 | 1·5 | 2·0 |
| 17 | 530 | 0·047 | 0·043 | 0·110 | 0·328 | 0·719 | 0·988 | 1·461 |
| 25 | 530 | 0·047 | 0·043 | 0·110 | 0·323 | 0·699 | 0·964 | 1·279 |
| 17 | 518 | 0·052 | 0·038 | 0·098 | 0·301 | 0·658 | 0·783 | 1·000 |
| 25 | 518 | 0·052 | 0·038 | 0·098 | 0·300 | 0·645 | 0·769 | 0·978 |

CONCLUSIONS ON THE ALDRIDGE METHOD—

(*i*) At concentrations up to 1·0 p.p.m., *viz.*, with a total of 2·0 μg of cyanide per ml, the optical density may be considered to be stable for a short period. This is possibly owing to the fact that the amount of fading of the colour is too small to be measured on a weakly coloured solution. At higher concentrations of cyanide, the colour is not stable.

(*ii*) The wavelength used for the range 0·1 p.p.m. to 1·0 p.p.m. is not critical, and any particular wavelength between 518 and 532 mμ or filters covering this range may be used without any noticeable disadvantage.

(*iii*) The Aldridge method without modification, although quite sensitive, is valid only for a limited concentration range.

EXTENSION OF THE RANGE OF MEASUREMENT BY MODIFYING ALDRIDGE'S METHOD

MODIFICATION A—

Baker, Foster, Post and Hiett[11] modified Aldridge's method by diluting the coloured solution with 5 ml of ethanol and then with water to a total volume of 25 ml, but they used different concentrations of benzidine and the pyridine reagent from those used by Aldridge.

The Aldridge method was therefore re-examined with the concentrations of reagents originally used, but the coloured solution was diluted with ethanol and water as in the above method before the optical density was measured. Each standard solution was so treated, and its optical density compared to that of water, measured at various intervals between 500 and 540 mμ as before. Measurements were also made by using a manually operated instrument.

RESULTS—

It was found that 15 minutes was an insufficient time for optical densities to reach a constant value and that 20 minutes was the required development time.

The results obtained by using the recording spectrophotometer are shown graphically in Fig. 2. The various spectral scans converge at the isosbestic point.

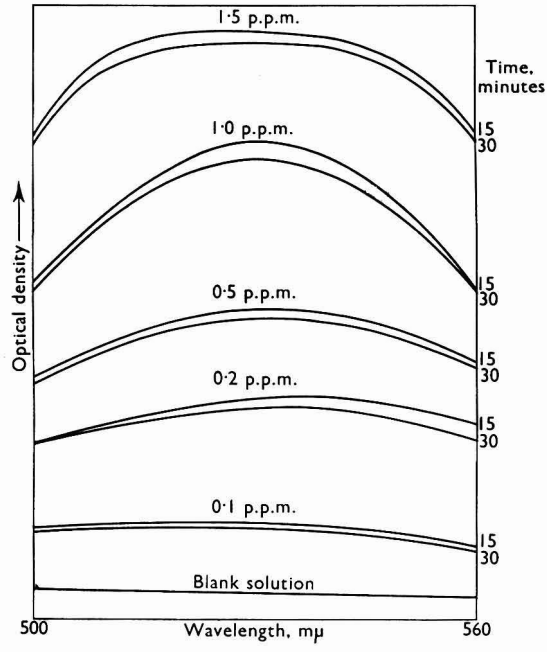



Fig. 1. The effect of time on the colour development of solutions containing varying amounts of cyanide

Measurements were made by using a manually operated instrument, operating at the wavelength of the isosbestic point and at a lower wavelength (514 mμ) for comparison purposes. The latter wavelength was selected arbitrarily at 10 mμ below the isosbestic point.

TABLE II

OPTICAL DENSITIES OF CYANIDE-CONTAINING SOLUTIONS

Results are corrected for the blank value

| Minutes after mixing | Wavelength, mμ | Optical density: Concentration of cyanide, μg per ml | | | | |
|---|---|---|---|---|---|---|
| | | 0·0 | 0·5 | 1·0 | 2·5 | 5·0 |
| 17 | 524 | 0·009 | 0·070 | 0·154 | 0·442 | 0·909 |
| 27 | 524 | 0·009 | 0·072 | 0·150 | 0·438 | 0·926 |
| 17 | 514 | 0·010 | 0·071 | 0·156 | 0·448 | 0·930 |
| 27 | 514 | 0·010 | 0·070 | 0·142 | 0·424 | 0·908 |

This modification of Aldridge's method extends the range of the original method from 1·0 μg per ml to 5·0 μg per ml for maximum measurable concentration.

For cyanide concentrations of less than 1 μg per ml it is recommended that the optical density be measured without diluting the solution. For cyanide concentrations greater than 1 μg per ml, it is better to dilute the solution as indicated before measuring the optical density.

The constancy of the optical density with respect to time is dependent upon the wavelength considered.

From a consideration of the graphs (see Fig. 2) obtained by plotting optical density at various time intervals against wavelength, it may be seen that there is one wavelength at

which there is no variation in optical density with time. This wavelength corresponds to the point at which the various spectral scans for a particular concentration of cyanide cross each other. Since variation of optical density with time is a minimum at this point, this wavelength is the optimum for measurement. For the concentrations used, the isosbestic point occurs at 524 mμ.

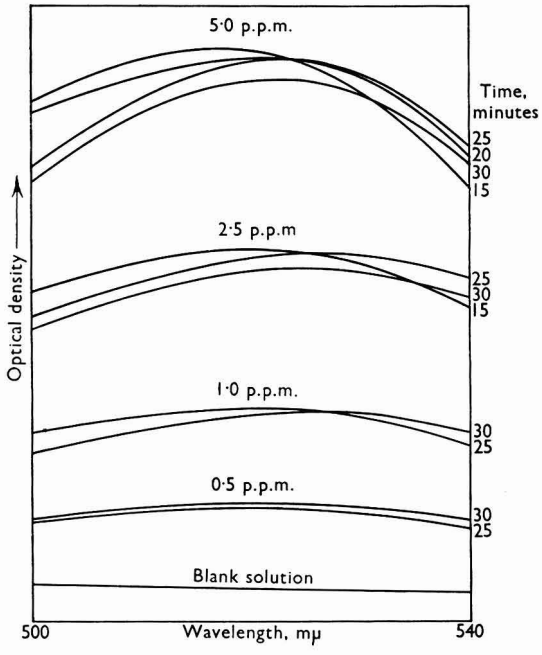



Fig. 2. The effect of time on the colour development of solutions containing varying amounts of cyanide

However, Baker, Foster, Post and Hiett[11] used a fixed wavelength of 530 mμ, which is higher and does not appear to be the optimum wavelength for accurate measurement. Since they did not use the same benzidine concentration as is used in Aldridge's method, and since Aldridge states that variations in concentrations of the reagents alters the time required for colour development, it is possible that variation in the concentrations of reagents produces a variation in the wavelength of the isosbestic point. This possibility was therefore investigated in modification B.

MODIFICATION B—

The revised reagent concentrations are given below—

*Pyridine - water - hydrochloric acid*—Mix 18 ml of pyridine, 12 ml of water and 3 ml of concentrated hydrochloric acid. This is reagent A, and is similar to the pyridine solution described by Aldridge.

*Benzidine*—Dissolve 0·5 g of benzidine in 50 ml of 0·5 N hydrochloric acid. This is reagent B.

*Pyridine - benzidine reagent*—Prepare a mixture of 33 ml of reagent A and 10 ml of reagent B. Use 4 ml of this combined reagent for each determination.

Solutions containing 0 to 10 p.p.m. of cyanide were examined by using the procedure of Baker, Foster, Post and Hiett,[11] but their optical densities were measured at 450 to 560 mμ against water, by using the spectrophotometers mentioned earlier.

The results obtained by using a recording spectrophotometer are shown graphically in Fig. 3, and are given in detail in Table III.

TABLE III

OPTICAL DENSITIES OF CYANIDE-CONTAINING SOLUTIONS

Results are corrected for the blank value

| Minutes after mixing | Wavelength,* mμ | Optical density: Concentration of cyanide, μg per ml | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 0·0 | 0·1 | 0·5 | 1·0 | 2·5 | 5·0 | 10·0 |
| 15 | 518 | 0·008 | 0·006 | 0·026 | 0·054 | 0·136 | 0·275 | 0·557 |
| 25 | 518 | 0·008 | 0·006 | 0·027 | 0·054 | 0·136 | 0·275 | 0·557 |
| 15 | 530 | 0·008 | 0·004 | 0·024 | 0·046 | 0·116 | 0·247 | 0·505 |
| 25 | 530 | 0·008 | 0·004 | 0·026 | 0·049 | 0·125 | 0·260 | 0·542 |

* The isosbestic point is at 518 mμ, and the wavelength used by Baker, Foster, Post and Hiett[11] was 530 mμ.

From Fig. 3 and Table III, it can be seen that the colour produced with the pyridine - benzidine reagent and cyanogen bromide takes time to develop, and it is only possible to use one particular wavelength (518 mμ for the concentration of benzidine used) for obtaining accurate analytical results, since the optical density at other wavelengths varies with time.

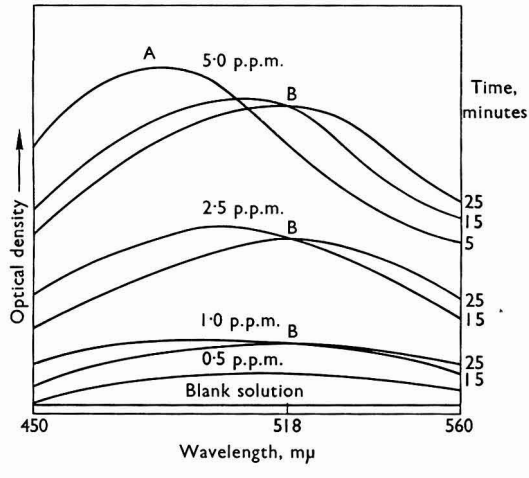



Fig. 3. The effect of time on the colour development of solutions containing varying amounts of cyanide: A, point of maximum absorbance at 493 mμ for a solution containing 5·0 p.p.m.; B, isosbestic points at 518 mμ.

A calibration curve obtained by plotting the optical densities measured at 518 mμ as ordinates *versus* the cyanide content as abscissae proved to be a straight line of gradient 0·056, that passed through the origin.

EFFECT OF CONCENTRATION OF BENZIDINE REAGENT—

The weaker reagent used in the latter work (1 per cent. benzidine) compared to the original (Aldridge's) reagent (5 per cent. benzidine hydrochloride, equivalent to approximately 3·25 per cent. benzidine) produces low optical densities. For 0·5 p.p.m. of cyanide, the optical density with the 3·25 per cent. reagent was 0·071, and with the 1·0 per cent. reagent it was

0·027. For 5·0 p.p.m. of cyanide, the optical density with the 3·25 per cent. reagent was 0·926, and with the 1·0 per cent. reagent it was 0·275.

By using the dilution technique and with the 1 per cent. benzidine reagent, the reproducibility and linearity are better. Further, the upper limit is extended to 10·0 p.p.m. of cyanide, for which the optical density is 0·557, and possibly even to higher concentrations of cyanide.

It was therefore decided to re-examine Aldridge's original method, varying only the concentration of benzidine used, and measuring the optical densities (*a*) between 500 and 550 mμ at different time intervals to establish a wavelength for possible isosbestic points, by using the recording spectrophotometer, and (*b*) by using a Unicam SP500 spectrophotometer for accurate optical-density measurements at particular wavelengths for each concentration of benzidine used.

RESULTS—

The results of these experiments are given in Table IV. These results were obtained after allowing the colour produced with 0·6 ml of benzidine reagent to develop for 15 minutes.

TABLE IV

OPTICAL DENSITIES OF CYANIDE-CONTAINING SOLUTIONS

Results are corrected for the blank value

| Benzidine concentration, % | Wavelength used for measurement, mμ | Optical density Concentration of cyanide, p.p.m. 0·5 | 1·0 | 2·0 |
|---|---|---|---|---|
| 3·25 | 530 | 0·326 | 0·709 | 1·370 |
| 2·0 | 530 | 0·298 | 0·596 | 1·192 |
| 1·0 | 518* | 0·192 | 0·405 | 1·010 |
| 0·2 | 518* | 0·110 | 0·227 | 0·563 |

* Isosbestic point during the period 15 to 20 minutes after mixing the reagents.

At concentrations of benzidine of 0·2 and 1·0 per cent., the optical densities increase with time at 530 mμ and vary slightly with time at 518 mμ. These concentrations of benzidine appear too weak to give full colour development in a reasonable time.

The 2 per cent. concentration of benzidine produces constant optical densities at 530 mμ between 20 and 50 minutes after mixing, and is preferable to the 3·25 per cent. benzidine solution, which for 2 p.p.m. of cyanide gives optical densities that decrease with time.

This is in agreement with the previous findings, *i.e.*, 1 ml of 1 per cent. benzidine or 0·6 ml of 2 per cent. benzidine solution gives better results than the amount of benzidine recommended by Aldridge, *viz.*, 0·6 ml of 3·25 per cent. benzidine solution.

For Aldridge's method, it is recommended that the benzidine concentration used should be 2·0 per cent. in 0·5 N hydrochloric acid.

MODIFICATION C—

Krawczyk[12] reported that when the Aldridge method was used, a perceptible orange colour could be obtained with as little as 0·01 μg of hydrogen cyanide per ml for a 100-ml sample.

Nusbaum and Skupeko[13] determined extremely low concentrations of cyanide in the presence of n-butanol by using Aldridge's method (2·0 per cent. benzidine hydrochloride solution), and measured the colour in the butanolic extract at 480 mμ. This wavelength is low compared to the wavelengths investigated previously, and this modification was therefore investigated.

A solution containing 0·4 p.p.m. of cyanide was treated according to the procedure outlined by Nusbaum and Skupeko.[13] The colour was permitted to develop for 15 minutes before the butanol layer was separated. The optical density of the butanolic extract was measured between 450 and 530 mμ against water in a stoppered 1-cm optical cell, over a period of 1 hour at different time intervals, with a recording spectrophotometer. The optical density of the solution was also measured with a manually operated instrument.

RESULTS—

*Recording instrument*—A single, broad peak was produced with a maximum at about 480 mμ. The peak did not change in wavelength for 1 hour. This peak is approximately 10 mμ wide, and 480 mμ is the approximate centre of the peak.

Initial difficulty was experienced owing to small bubbles that formed from the supersaturated water - butanol solution, initially causing high optical-density values. The difficulty was overcome by using 2 ml of a 5 per cent. solution of disodium hydrogen orthophosphate to break any emulsion formed and then separating the butanolic extract by centrifugation for 2 minutes. (Phosphate had no apparent effect on the colour when it was tested under the usual aqueous conditions.)

*Manual instrument* (Unicam SP500)—The results obtained by using this instrument are given in Table V.

TABLE V

OPTICAL-DENSITY MEASUREMENTS ON A SOLUTION CONTAINING 0·4 P.P.M. OF CYANIDE

Measurements were made at 480 mμ

| Time after mixing reagents, minutes | Initial optical density | Optical density after extraction with n-butanol |
|---|---|---|
| 25 | 0·387 | 0·359 |
| 30 | 0·372 | 0·357 |
| 38 | 0·362 | 0·360 |
| 43 | 0·358 | 0·359 |
| 53 | 0·356 | 0·357 |
| 60 | 0·356 | 0·358 |

## CONCLUSIONS

ALDRIDGE'S METHOD—

The method is valid for cyanide concentrations up to 2·0 μg per 2-ml sample (1 p.p.m. of cyanide). The use of a wavelength of 520 mμ, or of filters or wavelengths from 518 to 535 mμ has been confirmed. The concentration of benzidine reagent (3·25 per cent. benzidine) seems needlessly high, and would be better at the 2·0 per cent. level. The method does not extend over a large enough range of concentrations of cyanide to be of the maximum use.

MODIFICATIONS OF ALDRIDGE'S METHOD—

*Modification A*—(Dilution of the coloured solution with alcohol.) This modification extends the original method to 10·0 μg per 2-ml sample (5 p.p.m. of cyanide). Optical densities are measured at a wavelength of 524 mμ (the isosbestic point), which is the only wavelength that yields reproducible results with time. The benzidine concentration is the same as in Aldridge's method, and is needlessly high.

*Modification B*—(Method of Baker, Foster, Post and Hiett[11].) Optical densities should be measured at 518 mμ (the isosbestic point), and not 530 mμ as recommended by Baker, *et al.* (Optical densities measured at 530 mμ are less at any time than those measured at 518 mμ.) The concentration of benzidine is about a third of that used in the original Aldridge method. The method is applicable over the range 0·2 to 20 p.p.m. of cyanide. For samples containing less than 0·2 p.p.m. of cyanide, an initial sample size greater than 2 ml will improve the lower limits.

*Modification C*—(Method of Nusbaum and Skupeko.[13]) Optical densities are measured at 480 mμ, with the concentration of benzidine approximately a third of that used in the original Aldridge method. Although in principle this is a good method and is useful for concentrations of cyanide less than 1 p.p.m., relatively large samples are required, and it is necessary to prevent formation of emulsions during the time of measurement.

For trace amounts of cyanide (less than 0·5 p.p.m.) there are two alternatives: modifications B and C. In both modifications, 10-ml samples are preferable.

For cyanide concentrations of 0·5 to 1·0 p.p.m., the best method is modification B, without dilution if the sample is limited in size.

For cyanide concentrations of 1·0 p.p.m. to 20 p.p.m., the best method is modification B. The use of the correct wavelength is necessary for determinations of cyanide at the higher concentrations. This wavelength (that of the isosbestic point) is that at which the most satisfactory measurements are obtained, *i.e.*, 518 m$\mu$. For a larger concentration of benzidine (2·0 per cent. is to be preferred) the correct wavelength will be higher than 518 m$\mu$.

## References


1. Ministry of Housing and Local Government, "Methods of Chemical Analysis Applied to Sewage and Sewage Effluents," Second Edition, H.M. Stationery Office, London, 1956.
2. The Joint Committee of the Association of British Chemical Manufacturers and the Society for Analytical Chemistry, "Recommended Methods for the Analysis of Trade Effluents," W. Heffer & Sons Limited, Cambridge, 1958.
3. Analytical Methods Committee of the Society for Analytical Chemistry, "Official, Standardised and Recommended Methods of Analysis," W. Heffer & Sons Limited, Cambridge, 1963, p. 253.
4. Aldridge, W. N., *Analyst*, 1944, **69**, 262.
5. ——, *Ibid.*, 1945, **70**, 474.
6. The Joint Committee of the Institution of Water Engineers, The Royal Institute of Chemistry, The Society for Analytical Chemistry and the Society for Water Treatment and Examination, "Approved Methods for the Physical and Chemical Examination of Water Supplies," Third Edition, The Institution of Water Engineers, London, 1960.
7. Ludzack, F. J., Moore, W. A., and Ruchoft, C. C., *Anal. Chem.*, 1954, **26**, 1784.
8. Saltzman, B. E., *Ibid.*, 1961, **33**, 1100.
9. Tompsett, S. L., *Clin. Chem.*, 1959, **5**, 587.
10. Bruce, R. B., Howard, J. W., and Hanzal, R. F., *Anal. Chem.*, 1955, **27**, 1346.
11. Baker, M. O., Foster, R. A., Post, B. G., and Hiett, T. A., *Ibid.*, 1955, **27**, 448.
12. Krawczyk, D. F., *Sewage and Industrial Wastes*, 1954, **26**, 388.
13. Nusbaum, I., and Skupeko, P., *Ibid.*, 1951, **23**, 875.


Received *July 25th*, 1963

# The X-ray Fluorescence Determination of Zinc in Samples of Unknown Composition

By K. G. CARR-BRION

(*Warren Spring Laboratory, Department of Scientific and Industrial Research, Stevenage, Herts.*)

An X-ray fluorescence method has been developed for determining elements in powder samples from the processing of mineral ores, with special reference to zinc. The effect of sample films of known thickness on the fluorescent intensity from a zinc disc is used to compensate for absorption effects and to simplify corrections for enhancements. The method is flexible, sensitive, non-destructive and capable of operation on a routine basis.

In applying X-ray fluorescence methods to the products of mineral processing, the analyst is faced with the problem of determining elements in concentrations ranging from a few parts per million to almost 100 per cent. in constantly changing matrices. As small samples are often required for further examination, any method used should preferably be non-destructive and non-contaminating.

Several X-ray fluorescence methods were examined to find one that would not require constant correction and re-calibration and would give consistent results independent of the matrix composition. None proved entirely satisfactory. The use of radiation scattered from the sample to compensate for variations in fluorescent intensity[1] was found to be only semi-quantitative even when applied to a limited range of matrices. Dilution of the sample causes contamination and reduces sensitivity, and in its most effective form, *i.e.*, borax fusion,[2] it results in destruction of the sample. Internal standards[3] are inflexible, cause contamination, and cannot be relied upon to give adequate compensation, especially with powder samples. Thin-film methods are suitable in being non-destructive and permit small samples to be analysed, but they are only of general applicability when the film is so thin as to cause a major loss in sensitivity.[4]

It appeared possible, however, that if the films were thick enough to give little loss in sensitivity but still thin enough to permit the excitation and detection of the characteristic X-rays through them, then absorption effects could be calculated, as shown below, making correction for enhancement effects much simpler. It has been demonstrated[5] that the characteristic X-ray intensity, $I_F^\infty$, from a given w/w concentration of an element in an "infinitely" thick sample excited by a monochromatic beam is given by—

$$I_F^\infty = \frac{K}{(\mu_p \operatorname{cosec} \theta_p + \mu_s \operatorname{cosec} \theta_s)} \quad \ldots \quad (1)$$

where $\theta_p$ and $\theta_s$ are the angles made by the primary and secondary beams with the sample surface,

$\mu_p$ and $\mu_s$ are the mass-absorption coefficients of the sample for the primary and secondary X-rays, and

$K$ is a constant for a fixed primary X-ray intensity, a given element and a particular X-ray spectrograph.

With identical excitation conditions, the characteristic X-ray intensity, $I_T$, from a substance containing a given concentration of the same element, covered by a film of thickness $x$ cm and density $\rho$, is given by—

$$I_T = I_0 \exp. [-(\mu_p \operatorname{cosec} \theta_p + \mu_s \operatorname{cosec} \theta_s) \rho x] \quad \ldots \quad (2)$$

where $I_0$ is the fluorescent intensity from the substance with the film removed, and $\mu_p$ and $\mu_s$ refer to the film.

If $\rho$ is expressed as $W/Ax$, where $W$ is the weight of the disc in grams, and $A$ is its area, which is constant, then equation (2) becomes—

$$I_T = I_0 \exp. [-(\mu_p \operatorname{cosec} \theta_p + \mu_s \operatorname{cosec} \theta_s) W/A] \quad \ldots \quad (3)$$

Further, the fluorescent intensity, $I_F^x$, from a film of thickness $x$ cm is related to that from an "infinitely" thick sample by the equation[6]—

$$I_F^x = I_F^\infty \left(1 - \frac{I_T}{I_0}\right) \quad .. \quad .. \quad .. \quad .. \quad .. \quad (4)$$

As $I_T$ and $I_0$ can be measured, it is possible, by using equation (3), to obtain, for a sample in the form of a film, a value for $\frac{1}{A}(\mu_p \text{ cosec } \theta_p + \mu_s \text{ cosec } \theta_s)$, hereafter referred to as $M$. If $I_F^\infty$ is known for a given w/w concentration in one matrix of measured $M$, it should be possible to obtain from equation (1) the intensity for the same concentration of the element in any matrix. $I_F^\infty$ can simply be calculated from the characteristic fluorescent intensity of the film alone by using equation (4).

These equations have been derived from simplified models and their limitations in real systems had to be found experimentally. Zinc, a common element in mineral processing, was selected for the initial work.

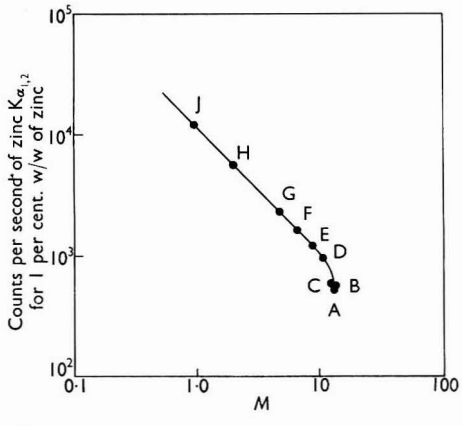



Fig. 1. Variation of the zinc X-ray intensity with measured $M$ of the matrix: A, iron oxide; B, barium carbonate; C, lead oxide; D, mercuric oxide; E, molybdenum oxide; F, gallium oxide; G, copper sulphate; H, sodium carbonate; J, carbon

## Experimental

### Preparation of films—

The first requirement was to make homogeneous films of uniform thickness from the powder samples produced by mineral processing. Several binding agents were tried, including hot- and cold-setting resins, Perspex cements, starch and various waxes, and films were prepared both by casting and pressing. The only successful method involved the use of a hard wax as a binder, the films being produced under pressure; this method also had the advantage that the sample could be readily recovered intact by dissolving the wax in light petroleum, and filtering off the sample.

### Calibration—

For finding the relationship between the intensity of the characteristic X-rays and $M$, standards were made from a number of zinc-free elements and compounds covering a range of elements from carbon to lead. These were ground in a high-speed ball-mill with ignited analytical-reagent grade zinc oxide, equivalent to 1 per cent. w/w of zinc in the final product. Each was ground for twice the time required to reach a constant zinc X-ray intensity. Films made from these mixtures were examined, and $I_F^\infty$ was plotted against $M$ on log-log graph paper. The two variables were almost inversely proportional except at values of $M$ greater than 10, where the intensity was less than predicted (see Fig. 1).

A series of films containing varying w/w concentrations of zinc was also made, and the ratios of the zinc intensity from each sample to the intensity from 1 per cent. w/w zinc in a matrix with the same $M$ was calculated. These ratios were plotted against per cent. w/w zinc on log - log graph paper, giving an approximately straight line calibration from the lowest values up to 80 per cent. w/w zinc. Finally, films were made from compounds of any element that was suspected of causing enhancement of the zinc intensity through absorption of its characteristic X-rays by zinc atoms in the sample, with consequent emission of an excess of zinc $K_\alpha$ radiation. The interference effects found under the conditions used were appreciable, but a simple percentage correction of the observed zinc X-ray intensity, proportional to the intensity of either the $K_{\alpha_{1,2}}$ or the $L_{\alpha_1}$ lines of the interfering elements, whichever could be detected, was found to be adequate. The presence of these elements in a sample can be quickly established by a manual scan over the required portion of the X-ray spectrum. The interfering elements and the correction factors found are given below—

| Interfering elements .. .. | Gold | Mercury | Germanium | Arsenic | Selenium | Bromine |
|---|---|---|---|---|---|---|
| Decrease in zinc $K_{\alpha_{1,2}}$ intensity, for $10^3$ counts per second of the interfering $K_{\alpha_{1,2}}$ or $L_{\alpha_1}$ emission, per cent. .. .. .. | 0·87 | 0·34 | 0·36 | 0·27 | 0·17 | 0·04 |

## Method

Reagents—

*Paraffin wax* (*so-called*)—Clearing-point 65 °to 71° C. (Obtainable from The British Drug Houses Ltd.)

*Light petroleum*—Boiling-range 60° to 80° C.

Instrumental conditions—

The conditions listed below were found suitable for a Philips PW1540 spectrograph—

X-ray tube: Tungsten target running at 30 kV, 6 mA.

Analysing crystal: Lithium fluoride.

Detector: Scintillation counter at 850 V.

Counting mode: Fixed time of 15 seconds, scaling factor × 256. Owing to the high intensities measured, up to $10^5$ counts per second, a graphical correction for the counting losses is necessary.

Procedure—

Grind the sample in a high-speed ball-mill until more than 80 per cent. w/w is less than 350 mesh. Weigh 0·333 g, or less if the sample is small, into an agate mortar and add powdered wax to give a total of 1 g. Mix the sample and powder with about 5 ml of light petroleum by using an agate pestle until a homogeneous cream, and finally a crumbly powder, is obtained. Set the powder aside for 5 minutes in order to evaporate the residual solvent.

Heap the powder on to a flat, steel plate covered in a double film of 6 $\mu$ Melinex. Press between this and a second similarly covered plate under a load of 10 tons, controlling the film thickness to 0·05 cm $\pm$ 0·005 cm with a steel spacer. Remove the steel plates, strip the Melinex from the film, and cut a $1\frac{1}{4}$-inch diameter circle from it with a cutter giving an area reproducible to within $\pm$1 per cent. and weigh the disc to $\pm$0·001 g.

Place the film in the rotating sample holder of the X-ray spectrograph and measure the zinc $K_{\alpha_{1,2}}$ intensity, $I_F^x$. Check by hand-scanning whether any elements causing enhancement are present. If so, measure either their $K_{\alpha_{1,2}}$ or $L_{\alpha_1}$ intensity. Carefully place a zinc metal disc about 0·5 cm thick on top of the film, and measure the total zinc intensity, $I_F^x + I_T$. Remove the film, replace it with an annular spacer of 0·05 cm, put back the zinc metal disc and measure the zinc intensity $I_0$.

Correct intensities, where necessary, for counting losses. Calculate $I_T/I_0$, obtain $\log_e (I_T/I_0)$ and divide by $W$ to obtain $M$. From the graph of $M$ against $I_F^\infty$ find the intensity produced by 1 per cent. w/w zinc in a matrix of the same $M$. Correct $I_F^x$ to $I_F^\infty$ by dividing by $(1 - I_T/I_0)$ and make any corrections necessary for enhancement. Calculate the ratio of $I_F^\infty$ to the intensity for 1 per cent. w/w zinc, and find the percentage w/w of zinc in the sample from the calibration graph. If less than 0·333 g of sample is used, make the necessary weight correction.

## Results

Table I gives the results obtained with typical analysed samples.

Table I

Examination of analysed samples

| Sample | *M* | Zinc found by routine analysis, per cent. | Zinc found by proposed method, per cent. |
|---|---|---|---|
| Arsenical pyrite cinder .. | 13·7 | 0·78 | 0·78 |
| | 12·7 | 2·33 | 2·30 |
| Silica and cinder .. .. | 8·25 | 0·78 | 0·82 |
| | 6·45 | 0·63 | 0·63 |
| Zinc ore fraction .. .. | 4·60 | 0·07 | 0·078 |
| | 5·01 | 0·36 | 0·35 |
| | 5·21 | 1·15 | 1·16 |
| | 5·72 | 6·1 | 6·3 |
| Zinc concentrate .. .. | 9·55 | 49·1 | 48 |
| | 10·3 | 62·0 | 62 |
| Lead concentrate .. .. | 12·8 | 1 | 0·84 |
| Dolomite concentrate .. | 8·6 | 34·1 | 35 |

### Reproducibility and accuracy—

The standard deviation in zinc concentration obtained from replicate films was less than 2 per cent., except when the counting error exceeded this. The results obtained from analysed samples suggest that an accuracy of $\pm 5$ per cent. or better can be achieved with the method in its present form.

Interference caused by emission of characteristic X-rays by different elements at nearly identical wavelengths are not eliminated, and must be taken into account in any analysis. Errors due to particle-size variations are also uncorrected. It was found that a regular relationship existed between per cent. w/w of the sample less than 350 mesh and the zinc intensity from it. This could be used for correcting observed intensities, but a more satisfactory course would be to ensure that more than 80 per cent. w/w of the sample was less than 350 mesh; under these conditions zinc gave consistent results.

## Conclusions

The proposed method is flexible, sensitive, non-destructive and suitable for routine operation and it can be applied to all powder samples. With simple adaptation it can be used for liquids and any homogeneous solid capable of preparation in film form.

Although, so far, only zinc has been examined, the method is applicable to a wide range of elements, the main limitation being the difficulty of making films sufficiently thin and homogeneous with elements of low atomic weights. For these elements a different method of preparation, such as blowing extremely thin films from a molten glass containing the sample in solution, would be necessary.

The work described in this Paper forms part of the research programme of the Warren Spring Laboratory and is published by permission of the Director.

## References

1. Andermann, G., and Kemp, J. W., *Anal. Chem.*, 1958, **30**, 1306.
2. Claisse, F., *Norelco Reptr.*, 1957, **4**, 3.
3. Adler, I., and Axelrod, J. M., *Spectrochim. Acta*, 1955, **7**, 91.
4. Salmon, M. L., *Advances in X-ray Analysis*, 1962, **5**, 389.
5. Liebhafsky, H. A., Pfeiffer, H. G., Winslow, E. H., and Zemany, P. D., "X-ray Absorption and Emission in Analytical Chemistry: Spectrochemical Analysis with X-rays," J. Wiley and Sons Inc., New York and London, 1960, p. 168.
6. Brissey, R. M., Liebhafsky, H. A., Pfeiffer, H. G., *Special Technical Publication No.* **157**, American Society for Testing Materials, Philadelphia, 1954, p. **43**.

Received *September 26th*, 1963

# The Response Properties of an Electrode Glass Sensitive to Potassium and Ammonium Ions*

BY G. MATTOCK AND R. UNCLES
(*Electronic Instruments Ltd., Richmond, Surrey*)

The properties of a new electrode glass, designated BH115, have been investigated; this glass shows a selective response to potassium and ammonium ions. The working range for response to both types of ions has been found to be from M to 0·0001 M, *i.e.*, down to 1 to 5 p.p.m.; the response was logarithmic to concentration, and the slope was in good agreement with that predicted from the Nernst equation. The effect of hydrogen ions, sodium ions and ions of the alkaline-earth elements on the response have been determined; only sodium ions exerted significant interference. Temperature changes produce the expected sensitivity (slope) shift, and zero shifts have been measured. Reproducibility, stability and speed of response of the glass are comparable to those of pH electrodes. An indication of the analytical potentialities is presented.

RECENT research on electrode-glass compositions has been directed towards the development of glasses with a specific response to various alkali-metal cations. Most work has been focused on systems responsive to sodium ions, following the stimulus provided by Eisenman, Rudin and Casby,[1] and we have recently published a study of two systems selectively responsive to sodium ions[2] and a note on responses to silver, lithium and thallium ions.[3] Bishop and Dhaneshwar[4] have also studied these systems. This Paper presents results on a glass evolved from an investigation of several systems responsive to potassium ions; the glass described, coded BH115, constitutes the best of those examined, in terms of stability, reproducibility, speed of response and selectivity. It has also been found that, consistent with the Eisenman theory of ion-specific response systems,[5] these glasses that are responsive to potassium ions also show an excellent response to ammonium ions; the response of BH115 to ammonium ions is slightly superior to its response to potassium ions.

Glasses responsive to potassium ions have been described by Eisenman, Rudin and Casby,[1] and by Portnoy, Thomas and Gurdjian.[6] In our experience these glasses are not easy to prepare in a form that shows consistent properties, and are not readily fused to generally available insulating glasses for the construction of electrodes. BH115 electrode glass shows improved stability and reproducibility as well as ease of preparation, consistency in properties, and ease of handling in the preparation of electrodes. A preliminary account of some of the analytical applications of BH115 electrodes has already been given[7] and they are now commercially available.†

## EXPERIMENTAL

### ELECTRODES—

Electrodes are readily constructed by using conventional techniques for blowing pH electrodes. In this study bulb types were used with the membranes sealed to G.E.C. L1 glass. The resistance of the membranes was of the order of 40 megohms. An inner reference system was made of silver - silver chloride dipping into a solution consisting of 0·04 M potassium dihydrogen orthophosphate, $KH_2PO_4$, 0·01 M disodium hydrogen orthophosphate, $Na_2HPO_4$ and 0·01 M potassium chloride. The external reference was a 3·8 M potassium chloride calomel electrode. To prevent potassium chloride leaking into the test solution an intermediate salt bridge solution was incorporated in tubing between the ceramic plugs

* This Paper was presented to the Electroanalytical Section of the I.U.P.A.C. Congress held in London in July, 1963.

† Now commercially available from Electronic Instruments Ltd., Richmond, Surrey (see E.I.L. Technical Data Sheet ELECT 13).

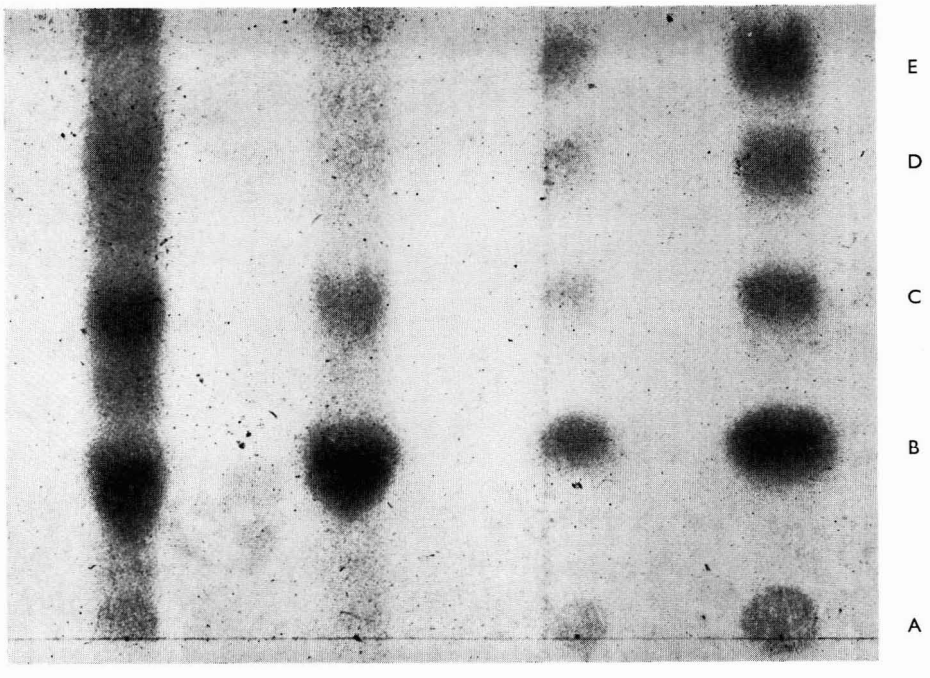



Fig. 1. Thin-layer chromatogram showing the alkali-hydrolysed feeding stuff and iodinated casein, similarly treated, alongside standard solutions of iodo compounds. The chromatogram was run in an ascending-solvent system of t-amyl alcohol - ammonia solution mixture, and then sprayed with FFCA reagent.

Series (*i*), 2 × 2 μl of iodinated casein; series (*ii*), 2 × 2 μl of feeding stuff; series (*iii*), 1 μl and series (*iv*), 2 μl of a standard solution of iodo compounds. Spots A, di-iodotyrosine; spots B, iodide; spots C, thyroxine; spots D, tri-iodothyronine; spots E, di-iodothyronine

[*To face p.* **330**

making contact with the reference and test solutions. The arrangement used and recommended is shown in Fig. 1. This scheme provides for the displacement of samples past the electrode, rather than the transfer of the electrode between samples held in different containers. Air between the samples should be avoided. The most convenient liquid for the bridge is the buffer medium that is used to maintain the ionic strength and the pH conditions in the sample at a constant value. It was found that to obtain good results it was essential to use this intermediate solution, and as it is slowly contaminated by potassium chloride from the reference electrode it should be renewed in each daily series of measurements. When a buffer medium is not being used, the intermediate salt bridge can be lanthanum or barium chloride.

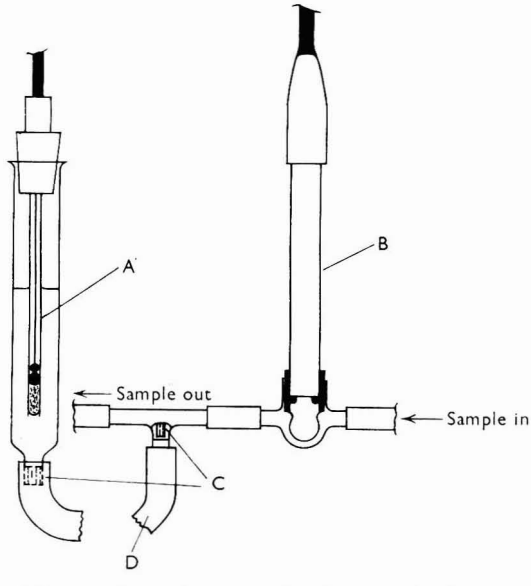



Fig. 1. Preferred experimental arrangement for the use of alkali-sensitive glass electrodes

While not in use, the electrodes were stored in 0·1 M potassium chloride when experiments on the response to potassium ions were being carried out, and in 0·1 M ammonium chloride when experiments on the response to ammonium ions were being carried out. It is advisable not to let the electrodes dry out.

SOLUTIONS—

These were prepared from analytical-reagent grade materials whenever possible, and were stored and used in polythene containers. To avoid variations in activity coefficients, media of constant ionic strength were used; these were ethanolamine or triethanolamine that had been adjusted to the appropriate pH by the addition of hydrochloric acid or nitric acid. Eisenman, in a private communication, said that some response to ethanolammonium or triethanolammonium cations might be observable with glass responsive to potassium ions, and may be responsible for limiting the response range to potassium or ammonium ions, but this was not found particularly serious with BH115, and the advantages of an organic-cation buffer outweighed the possible disadvantages.

MEASUREMENT TECHNIQUE—

This was the same in principle as that described for electrodes responsive to sodium ions,[2] and a pH meter provided measurements of an apparent pK or $pNH_4$, defined as—

$$pK = -\log c_{K^+} \text{ and } pNH_4 = -\log c_{NH_4^+}$$

for the formal equivalence of the pH, pK and $pNH_4$ scales. The pH scale of a pH meter is calibrated in terms of the known potassium or ammonium ion concentrations. These are expressed as pK or $pNH_4$ units according to the equations given above. A high-impedance electrometer that gives mV values, or the mV scale of a pH meter can alternatively be used to calibrate mV against pK or $pNH_4$: the pH scale was used in this work both to demonstrate the fundamental analogy and to clarify the presentation of results.

The best technique of handling the electrodes when transfer is made from one solution to the other is by washing the electrode in a sample of the second solution. Washing the electrodes with water causes sluggishness, and so does wiping, *e.g.*, with a filter-paper (light wiping with a tissue is acceptable). For the best results it is preferable to mount the electrode in a small chamber (see Fig. 1) and to flow liquid past it, a new solution replacing the original (without air bubbles intervening if possible).

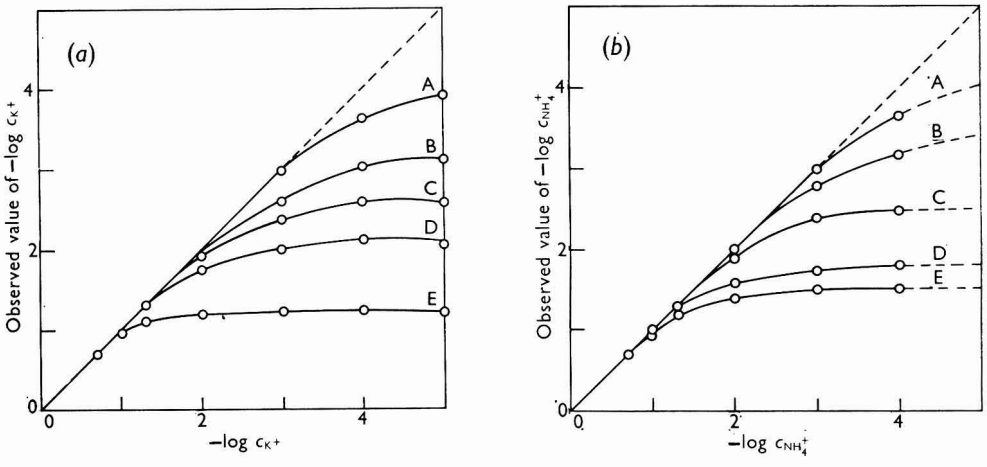



Fig. 2. The response of BH115 electrodes to (*a*) potassium and (*b*) ammonium ions, in the presence of differing concentrations of sodium ions: curves A, nil; curves B, 0·001 M; curves C, 0·01 M; curves D, 0·05 M; curves E, 0·1 M. The broken lines show the theoretical response. The background buffer medium was 0·1 M triethanolamine *plus* hydrochloric acid, at pH 7·0

Most of the measurements described in this Paper were made with an E.I.L. 33B Vibron electrometer with a C33B pH accessory unit; this gave an instrumental significance of $\pm$0·002 pK or $pNH_4$ units, equivalent to 0·5 per cent. in concentration terms.

## Results

Most of the results obtained are presented graphically in Figs. 2 (*a*), 2 (*b*), 3 and 4. In common with other glass electrodes, BH115 electrodes show a slight variability from one to another, but the results given are representative of the performance of the many electrodes examined. The form of the investigation consisted mainly of an examination of the effects of possible interfering substances on the fundamental response curves.

### Response characteristics to potassium and ammonium ions—

The response characteristics of BH115 to potassium and ammonium ions are shown in the curves marked A in Figs. 2 (*a*) and 2 (*b*). The ranges are similar, and cover the region approximately M to 0·0001 M, *i.e.*, pK (or $pNH_4$) = 0 to pK (or $pNH_4$) = 4. A linear and nearly theoretical response (95 to 99 per cent.) was observed in ethanolamine and triethanolamine medium over the range 0·5 M to 0·001 M. In the higher concentration regions the constancy of ionic strength was obviously not maintained, but in a few experiments on response at pK = 0 and $pNH_4$ = 0 the observed values were nearly theoretical (100 to 105 per cent.). As noted above, this range may be limited by some slight interference caused

by response to the medium. The practical low concentration limit for useful reproducibility and sensitivity is about 0·0001 M (4 p.p.m. for potassium ions and 1 p.p.m. for ammonium ions), but response does not actually cease at this level. The co-presence of potassium and ammonium in a solution causes strong interference in the observation of either one, although in principle it is possible to construct empirical calibration curves if the interfering species is at a constant concentration and always less than one tenth of the measured species. Otherwise, the concentration of the ion to be measured must exceed that of the other by a factor of at least 30.

THE EFFECT OF HYDROGEN IONS—

When the pH is changed in a buffered medium at a constant pK level, the observed pK value is affected to extents shown in Figure 3. Similar results are obtained with $pNH_4$ responses. It can be seen that the greater the concentration of potassium or ammonium ions, the less effect pH has, and that its influence is generally of a small order above a pH value of 6, except for the most dilute solutions of potassium and ammonium ions. However, for the most precise results, it is desirable to add buffer to bring the solution to a pH of 7, although any constant pH up to 9 is satisfactory.

THE EFFECT OF SODIUM IONS—

Figs. 2 (*a*) and 2 (*b*) show the response curves in the absence of sodium salts and with various backgrounds of constant sodium ion concentration. The rule deducible from the Nernst electrode-potential equation is that it is the ratio of concentrations of interfering ion to measured ion that is the variable of primary importance for an electrode responding to both ions[1,2]: the stronger the potassium or ammonium concentration with respect to a given

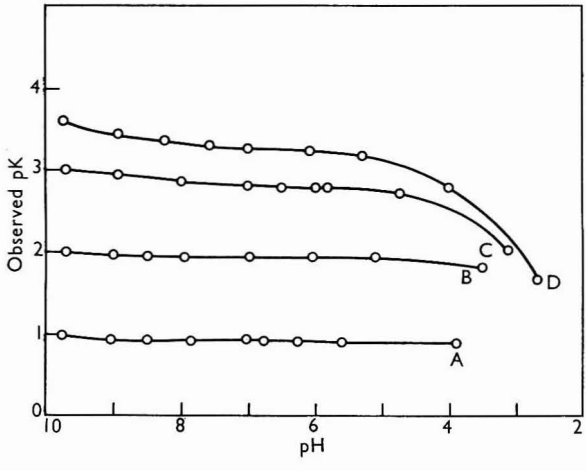



Fig. 3. The effect of pH on the response of BH115 electrodes to potassium ions. Measurements were made by changing the pH in solutions with a constant pK value and a background buffer of 0·1 M ethanolamine *plus* hydrochloric acid (the latter added in the concentrated form to adjust the pH without significantly affecting the volume of the solution). Curves A, B, C and D at pK values of 1, 2, 3 and 4, respectively

sodium concentration, the less interference. The curves of Figs. 2 (*a*) and 2 (*b*) show that up to pK or $pNH_4$ values of 3, interference will occur when the concentration ratio of 1 sodium ion to 5 potassium ions (or 1 sodium ion to 5 ammonium ions) is exceeded. In practice, it

is possible to exceed this ratio by applying a correction based on the sodium content. Two approaches are possible—

(*i*) If the background sodium ion concentration is constant in a series of solutions to be measured, the calibrating solutions themselves can incorporate the same sodium background level.

(*ii*) If the sodium ion concentration is likely to vary, the electrode can be standardised in potassium or ammonium salt solutions containing varying amounts of sodium ion and a series of pseudo sodium ion calibration lines can be drawn.

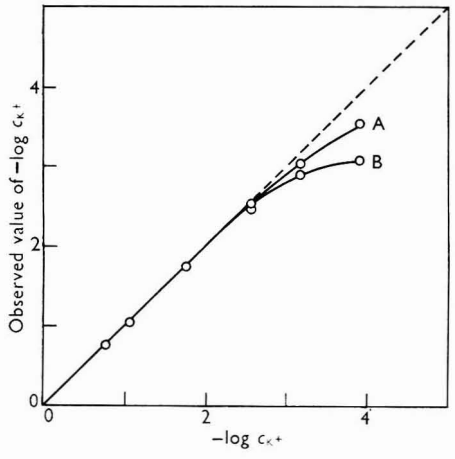



Fig. 4. The response of BH115 electrodes to potassium ions in the presence of differing concentrations of calcium ions: curve A, nil; curve B, 0·1 M The broken line shows the theoretical response. The background buffer was 0·1 M triethanolamine *plus* nitric acid, at pH 7·0

Considerable interest attaches to the possibility of potassium ion and sodium ion measurements on physiological fluids, such as blood, where the ratio of sodium to potassium ions is approximately 30 to 1. Correction techniques based on the assumption of constant correction factors have been applied,[8] but a more satisfactory method involves the construction of a series of response curves for potassium ions in Ringer solutions having various constant backgrounds of sodium salts. The family of curves thus obtained is used to obtain an unknown potassium-ion concentration by reference to the calibration curve for the particular sodium-ion concentration (itself found by measurement of the response of an electrode to sodium ions). In the course of a series of measurements of this kind it is advisable to check the calibration of the electrode by observing its reading in 0·1 M potassium chloride, and if necessary adjusting the pH meter back-off controls to bring the reading back to a given value. Re-calibration is necessary because the presence of a large excess of sodium over potassium causes slow drift, and reference back to a strong, pure potassium solution not only provides the opportunity for checking zero drift but also renews the potassium activity of the surface.

THE EFFECT OF IONS OF ALKALINE-EARTH ELEMENTS—

This effect has been examined with principal reference to calcium ions, and the results are given in Fig. 4. An apparent shift of reading occurs when the ratio $c_{Ca^{2+}}$ to $c_{K^+}$ exceeds about 20 to 1, when a lowering of the pK value of approximately 0·05 unit occurs at about 0·005 M potassium ion concentration. Magnesium ions exert a similar influence.

THE EFFECT OF TEMPERATURE—

The slope factor $\left(\frac{2{\cdot}303RT}{F}\right)$ in the theoretical equation—

$$E = E^0 - \frac{2{\cdot}303RT}{F} \text{ pK (or pNH}_4\text{)}$$

is temperature dependent. The theoretical dependence on temperature has been observed in the pK (or $pNH_4$) range, 0 to 3, so it is possible to use the slope-factor compensation normally available on commercial pH meters (since identical temperature dependence applies in pH measurements). The zero shift $\left(\frac{dE^0}{dT}\right)$ is a $-0{\cdot}002$ pK per °C rise when the reference electrode is at a constant temperature, and approximately a $+0{\cdot}006$ pK per °C rise when the glass electrode and a 3·8 M potassium chloride calomel electrode, used for reference, are both at the changing solution temperature. In the former conditions, zero shifts are obviously quite small in the 15° to 25° C range, but, to obtain the most reproducible results, temperature control should be used.

SPEED OF RESPONSE—

In potassium or ammonium salt solutions containing no, or relatively small, amounts of sodium salts the response time of BH115 electrodes is comparable with that found with pH electrodes, and less than response times normally observed with electrodes responsive to sodium ions. The presence of sodium ions in the solutions causes an increase in these response times, and with an excess of sodium ions they may increase by a factor of 2 or 3.

A reading to within 0·01 pK unit of the equilibrium reading (assumed to be after 10 minutes) is reached in less than 30 seconds when a solution of pK value 1 is displaced by a solution of pK value 3 by using an arrangement such as that shown in Fig. 1, whereas a reading to within 0·005 pK unit is obtained after approximately 1 minute.

It should be noted that these times apply where the technique indicated under "Experimental," p. 350, is used. With wiping or water-washing methods, the times increase markedly.

REPRODUCIBILITY AND STABILITY—

Reproducibility and stability also depend on whether or not an excess of sodium ions is present in the solutions being measured. In a series of 20 measurements on solutions with pK values of 1, 2 and 3, observed in a factorial sequence (to allow for memory effects), reproducibilities in solutions containing no sodium ions were within $\pm 0{\cdot}01$ pK unit for all three solutions. Similar results were obtained in the presence of sodium ions, but some drifting occurred (about 0·05 pK unit in the stronger solutions over a period of 3 to 4 hours). Readings for solutions free from sodium ions drifted by less than 0·01 pK unit even over 2 to 3 days. Curiously, in ammonium salt solutions free from sodium ions, there was a strong tendency to drift (0·1 $pNH_4$ unit in 30 minutes being representative), but behaviour in ammonium salt solutions containing 0·01 M sodium ions was excellent, and comparable with that in potassium salt solutions free from sodium ions. It is a good general practice to store the electrodes in 0·1 M solutions of the ion to be measured. (It should be noted that the results given here apply to those obtained by using the recommended measuring technique.)

FLOW EFFECT—

A flow effect is generally observable with electrodes sensitive to alkali ions,[2,9] and BH115 is no exception. With normal static laboratory measurements or in constant-flow conditions it does not constitute a limitation.

## NOTE ON APPLICATIONS

In addition to direct analytical applications,[7] electrodes responsive to potassium ions are finding some use in physiological studies.[6 to 10] Their use as indicator electrodes in titrations has been shown by Geyer and Frank,[11] who used calcium tetraphenylboron as titrant;

sodium tetraphenylboron is unsatisfactory, owing to the development of an undesirably high ratio of sodium ions to potassium ions in the solution as the titration proceeds.[7] Application to the determination of ion-association constants of potassium or ammonium ions with various anions can also be envisaged, since the response is essentially to ionic activities, and not to total concentrations.

We thank the Directors of Electronic Instruments Ltd., for permission to publish this Paper.

## References

1. Eisenman, G., Rudin, D. O., and Casby, J. U., *Science*, 1957, **126**, 831.
2. Mattock, G., *Analyst*, 1962, **87**, 930.
3. Mattock, G., and Uncles, R., *Ibid.*, 1962, **87**, 977.
4. Bishop, E., and Dhaneshwar, R. G., *Ibid.*, 1963, **88**, 424.
5. Eisenman, G., *Biophys. J.*, 1962, **2**, 259, Supplement.
6. Portnoy, H. D., Thomas, L. M., and Gurdjian, E. S., *Talanta*, 1962, **9**, 119.
7. Mattock, G., *in* West, P. W., Macdonald, A. M. G., and West, T. S., *Editors*, "Analytical Chemistry, 1962," Elsevier Publishing Company, Amsterdam, New York and London, 1963, p. 247.
8. Friedman, S. M., Jamieson, J. D., Nakashima, N., and Friedman, C. L., *Science*, 1959, **130**, 1252.
9. Hinke, J. A. M., *J. Physiol.*, 1961, **156**, 314.
10. Friedman, S. M., and Friedman, C. L., *Anat. Rec.*, 1960, **138**, 129.
11. Geyer, R., and Frank, H., *Z. anal. Chem.*, 1961, **179**, 99.

Received *August 20th*, 1963

# The Copper-catalysed Oxidation of Hydroxylamine

By J. H. ANDERSON*
(*Long Ashton Research Station, University of Bristol*)

The oxidative loss of hydroxylamine at pH 8 in the presence of low concentrations of copper ions and air was associated with the formation of traces of nitrite and only a small uptake of oxygen. The loss was inhibited by metal-binding agents or by the absence of air. In the absence of air and with excess of cupric sulphate solution, a gas (probably nitrous oxide) was formed, sufficient to account for the complete oxidation of hydroxylamine. These results suggested that the catalysis in air involved the oxidation of hydroxylamine mainly to nitrous oxide by the reduction of cupric ions to the cuprous state; cuprous ions were then oxidised by the reduction of oxygen. A possible mechanism for the formation of nitrous oxide and traces of nitrite has been suggested.

THE inorganic nitrogen compound hydroxylamine is somewhat unstable in neutral or alkaline solution. Moews and Audrieth[1] found that 400 mM solutions of hydroxylamine at pH 9 decomposed in the presence of oxygen, and that the rate of oxidative loss was increased by adding metal ions, particularly copper. The loss of hydroxylamine in the presence or absence of copper was inhibited by adding metal-binding agents in the presence of oxygen, or by the absence of air. Other work,[2] in which copper ions and lower concentrations of hydroxylamine (1 mM) buffered at pH 8 were used gave essentially the same results. Since the loss of hydroxylmine in air in the presence of low concentrations of copper was accompanied by a negligible uptake of oxygen and the formation of only traces of nitrite, but no ammonia, it was concluded[2] that the main reaction was the oxidation of hydroxylamine to nitrous oxide. In studies on the biological transformations of hydroxylamine (at pH 6 to 9), non-enzymic reactions are sometimes encountered; these may be due to metal-ion impurities (copper) in the reagents. The purpose of the present work was to investigate more closely the products of the copper-catalysed oxidation at pH 8.

## METHODS

REAGENTS—

All reagents should be of analytical-reagent grade whenever possible.

*De-ionised water.*

*Hydrochloric acid*—Purify by distilling a constant boiling-point mixture.

*Sodium hyponitrite*—Prepare the solution according to the method of Addison, Gamlen and Thompson.[3] Solutions in 0·1 N sodium hydroxide are stable.

*Succinate buffer*—Prepare a solution of succinic acid and adjust the pH to 4 with sodium hydroxide. The final concentration should be 0·1 M.

*Phosphate buffer*—Prepare a solution of potassium dihydrogen orthophosphate and adjust the pH to 8·0 with sodium hydroxide. The final concentration should be 0·2 M.

*Tris-hydrochloric acid buffer*—Prepare a solution of 2-amino-2-hydroxymethylpropane-1,3-diol at pH 8·0. The final concentration should be 0·25 M.

*Tris - hydrochloric acid buffer containing copper sulphate*—Prepare solutions at pH 8·0 containing 1 to 25 mM copper sulphate and 0·25 M tris buffer.

*2-Amino-2-methylpropane-1,3-diol - hydrochloric acid buffer*—Prepare a solution at pH 8·0 so that the final concentration is 0·2 M.

*Potassium hydroxide*, 2N.

*Suspension of calcium hydroxide in calcium cyanide*—Prepare a suspension containing 10 per cent. of calcium hydroxide in 0·42 M calcium cyanide.

*Sodium sulphite in sodium hydroxide*—Prepare a solution containing 10 per cent. of sodium sulphite in 0·5 N sodium hydroxide.

* Present address: Department of Physiology, University of Bristol.

PROCEDURES—

Ammonia was determined by the method of Conway.[4] Hydroxylamine, by oxidation with iodine, and nitrite were determined by previously described methods.[5]

Reactions were carried out at 30° C. Those used for studying the rate of oxidative loss of hydroxylamine were performed in 100-ml conical flasks. In these conditions the mixtures, whose volume was 5 ml, were shallow and well aerated.

Reactions used for investigating the stability of hydroxylamine under reduced pressure were carried out in 40-ml Thunberg tubes.[6] The volume of the reaction mixture was 5 ml. The vessels were evacuated with a rotary oil vacuum-pump until the saturated water-vapour pressure of 10 to 15 mm of mercury was attained. Smaller tubes (18 ml) were used for studying the rate of reduction of copper sulphate by hydroxylamine under reduced pressure. A calibration curve for solutions at pH 8·0 of 5 to 100 $\mu$moles of copper sulphate in 5 ml of 200 mM tris buffer was prepared by using an Evans Electroselenium Ltd. colorimeter having a green filter (OGRI) with a maximum transmission at 530 m$\mu$.

Changes in the volume of gases, resulting from the oxidation of hydroxylamine, were measured by performing the reactions in Warburg constant-volume respirometers[6] having 16-ml flask-volumes. The centre well contained 0·2 ml of 2 N potassium hydroxide for absorbing carbon dioxide, or for cyanide inhibitions, 0·2 ml of a suspension of calcium hydroxide in calcium cyanide solutions.[7] An inert atmosphere was obtained by de-gassing the apparatus for 20 minutes with oxygen-free nitrogen. A differential method was used[5] for determining the nitric oxide content of nitrogenous gases, in which the centre well contained 0·2 ml of 10 per cent. of sodium sulphite in 0·5 N sodium hydroxide, which converted nitric oxide to the stable dinitrososulphite. Reactions were initiated by adding hydroxylammonium chloride from the side arm, bringing the total reaction volume to 2·5 ml. The flasks were shaken at 80 cycles per minute.

The fall in pH of reactions caused by the addition of hydroxylamine as its hydrochloride (hydroxylammonium chloride) was 0·2 to 0·3 unit; the pH values given are those measured after the reactions.

## RESULTS

COPPER CATALYSIS IN AIR—

Table I shows that mM hydroxylamine was stable for 60 minutes at pH 7·7 to 7·8 in the presence of air. In the presence of 1 $\mu$M cupric sulphate, hydroxylamine was lost and traces of nitrite appeared. Increasing the copper concentration from 1 to 1000 $\mu$M increased the rate of loss of hydroxylamine. There was no loss of hydroxylamine when the copper was replaced by one of the salts listed below at a concentration of 0·1 mM—

Zinc, manganese$^{II}$, magnesium, cobalt$^{II}$, nickel, aluminium, iron$^{II}$, iron$^{III}$, cadmium, vanadyl$^{IV}$ or chromium$^{III}$ sulphates
Potassium iodide or potassium bromide
Calcium, strontium, barium, tin$^{IV}$ or titanium$^{IV}$ chlorides
Sodium molybdate, sodium arsenite, or sodium tetraborate

Many of the cations formed precipitates of hydroxide or phosphate during the reaction, as did copper at the same concentration. After incubation for 5 minutes in the presence of mercuric chloride or silver sulphate, small losses of hydroxylamine (approximately 200 m$\mu$moles) and small productions of nitrite occurred, but there was no further reaction. These results suggested that cupric, mercuric and silver salts were reduced by hydroxylamine, but that the loss of hydroxylamine was continuous only in the presence of copper ions.

Millimolar hydroxylamine solution was stable in the presence of 10 $\mu$M copper sulphate with 20 mM succinate as buffer at pH 4·0. Since the p$K$ value for the dissociation—

$$NH_3OH^+ \rightleftharpoons NH_2OH + H^+$$

is 5·9, the oxidative loss of hydroxylamine was due to the reaction of copper with the uncharged form.

BINDING OF COPPER—

In the presence of catalytic amounts of copper, the loss of hydroxylamine was partially inhibited by pork-liver catalase, serum albumin or by heat-inactivated cells of the micro-organisms *Pseudomonas aeruginosa* or *Nitrosomonas* (see Table I). Before use, the cells were

heated at 100° C for 20 minutes to destroy their enzyme activities. Nitrite was still produced in the inhibited reactions. The inhibition was probably caused by protein binding the copper ions. The oxidative loss was partially inhibited by tris buffer and completely inhibited by cyanide, EDTA or pyrophosphate. These compounds probably bind the copper ion.

TABLE I

INHIBITORS OF THE COPPER-CATALYSED OXIDATIVE LOSS OF HYDROXYLAMINE

Reactions were carried out in 100-ml conical flasks containing 100 μmoles of phosphate buffer solution at pH 8·0, cupric sulphate solution and other additions as indicated, in 5 ml of solution. The reactions were started by adding 1000 mμmoles of hydroxylammonium chloride per ml of solution. Final pH values of the solutions were 7·7 to 7·8

| Concentration of cupric sulphate, μM | Additions | Recovery of hydroxylamine and production of nitrite, mμmoles per ml | | | | | |
|---|---|---|---|---|---|---|---|
| | | after 5 minutes | | after 30 minutes | | after 60 minutes | |
| | | Hydroxylamine | Nitrite | Hydroxylamine | Nitrite | Hydroxylamine | Nitrite |
| 0 | None | 1000 | 0 | 1000 | 0 | 975 | 0 |
| 1 | None | 990 | 0 | 770 | 12 | 700 | 15 |
| 10 | None | 793 | 7 | 228 | 32 | 100 | 40 |
| 100 | None | 718 | 12 | 125 | 35 | 60 | 35 |
| 1000 | None | 688 | 12 | 93 | 42 | 75 | 72 |
| 10 | Heat-inactivated cells of *Pseudomonas aerugina*, 0·2 mg dry weight per ml | 1000 | 0 | 1000 | 0 | 960 | 13 |
| 10 | Heat-inactivated cells of *Nitrosomonas*, 0·2 mg, dry weight per ml | 1000 | 0 | 960 | 5 | 950 | 17 |
| 10 | Catalase, 100 μg per ml | 1000 | 0 | 850 | 2 | 830 | 2 |
| 10 | Albumin, 100 μg per ml | 1050 | 0 | 1000 | 0 | 930 | 2 |
| 10 | Tris buffer, 20 mM at pH 8·0 | 1000 | 0 | 900 | 0 | 760 | 10 |
| 10 | Pyrophosphate, 10 mM at pH 8·0 | 1050 | 0 | 1000 | 2 | 1000 | 2 |
| 100 | Potassium cyanide, 1 mM | 1000 | 0 | 1000 | 0 | 980 | 2 |
| 100 | EDTA, 1 mM | 1000 | 0 | 970 | 0 | 1000 | 0 |

REACTION PRODUCTS IN AIR—

Twenty μmoles of hydroxylammonium chloride, adjusted to pH 8 with sodium hydroxide solution, were added to 2·5 ml of 0·2 mM cupric sulphate solution in the presence of air, in the Warburg respirometers, with 400 mM phosphate solution to buffer the solutions at pH 6·5, 7·5, 8·0 and 9·0. The loss of hydroxylamine was complete within 3 hours. Small uptakes of oxygen (0·5 to 1·4 μmoles) were attained slowly, showing that peroxide or hyponitrite did not accumulate. Nitrite (0·25 to 0·4 μmole), but no ammonia, was found in the residual solutions. Since the nitrite productions were small and the equation—

$$2NH_2OH + O_2 = N_2O + 3H_2O \quad .. \quad .. \quad .. \quad .. \quad (1)$$

does not involve a change in the volume of the gases, it follows that most of the hydroxylamine was oxidised to nitrous oxide. Taking into account the uptake of oxygen (calculated to be approximately 1·9 μmoles), which resulted from the greater solubility of nitrous oxide compared with that of oxygen, the oxygen consumption was in agreement with the production of nitrite according to the equation—

$$NH_2OH + O_2 \rightleftharpoons NO_2^- + H_2O + H^+ \quad .. \quad .. \quad .. \quad (2)$$

STABILITY OF HYDROXYLAMINE IN THE ABSENCE OF AIR—

The recovery of 5 μmoles of hydroxylammonium chloride that were added under reduced pressure to 5 ml of 0·1 mM copper sulphate buffered at pH 6·5 to 9·0 with 200 mM phosphate buffer or at pH 9·5 with 40 mM 2-amino-2-methylpropane-1,3-diol buffer was greater than 90 per cent. throughout a period of 2 hours, and no nitrite was formed. There was no loss of hydroxylamine, probably because the oxidation of cuprous ions did not proceed.

The recoveries of 5 μmoles of hydroxylamine and 2·5 μmoles of nitrite present together at pH 7·8 in 5 ml of 40 mM phosphate buffer with or without 0·1 mM copper sulphate were

quantitative (95 to 103 per cent.) throughout a period of 2 hours. In air, 0·5 mM nitrite did not affect either the rate at which mM hydroxylamine was lost in the presence of 10 $\mu$M copper, or the production of nitrite.

FORMATION OF GAS FROM HYDROXYLAMINE AND COPPER SULPHATE—

A nitrogenous gas was evolved when 15 $\mu$moles of hydroxylammonium chloride were added to solutions of 500 $\mu$moles of tris buffer and 5 to 50 $\mu$moles of copper sulphate solution at pH 8·0 in the Warburg respirometer in the absence of oxygen. The gas formations were complete in 30 to 60 minutes; the final pH values were 7·8. No nitric oxide was formed

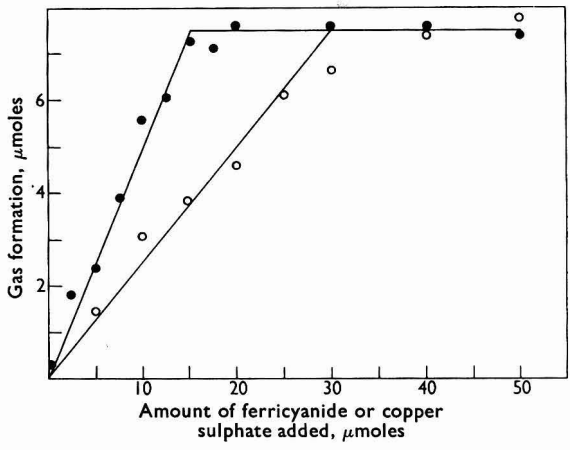



Fig. 1. The relationship between the gas formed from hydroxylamine and the amount of electron acceptor added. Gas formations ($\mu$moles): ●, with ferricyanide as the electron acceptor; ○, with copper sulphate as the electron acceptor

because the same amount of gas was produced when alkaline sulphite was present in the centre well. The amount of gas produced with 5 to 30 $\mu$moles of copper sulphate was approximately equivalent to a quarter of the copper added as an electron acceptor (see Fig. 1), suggesting that the gas was mainly nitrous oxide, produced according to the equation—

$$2NH_2OH + 4Cu^{2+} = N_2O + H_2O + 4Cu^{+} + 4H^{+} \quad .. \quad .. \quad (3)$$

The theoretical yield of gas was obtained with 30 to 50 $\mu$moles of copper sulphate.

The reduction of 100 $\mu$moles of copper by 30 $\mu$moles of hydroxylamine in 5 ml of similar mixtures was followed colorimetrically in Thunberg tubes under reduced pressure at 20° C. The rate of reaction was parallel to the formation of gas, and after 50 minutes the stationary value, representing a quantitative reduction of 56 $\mu$moles of copper, was obtained. After the mixture had been shaken in air, the recovery of copper$^{II}$ was quantitative.

When the cupric sulphate was replaced by 50 $\mu$moles of a suspension of silver acetate in tris buffer at pH 8·0 in the Warburg respirometer, 1 $\mu$mole of gas was formed in 20 minutes; when the cupric sulphate was replaced by 50 $\mu$moles of a solution of mercuric chloride in the tris buffer at pH 8·2 in the Warburg respirometer, 2·8 $\mu$moles of gas were formed in 20 minutes; nitric oxide was not formed in either experiment. Traces of nitrite were found in the residual reaction mixtures. These reactions confirmed that mercuric and silver ions oxidised hydroxylamine.

FORMATION OF GAS FROM HYDROXYLAMINE AND FERRICYANIDE—

Gas was also produced in the Warburg respirometer, in the absence of air, from hydroxylamine at pH 8, with ferricyanide as the electron acceptor. The gas formations were complete in 20 minutes and no nitric oxide was formed. The reaction was probably catalysed by a

metal ion because the gas formation was markedly inhibited by EDTA, pyrophosphate and heat-inactivated cells of *Nitrosomonas*, and to a lesser extent by cyanide (see Table II). Low concentrations of copper sulphate increased the rate of gas formation, and this reaction was

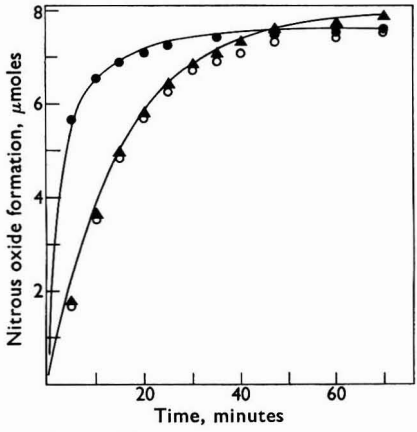



Fig. 2. The course of nitrous oxide formation at pH 8 from (*i*) hydroxylamine and copper, (*ii*) the decomposition of hyponitrite. Nitrous oxide formations (μmoles): (*i*) when 7·6 μmoles of hyponitrite in 0·2 ml of 0·1 N sodium hydroxide solution were added from the side-arm to: ▲, 500 μmoles of tris buffer at pH 8·0; ○, a solution at pH 8·0 of 500 μmoles of tris buffer *plus* 50 μmoles of copper sulphate; (*ii*) ●, when 15 μmoles of hydroxylammonium chloride were added to a solution at pH 8·0 of 500 μmoles of tris buffer *plus* 50 μmoles of copper sulphate

## TABLE II

### INHIBITION OF THE RATE OF GAS FORMATION FROM HYDROXYLAMINE AND FERRICYANIDE

Reactions were carried out in Warburg flasks containing 200 μmoles of tris buffer solution, pH 8·0, 100 μmoles of ferricyanide and other additions as indicated, in 2·5 ml of solution. Hydroxylammonium chloride (15 μmoles) was added from the side-arm. The centre well contained 0·2 ml of 2 N potassium hydroxide. Atmosphere nitrogen. Final pH value, 7·8

| Additions | Rate of gas formation, μmoles per 8 minutes |
|---|---|
| None | 4·9 |
| 1 mM EDTA | 1·5 |
| 0·1 mM copper sulphate | 7·8 |
| 0·1 mM copper sulphate *plus* 1 mM EDTA | 1·7 |
| 1 mM cyanide | 3·8 |
| Heat-inactivated cells of *Nitrosomonas*, 0·8 mg dry eight per ml | 2·2 |
| 10 mM pyrophosphate | 1·6 |

inhibited by EDTA. The catalytic ion was probably replaceable by copper. The gas produced from 15 μmoles of hydroxylamine when the ferricyanide concentration was increased from 2·5 to 15 μmoles was approximately equivalent to half the ferricyanide added (see Fig. 1), suggesting that the main gaseous product was nitrogen, produced according to the equation—

$$2NH_2OH + 2Fe(CN)_6^{3-} = N_2 + 2Fe(CN)_6^{4-} + 2H_2O + 2H^+ \quad .. \quad .. \quad (4)$$

Ferricyanide possibly maintained the catalytic metal ion in the oxidised state. At pH 8, however, 50 μmoles of ferrocyanide were not oxidised in the presence of air in the Warburg respirometer, with or without 0·1 mM copper sulphate.

FORMATION OF NITROUS OXIDE FROM HYPONITRITE—

The rate at which hyponitrite decomposes spontaneously to nitrous oxide suggested that the decomposition might be involved in the formation of the gas from hydroxylamine and copper: at pH 8, however, the formation of nitrous oxide from hyponitrite, which is a first-order reaction,[8] was less rapid (see Fig. 2). It was therefore necessary to find the effect of copper on the rate of decomposition of hyponitrite. The presence of 50 $\mu$moles of copper sulphate in tris buffer at pH 8 did not affect the rate of decomposition of hyponitrite under nitrogen (see Fig. 2). It therefore seems unlikely that hyponitrite is involved in the formation of nitrous oxide from hydroxylamine. In air, at pH values between 7 and 9, the rate of decomposition of hyponitrite was not affected by the presence of 0·1 mM copper sulphate, and no nitrite was formed. The rate of formation of gas from hyponitrite at pH 8 was not affected by the presence of 50 $\mu$moles of ferricyanide with or without 0·1 mM copper sulphate.

## DISCUSSION

In the absence of oxygen, the amounts of copper$^{II}$ reduced and of gas produced from hydroxylamine with limited amounts of copper were sufficient to account for the formation of nitrous oxide as the main product; see equation (3). The amount of nitrous oxide formed in air was equivalent to the oxygen required for the oxidation of cuprous ions according to the equation—

$$4Cu^{+} + O_2 + 4H^{+} = 4Cu^{2+} + 2H_2O \quad .. \quad .. \quad .. \quad (5)$$

It seems that the copper-catalysed oxidation of hydroxylamine in air to nitrous oxide is due to the reactions represented by equations (3) and (5).

Since the recovery of hydroxylamine incubated with low concentrations of copper ions under reduced pressure was quantitative, and no ammonia was formed by the catalysed oxidation of hydroxylamine in air, the reduction of hydroxylamine by cuprous ions did not proceed. The findings that silver acetate and mercuric chloride slowly oxidised hydroxylamine, but did not catalyse the oxidative loss, is probably owing to the lack of oxidation of silver and of the mercurous ion by oxygen. In contrast with the present work, Moews and Audrieth[1] found that metal ions other than copper catalysed the oxidative loss of hydroxylamine in oxygen. They did not, however, investigate the products of the reaction. Possibly in their experimental conditions (pH 9) these metal ions were reduced by the oxidation of hydroxylamine and slowly oxidised by oxygen, or by the reduction of hydroxylamine to ammonia.

Two facts suggest that hyponitrite is not involved in the copper-catalysed oxidation of hydroxylamine to nitrous oxide at pH 8. Firstly, the formation of nitrous oxide from hydroxylamine and excess of copper in an inert atmosphere is more rapid than the spontaneous decomposition or hyponitrite to nitrous oxide, a reaction that proceeds at the same rate in the presence of absence of copper. Secondly, in air the catalysed oxidation of hydroxylamine gave rise to small amounts of nitrite, but the decomposition of hyponitrite in the presence or absence of copper did not.

The oxidation of hydroxylamine by copper as represented by reaction (3) is a complicated reaction since it involves the interaction of six atoms and molecules. Possibly the copper-catalysed oxidation of hydroxylamine involves a transient intermediate compound containing a single nitrogen atom, such as nitroxyl, NOH. This might decompose spontaneously to nitrous oxide with such speed that only small amounts are oxidised to nitrite.

## REFERENCES

1. Moews, P. C., and Audrieth, L. F., *J. Inorg. Nucl. Chem.*, 1959, **11**, 242.
2. Anderson, J. H., Ph.D. Thesis, University of Aberdeen, 1959.
3. Addison, C. C., Gamlen, G. A., and Thompson, R., *J. Chem. Soc.*, 1952, 338.
4. Conway, E. J., "Microdiffusion Analysis and Volumetric Error," Fourth Edition, Crosby Lockwood & Son Ltd., London, 1957.
5. Anderson, J. H., *Biochem. J.*, 1964, **91**, 8.
6. Umbriet, W. W., Burris, R. H., and Stauffer, J. F., "Manometric Techniques," Third Edition, Burgess Publishing Co., Minneapolis.
7. Robbie, W. A., *Meth. Med. Res.*, 1948, **1**, 307.
8. Anderson, J. H., *Analyst*, 1963, **88**, 494.

First received *May* 3*rd*, 1963
Amended *October* 15*th*, 1963

# SHORT PAPERS

## The Estimation of Volatile Fatty Acids by Gas Chromatography with Automatic Titration

By J. M. A. TILLEY, R. J. CANAWAY and R. A. TERRY
(*The Grassland Research Institute, Hurley, Maidenhead, Berkshire*)

In their first Paper on gas chromatography, James and Martin[1] described a technique for determining the relative proportions of volatile fatty acids in solution. This method was soon applied to the analysis of the proportions of these acids in sheep-rumen liquor,[2] and for this purpose it still has advantages over alternative gas-chromatographic procedures that use more elaborate detector systems. This is because a relatively large amount (0·03 milliequivalents of total acid) can be applied to the column, sample preparation is simple and the result is obtained directly in terms of acid equivalents that do not require correction or calibration of the instrument. Unfortunately, the detector equipment is not available commercially; this Paper describes modifications to the original technique of sample preparation and to the design of the titrator that have been in use here for several years.

Titration with a Titrigraph (Radiometer Ltd.) at constant pH has been described.[3] The method outlined below seems to compare favourably in sensitivity; the minimum addition of titrant is about 0·01 to 0·02 ml of 0·01 N sodium hydroxide, though the volume varies according to the mixing conditions within the cell.

### Method

Apparatus—

The automatic burette is based on that described by James and Martin,[1] with the modifications described below. A magnetic clutch is fitted between the lead screw and its motor. Instead of starting and stopping the motor, the photoelectric cell signal is used to actuate the clutch and make or break the mechanical link between the motor and the screw. The burette was built from parts of a surplus United States Army Signal Corps Recorder RD-7/APA 23 (Gamewell Company) and incorporated the lead screw and the magnetic clutches, and also the mechanism for feeding chart paper from a reel; however, suitable magnetic clutches are now available here from Westool Ltd. or Crofts (Engineers) Ltd. The lead screw has an effective length of 5·5 inches of chart paper, has 14 turns per inch and rotates at 21 r.p.m. The speed of the chart paper is 30 inches per hour. The burette rod has a diameter of 0·219 inch. By using 0·01 N sodium hydroxide, a total of 3 ml of titrant can be added at a maximum rate of 0·8 ml per minute. At a gas flow-rate of 40 to 50 ml of nitrogen per minute, acetic acid can be titrated in full before the appearance of the next acid to be eluted (propionic); about 20 minutes will be required for the titration of these and the two butyric acids.

Fig. 1 shows the photo-electric control unit. The sensitivity of the circuit can be altered by varying the ratio of the resistors, $R_1$ to $R_2$, so as to change the operating voltage of the photo-electric cell. The high-tension secondary voltage is not critical between, say, 125 and 220 V, but the values of $R_1$ and $R_2$ should be chosen to maintain about 70 V r.m.s. at the photo-electric cell, and of $R_5$ to allow about 10 mA a.c. to pass through the 1000-ohm variable resistor, $R_6$.

A separate 8-V transformer supplies the 12-V, 21-watt lamp. The lamp and photo-electric cell are mounted in an aluminium housing that straddles the titration cell, so that the tip of the burette lies in the centre of the light-path. The housing need not be completely light-tight and, provided the aperture is of a suitable size, an adjustable shutter is not required. Minor adjustment of the sensitivity of the unit can be made through $R_6$.

Of the other parts of the apparatus described by James and Martin,[1] the manostat is not necessary, and a simple two-stage gas-pressure regulator is adequate. The vapour jacket and the titration cell are unmodified, except that a drain-tap is provided for the cell. Fresh, slightly acidified indicator solution at 100° C is added before each analysis. The solution is then titrated to the end-point in order to give the base-line for the analysis.

PROCEDURE—

Take rumen contents from an animal and strain them immediately through butter muslin. Spin the expressed liquid in a centrifuge at 1800 *g* for 15 minutes, and by using a pipette transfer 2 ml of the supernantant liquid into a 50-ml glass-stoppered test-tube. Add 0·5 ml of 88 per cent. orthophosphoric acid and 20 ml of diethyl ether, stopper the tube and shake it well. Add 15 g of anhydrous sodium sulphate, stopper again, shake the tube and leave it overnight at room temperature. The sample is now in a stable form.

Prepare the column as described by James and Martin,[1] but use 120-mesh Celite as a column support without further preparation. To introduce the acids on to the column, place 1 or 2 ml of the dried ether extract in a test-tube held in a water-bath at 35° C, and evaporate the ether through the column with a stream of air. For this operation, connect the test-tube to a glass T-piece; slide the column through a rubber sleeve and through the T-piece so that its inlet end is just above the level of the ether in the test-tube. Draw air that has previously been dried over concentrated sulphuric acid through the side-arm of the T-piece, over the surface of the ether and up through the column (which is held vertically). After the ether has evaporated, continue the flow of air while heating the test-tube at 100° C for 5 minutes; this evaporates the acids on to the column. This completes the preparation[4]; carry out the analysis within a few hours.

ANALYSIS—

Maintain the vapour jacket at 100° C with steam, and elute the acids from the column with nitrogen. Acetic, propionic, iso- and n-butyric and iso- and n-valeric acids separate in that order. The trace on the chart consists of a series of steps, the height of each being equivalent to the titration of an acid. From the relative height of the steps, the proportions of the acids can be calculated in acid equivalents (see Fig. 2). The absolute content of each acid can be calculated if the normality of the alkali is known, or may be estimated by determining the total steam-volatile acids by distillation.

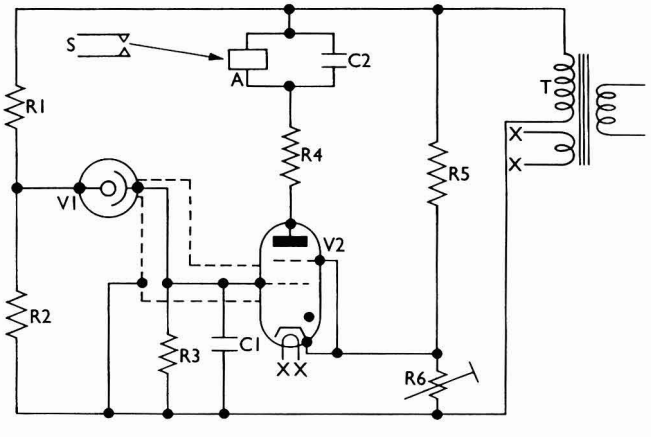



Fig. 1. Circuit of photoelectric control unit

$R_1$ = 150,000-ohm high stability resistor
$R_2$ = 68,000-ohm high stability resistor
$R_3$ = 10-megohm high stability resistor
$R_4$ = 10,000-ohm 2-watt resistor
$R_5$ = 20,000-ohm wire-wound resistor
$R_6$ = 1000-ohm wire-wound resistor
T = 220-watt transformer with a 6·3-V heater supply

$C_1$ = 0·005-$\mu$F capacitor
$C_2$ = 1-$\mu$F capacitor
$V_1$ = VA26T photoelectric cell
$V_2$ = 2D21 valve
A = 3000-type relay (5000-ohm resistance)
S = Lead-screw clutch switching
X = Leads to heater on $V_2$

## Results

In rumen liquor, the content of the valeric acids is small (less than 6 per cent.). Since the total titration with 0·01 N sodium hydroxide cannot exceed 3 ml and the minimum titration is 0·01 to 0·02 ml, the error of the determination of such minor components is thus quite large.

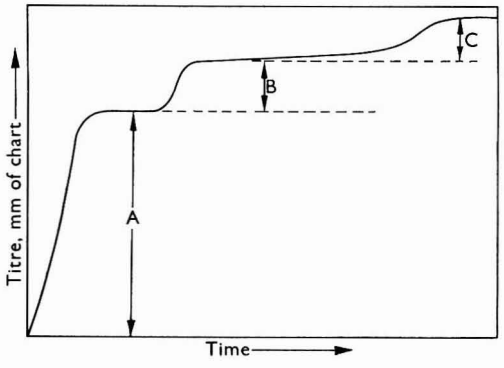



Fig. 2. Typical titration curve for a fatty acid; A, acetic acid, 67·3 mm equivalent to 71·8 per cent.; B, propionic acid, 15·5 mm equivalent to 16·5 per cent.; C, iso- and n-butyric acid, 11·0 mm equivalent to 11·7 per cent.

Results have therefore been reported only in terms of the relative contents of acetic, propionic and the sum of the butyric acids[5]; when expressed as a percentage of their total titration, the relative proportion of each of the three acids, measured in duplicate analyses of the same sample of rumen liquor, should not vary by more than 1 per cent. These acids represent only the most volatile of the fatty acids in rumen liquor; less volatile acids can be removed from the column by purging with nitrogen for 1 hour at 100° C. The column is then ready for re-use; owing to displacement of the liquid phase, the efficiency of separation gradually drops so that only about twenty analyses can be performed before re-packing becomes necessary.

## References

1. James, A. T., and Martin, A. J. P., *Biochem. J.*, 1952, **50**, 679.
2. El-Shazly, K., *Ibid.*, 1952, **51**, 640.
3. Aunstrup, K., and Djurtoft, R., *Radiometer News*, 1960, No. 1, 4.
4. Halse, K., and Velle, W., *Acta Physiol. Scand.*, 1956, **37**, 380.
5. Grassland Research Institute, *Exps. Prog. Grassld. Res. Inst.*, 1961, No. 13, 79.

Received *October 24th*, 1963

# A Rapid Titan Yellow Method for Determining Magnesium in Plant Material in the Presence of an Excess of Manganese

By E. M. CHENERY

(*The Tea Research Institute of East Africa, P.O. Box* 91, *Kericho, Kenya, East Africa*)

The method described in this Paper was developed in the first instance for tea leaves, but it will, no doubt, be suitable for many other materials once they are in solution.

Tea bushes are notorious for their propensity to take up abnormal amounts of manganese and aluminium from the soil. Most soils where African tea is grown bear large amounts of manganese, and consequently tea leaves from bushes grown in these soils may contain up to 1 per cent. of manganese.[1,2] The determination of magnesium in tea leaves by any of the customary chemical methods necessitates the removal of manganese by using time-consuming procedures. The well known EDTA titration method[3,4] gave consistently high results, despite repeated extractions with

dithizone and chloroform. The Titan yellow method for magnesium offered little hope, initially, since the technique for removing the excess of manganese described by Hunter[5] was found not to work for old tea leaves. Variants of Allen's method[6,7] were tried, and a reaction (believed to be hitherto unrecorded) was accidentally discovered that solved the manganese problem satisfactorily. This was simply to add to the ash solutions a Titan yellow reagent containing a large excess of triethanolamine, an excess of potassium ferricyanide and then a strong solution of sodium hydroxide. Under these conditions manganese produces a brown colour whose intensity is proportional to the concentration of manganese. More than 500 μg of manganese completely obscures the red magnesium complex. After the solution has been set aside for 10 to 15 minutes the brown colour disappears leaving the magnesium colour unimpaired. The manganese brown colour reappears if the solution is set aside overnight.

Triethanolamine[8,9] minimises aluminium interference to a constant intensification[4] of the red magnesium complex. Bradfield's technique of using 1,2-bis-(2-aminoethoxy)-ethane-*NNN'N'*-tetra-acetic acid, EGTA, to inhibit the precipitation of calcium phosphate, worked most efficiently. Hydroxylamine hydrochloride[11] improves recovery of magnesium in the presence of manganese by about 10 per cent. The method described below is for rapid routine determinations of 10 to 60 μg of magnesium in 5 ml of ash solution, in the presence of up to 400 μg of manganese II, 1500 μg of aluminium, 1000 μg of iron III, 1000 μg of calcium, 1000 μg of phosphorus and 1000 μg of fluorine. The recovery achieved is 90 to 100 per cent. of magnesium with these amounts of single accompanying ions, all of which are far in excess of the amount normally present in portions of ash solutions.

The results of a typical recovery trial in which up to 800 μg of manganese were added to an ash solution containing 22·5 μg of magnesium are given below—

| | | | | | | |
|---|---|---|---|---|---|---|
| Manganese present, μg .. | .. | 23 | 223 | 423 | 623 | 823 |
| Magnesium found, μg .. | .. | 22·5 | 22·5 | 21·5 | 21·0 | 21·0 |
| Recovery, per cent. .. | .. | — | 100 | 95·5 | 93·3 | 93·3 |

## Method

### Preparation of samples—

Weigh 0·1-g samples of oven-dried leaves into specimen tubes (25 mm × 50 mm) and heat them overnight, or for 6 hours, at 475° C in a muffle furnace. To the cold ashes (usually brown in colour) add 4 drops of hydrochloric acid (1 + 1) and 4 drops of hydrogen peroxide (25 volumes), while holding the tubes in a slantwise position; evaporate the solutions to dryness on a water-bath. By using a pipette, place 10 ml of 0·05 N hydrochloric acid into each tube, stopper the tubes tightly with corks covered with a thin polythene film and shake them in a reciprocating machine for 30 minutes. These stock solutions can be used for determining calcium, potassium, magnanese and phosphorus as well as magnesium.

### Reagents—

*Potassium ferricyanide solution*—Prepare a 25 per cent. w/v solution from analytical-reagent grade material.

*Sodium hydroxide solution*—Prepare a 50 per cent. w/v solution from analytical-reagent grade material, and store the solution in a polythene bottle.

*Compensating solution*—Dissolve 10 g of EGTA, 1·34 g of hydroxylamine hydrochloride and 1·48 g of hydrated aluminium sulphate $Al_2(SO_4)_3.18H_2O$, in 50 ml of water. Add 20 ml of triethanolamine and dilute to 100 ml with water.

*Standard magnesium solution*—Make up standard solutions containing 10 to 60 μg of magnesium as magnesium sulphate ($MgSO_4.7H_2O$) or as Specpure magnesium metal dissolved in 0·05 N hydrochloric acid.

*Titan yellow stock solution*—Dissolve 2·5 g of Titan yellow dye (this weight varies with the batch and must be adjusted to give maximum absorption for 60 μg of magnesium) and 6 g of boric acid in water. Mix the solution well, add 8 ml of 50 per cent. sodium hydroxide solution and dilute to 2½ litres with water. Store the solution in an amber-glass bottle in the dark.

*Titan yellow working reagent*—Transfer 25 ml of Titan yellow stock solution into a 100-ml calibrated flask, add 25 ml of freshly filtered 2 per cent. w/v poly(vinyl alcohol) and dilute the solution to the mark with triethanolamine.

All working reagents should be dispensed with rubber-bulb dropping-tubes from 50-ml bottles, so that the drops are 0·05 ml in volume.

PROCEDURE—

Transfer 1-ml portions of stock ash solution with a pipette into calibrated 5-ml hard-glass test-tubes (15 mm × 150 mm). Add 3 drops of compensating solution to each tube and mix the contents well by swirling. To each tube add as quickly as possible 1 ml of Titan yellow working reagent, 2 drops of potassium ferricyanide (if over 200 μg of manganese are present, 4 drops of potassium ferricyanide are necessary), and 6 drops of sodium hydroxide solution: mix the solutions by swirling, dilute them to 5 ml with water and swirl them again. Set them aside for 30 minutes and then measure the optical density of the solutions in 1-cm cells by using an Ilford green (404) filter in a suitable absorptiometer. Greater precision can be achieved with a spectrophotometer, such as the Unicam SP500, operating at a wavelength of 545 mμ, and by using pipettes and calibrated flasks rather than droppers and test-tubes. The working range is 10 to 60 μg of magnesium in 5 ml. New calibration curves are usually made for each batch of 24 test-tubes.

CALCULATION—

The percentage magnesium equals the number of micrograms of magnesium found in a 1-ml portion of ash solution divided by 100.

## RESULTS

Magnesium was determined in samples of tea, sisal and soils with a simple absorptiometer and by atomic-absorption spectrophotometry. Agreement in the figures obtained was good, as can be seen from Table I.

TABLE I

COMPARISON OF MAGNESIUM FOUND BY PROPOSED METHOD AND ATOMIC-ABSORPTION SPECTROPHOTOMETRY (A.A.S.)

Results are expressed as percentages

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *Tea leaves*— | | | | | | | | | |
| Titan yellow method | Mg | 0·19 | 0·19 | 0·24 | 0·17 | 0·17 | | | |
| A.A.S. method .. | Mg | 0·18 | — | 0·22 | 0·17 | — | | | |
| | Mn | 0·23 | 4·23* | 0·27 | 0·25 | 6·25* | | | |
| *Sisal leaves*— | | | | | | | | | |
| Titan yellow method | Mg | 1·12 | | 0·98 | 0·88 | | 1·06 | 0·72 | 0·78 |
| A.A.S. method .. | Mg | 1·12 | | 1·01 | 0·94 | | 1·04 | 0·76 | 0·86 |
| *Soils*†— | | | | | | | | | |
| Titan yellow method | Mg | 1·63 | | 2·25 | 0·06 | | 0·29 | 0·94 | 0·06 |
| A.A.S. method .. | Mg | 1·60 | | 2·25 | 0·10 | | 0·33 | 0·86 | 0·08 |

* With added manganese.

† Normal ammonium acetate extracts and results expressed as milliequivalents per 100 g of soil.

I thank Mr. M. T. Friend of the East African Agriculture and Forestry Research Organisation for the atomic-absorption figures and Mr. J. Wasswa for undertaking a great deal of the practical work in developing this method.

## REFERENCES

1. Coleman, D. R. K., and Gilbert, F. C., *Analyst*, 1939, **64**, 726.
2. Chenery, E. M., *Plant & Soil*, 1955, **6**, 174.
3. Cheng, K. L., and Bray, R. H., *Soil Sci.*, 1951, **72**, 449.
4. Cheng, K. L., Melsted, S. W., and Bray, R. H., *Ibid.*, 1953, **75**, 37.
5. Hunter, J. G., *Analyst*, 1950, **75**, 91.
6. Allen, M., *Report of the East Malling Research Station*, 1959, 116.
7. Mason, A. C., *Ibid.*, 1951, 126.
8. Ritchie, J. A., *Analyst*, 1955, **80**, 402.
9. Bould, C., Bradfield, E. G., and Clarke, G. M., *J. Sci. Food Agric.*, 1960, **11**, 229.
10. Bradfield, E. G., *Analyst*, 1961, **86**, 269.
11. Mehlich, A., *J. Ass. Off. Agric. Chem.*, 1956, **39**, 518.

Received *May 13th*, 1963

# Polarographic Determination of Zinc in Plant Materials Containing Phosphate

By G. ROBERTSON

(*Ministry of Agriculture, Fisheries and Food, National Agricultural Advisory Service, Shardlow Hall, nr. Derby*)

In the course of determining zinc in a series of ashed samples of plant material, by using a manual polarograph, it was found that, compared with the dithizone colorimetric method, the results were anything between 10 to 40 per cent. lower. Since the determination was carried out under alkaline conditions, a precipitate was formed when the base solution was added. Filtering off the precipitate had no effect on the level of agreement. Analysis of the precipitate showed that it contained calcium and magnesium phosphates and a small amount of zinc, either as entrained zinc phosphate or as adsorbed zinc on the calcium phosphate precipitate. Therefore it appeared necessary either to remove the phosphate at an early stage in the procedure, or to prevent the formation of the phosphate precipitate of calcium, etc., preferably without increasing the number of manipulations.

Orthophosphate has been removed from plant digests and acid solutions of ashed material by precipitation with zirconium salts,[1,2] but it has been suggested that the gelatinous precipitate so formed has the ability to remove heavy metals by adsorption.[2] However, it was considered that a slight modification of this technique, embodying evaporation of the solution containing the zirconium phosphate precipitate to dryness and subsequent treatment with dilute hydrochloric acid, would bring virtually all the zinc into the acid phase. Results given in Table I show good agreement with those obtained by the dithizone method and would suggest negligible adsorption of zinc by this modified procedure. Some results without zirconuim precipitation are also given.

TABLE I

COMPARISON OF RESULTS FOR ZINC CONTENT DETERMINED BY ORIGINAL AND MODIFIED PROCEDURES

| | Zinc content, p.p.m. | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Lettuce | | | | | | Radish tops | | | | | Pig meal |
| Dithizone method .. .. | 148 | 155 | 260 | 160 | 198 | 140 | 70 | 220 | 680 | 108 | 82 | 160 |
| Modified method with zirconium precipitation .. | 149 | 156 | 252 | 182 | 190 | 140 | 75 | 230 | 650 | 96 | 76 | 160 |
| Modified method without zirconium precipitation.. | 107 | 117 | 208 | — | — | — | 50 | 126 | — | — | — | 130 |

In view of the good agreement between the colorimetric and the polarographic method for a large number of samples, the method given in this Paper has now been adopted for the determination of zinc.

## METHOD

REAGENTS—

*Hydrochloric acid*, M.

*Zirconium oxychloride solution*—Dissolve 3·54 g of zirconium oxychloride, $ZrOCl_2.8H_2O$, in 100 ml of water.

1 ml of solution ≡ 10 mg of zirconium.

*Base solution*—Mix together 30 ml of 5 M ammonium hydroxide and 7·5 ml of 5 M ammonium chloride and dilute to 55 to 60 ml. Dissolve 1·26 g of anhydrous sodium sulphite and 0·01 to 0·012 g of gelatin or peptone in about 30 ml of water. Mix the two solutions and dilute to 100 ml. Set it aside for 1 day before use. Make a fresh solution weekly.

When 8·0 ml of this solution are added to the acid solution of the ash, the final concentrations in the cell are M ammonium hydroxide, 0·5 M ammonium chloride, 0·1 per cent. sodium sulphite and approximately 0·01 per cent. gelatin or peptone.

PROCEDURE—

Ash 1·0 g of oven-dried plant material at 450° to 500° C. Dissolve the ash in 5·0 ml of M hydrochloric acid and warm it to effect dissolution. Add 1·0 ml of zirconium oxychloride solution, stir the mixture and evaporate it to dryness on a water-bath. Add 2·0 ml of M hydrochloric acid with slight warming, then add 8 ml of base solution, mix, and transfer the solution to the polarograph cell. Record the polarogram between −0·9 and −1·35 V. For the standard zinc polarogram, transfer a volume of zinc solution containing 50 μg of zinc to a small basin and evaporate the solution to dryness. Then proceed as for the ash. Compare the step height of the sample with that of the standard.

The equipment used was a Southern A 1650 manual polarograph with a Cambridge "Spot" galvanometer giving a deflection of 160 mm for 0·95 mA. With the proposed method, zinc gave a half-wave potential of −1·2 V *versus* the mercury pool and a response of 0·006 μA per μg of zinc.

REFERENCES

1. Padhye, V. P., *Analyst*, 1957, **82**, 634.
2. Derderian, M. D., *Anal. Chem.*, 1961, **33**, 1796.

Received *October 9th*, 1963

# The Semi-micro Determination of Fluorine and Chlorine in Organic Compounds

## Part V.* Details of the Preparation of the Biphenyl - Sodium - Dimethoxyethane Complex

BY R. D. CHAMBERS, T. F. HOLMES AND W. K. R. MUSGRAVE
(*Department of Chemistry, The University, South Road, Durham*)

IN Part III of this series[1] we described the decomposition of halogenated organic compounds by using the biphenyl - sodium - dimethoxyethane complex, which was prepared as described by Liggett.[2] Whereas the reagent, as prepared in this way, is satisfactory if the solution is decanted carefully from undissolved sodium and biphenylsodium and is used within a few days of preparation, we have found that when it is stored in a refrigerator at 0° to 5° C it is thrown out of solution and will neither re-dissolve nor effectively decompose halogenated organic compounds. Consequently, we have modified the preparation to give a more dilute solution of the complex, about 0·5 M, that contains less toluene. Because of the lower proportion of toluene, it is easier to form the original sodium dispersion. Our experience over the last three years has proved that this more dilute solution can be stored at 0° to 5° C for several months without deterioration, but three to four times the volume of it is required to decompose the suggested amount of halogen-containing compound.

### METHOD

DRYING OF MATERIALS—

*Biphenyl*—Recrystallise the solid from methylated spirits, and dry it at room temperature and 0·001 mm pressure by using an oil-pump.

*Toluene*—Distil the liquid over sodium in an atmosphere of dry nitrogen. Use bubblers containing liquid paraffin on the nitrogen inlet and outlet. It is unnecessary to pass a stream of nitrogen once the atmosphere of nitrogen has been established. Dry the paraffin by heating it to 120° C until it no longer crackles.

*Dimethoxyethane (ethylene glycol dimethyl ether)*—Heat the liquid under reflux over potassium and then distil it, all in an atmosphere of dry nitrogen. Repeat the process, adding a few crystals of benzophenone and heating the mixture under reflux over potassium until a violet colour develops. The violet colour is due to the formation of the potassium derivative of benzophenone, $Ph_2\dot{C}—\bar{O}\,\dot{K}$, which is highly sensitive to traces of water. It is advisable to repeat the distillation a third time.

* For details of Part III of this series, see reference list, p. 370.

PREPARATION OF THE BIPHENYL - SODIUM - DIMETHOXYETHANE COMPLEX—

Place 70 ml of dry toluene and 23 g of sodium in a dry 250-ml three-necked flask fitted with a high-speed stirrer, reflux condenser and nitrogen inlet. Pass a slow stream of nitrogen and heat the toluene under reflux by using a heating mantle, until the sodium is molten. Stir the mixture vigorously until the sodium is finely dispersed, stop the stirrer immediately and allow the flask to cool at room temperature. While maintaining an atmosphere of dry nitrogen in both flasks, transfer the suspension to a 3-litre, three-necked flask fitted with a stirrer, and wash in the last traces of the suspension with a little of the dry dimethoxyethane. Add dimethoxyethane to give a total volume of 1750 ml. Cool the mixture to 5° C in an ice-bath, stir it gently and add a solution of 160 g of biphenyl in 250 ml of dimethoxyethane while maintaining an atmosphere of nitrogen in the flask and taking care that the temperature does not rise above 10° C. The reaction starts almost immediately after addition of the first biphenyl, and a green colour, which gradually darkens to black, develops. When the addition is complete, stir the mixture for 1 hour at 0° to 10° C, and then store the reagent under nitrogen, at 0 to 5° C, in two-necked flasks. If one neck is fitted with a nitrogen lead and a stop-cock, it is easy to maintain the dry nitrogen atmosphere while removing the required volume (30 to 40 ml) of reagent by means of a safety syringe pipette. This volume of reagent will decompose a sample of organic compound equivalent to about 8 mg of total halogen as described in Part III.

We thank Mr. T. Caygill for technical assistance.

REFERENCES

1. Johncock, P., Musgrave, W. K. R., and Wiper, A., *Analyst*, 1959, **84**, 245.
2. Liggett, L. M., *Anal. Chem.*, 1954, **26**, 748.

NOTE—Reference 1 is to Part III of this series.

Received *November 25th*, 1963

# Book Reviews

ADVANCES IN MASS SPECTROMETRY. Volume 2. Edited by R. M. ELLIOTT. Pp. xviii + 628. Oxford, London, New York and Paris: Pergamon Press. 1963. Price 140s.

The Mass Spectrometry Panel of the Hydrocarbon Research Group of the Institute of Petroleum held its first meeting on 10th March, 1949, and soon decided that one of its principal tasks must be to provide a channel for the interchange of information between users of mass spectrometers. Hence, a conference was arranged to take place in Manchester in April, 1950. The Proceedings of this conference were published in the summer of 1952. It was clear that the experts of the day welcomed the opportunity to present the results of their most recent experimental work in various branches of mass spectrometry, and to discuss many current problems with other workers. Accordingly, it seemed desirable to arrange further conferences. A second conference was held in London in 1953 and a third conference was held again in London in 1958. The volume under review records the Proceedings of the fourth conference held in Oxford in September, 1961.

Since preparations are now under way for a fifth conference to be held in Paris in 1964, it seems that such conferences are likely to become a triennial event. Over the years the conferences have become more and more international in character. The conferences in 1958 and 1961 were, in fact, organised jointly by the Mass Spectrometry Panel and the equivalent committee in the U.S.A., American Society for Testing Materials, Committee E-14. Next year, the organisation is to be extended further by collaboration with the corresponding French organisation, G.A.M.S.

The 1961 conference proved to be rather similar in scope and organisation to the 1958 conference, and, in consequence, the same title has been used for the proceedings of both conferences. Forty-four papers, including 11 from workers in Great Britain and 13 from workers in the U.S.A., were read and discussed. The other participating countries were Germany, France, Canada, Australia, Belgium and Holland. All these papers are now published in full (in English) in the volume under review, together with edited reports of the discussions on the various papers. A feature of earlier publications in this series has been the inclusion of a bibliography containing references to published papers and books on mass spectroscopy. The bibliography in the first volume of Advances in Mass Spectrometry covered the years 1938 to 1957. The bibliography

in the present volume is intended to supplement the earlier bibliography and extends from January, 1958, to December, 1960. It is rather frightening to find that during this period, nearly 900 papers and books have been published, averaging almost one a day. A careful study of all these published works would clearly leave no time at all for one's own researches. The versatility of the mass spectrometer is such that no worker can take part in more than a few of the many fields of research. One is, in fact, not just a mass spectrometrist, but a particular kind of mass spectrometrist.

With a few exceptions, for example, the paper by Beynon reviewing four years' experience in operating a high-resolution (up to 10,000) mass spectrometer on problems of organic chemical analysis, and the paper by Rosenstock and Krauss reviewing the present situation and the new problems in the statistical theory of mass spectra, all these papers are not be to regarded as reviews or general surveys of recent developments, but rather as original contributions to a rapidly expanding subject. In common with most scientific work newly published, one cannot assess immediately how much will survive more than a few years and how much will be superseded rapidly by later experimental and theoretical data. A critical assessment of many of these contributions could scarcely be undertaken without a sound knowledge of earlier work. Hence, although there is, indeed, a great deal of value in this volume, it is not easy to decide on the type of reader to whom the work is addressed and for whom the reviewer ought to be offering comment and opinion. Obviously, it is not specially for those who participated in this conference. Many participants may wish to have a bound volume on their shelves in addition to the full set of preprints of the papers already presented to them. Obviously, also, it is not for the physicist or chemist having only a superficial knowledge of mass spectrometry. Such a worker would gain more from a study of one or other of the earlier monographs on this subject.

However, there is a certain continuity in the published proceedings of all these conferences. This is because possible basic lines of research with the mass spectrometer were delineated a long time ago. Subjects under discussion today were being discussed ten or more years ago, albeit with less detail and erudition. Instruments and experimental techniques have naturally improved substantially in the last decade. Inevitably, then, one expects to find, as one does, many papers devoted to new instruments and techniques. In this section, it is interesting to note that the precise measurement of the isotopic ratio of uranium-235 to uranium-238 in uranium hexafluoride appears to be as important today as it was twenty years ago in the measurements being undertaken with a Nier-type mass spectrometer at Liverpool University.

Not all possible lines of research have received the same measure of attention over the years. For example, Dr. Meyerson makes the comment that "the potential of mass spectroscopy for structure work has scarcely been touched." However, Prof. Biemann presents an interesting paper on the structure of some alkaloids occurring in various plants. Another paper deals with the mass spectra of some steroids. The mass spectrometer was applied more than ten years ago to studies of materials of medical and biological interest: fatty acids, cholesterol, vitamin A, nicotine, carotene, etc.

Most of the expected features of such an extended conference are to be found in these published proceedings: field-ionisation mass spectrometry; studies of solid surfaces; ionisation in flames; free-radical reactions; nuclear chemistry; studies of inorganic compounds at high temperatures; spark-source mass spectrometry for solid analyis; ion - molecule reactions; chemical kinetics; appearance potentials and molecular-energy states. Clearly, there is an abundance of material for prolonged study.

Finally, the book is produced according to the high standards one has come to expect of the Pergamon Press.

G. P. BARNARD

LECTURES ON GAS CHROMATOGRAPHY 1962. Edited by H. A. SZYMANSKI. Pp. vi + 282. New York: Plenum Press. 1962. Price $10.00.

*Based on Lectures Presented at the Advanced Sessions of the Fourth Annual Gas Chromatography Institute held at Canisius College, Buffalo, New York, April 23rd to 26th, 1962.*

There has always been a trend in gas chromatography for the proceedings of meetings and symposia on the subject to be published as self-contained volumes, and an unusually high proportion of the significant references in the field occurs in such proceedings.

It seems an unwelcome extension of this practice to publish lectures given in formal courses, as inevitably much material is included, for the sake of completeness, that would not otherwise be considered worthy of publication in a recognised journal.

This volume provides an effective illustration. Most of the lectures, while eminently suitable for a regional annual course, are just not prepared for international consumption in 1962, as might well be expected. The ground covered is about what might be anticipated including sample injection, column packings, detection, capillaries, auxiliary techniques and some considerable emphasis on the instrumental side of temperature programmming. The sprinkling of original ideas, such as the proposal by Szymanski to use a heterogeneous packing support, one component of which is susceptible to induction heating, does little to compensate for the lack of integration and balance in the book. The contribution by McCullum on auxiliary analytical techniques is interesting, particularly the potentiality of far-ultraviolet spectroscopy for both sensitive detection and identification.

The book is well printed with clear diagrams and includes a verbatim recording of discussion periods. At its somewhat high price it is not to be recommended except for well stocked libraries that must have everything. D. H. DESTY

INFRA-RED SPECTROSCOPY AND MOLECULAR STRUCTURE: AN OUTLINE OF THE PRINCIPLES. Edited by MANSEL DAVIES. Pp. xiv + 468. Amsterdam, London and New York: Elsevier Publishing Company. 1963. Price 75s.

This book consists of an introduction by the editor, followed by 12 essays by experts on topics in which they have specialised. It is inevitable that some overlapping should occur in a work of this kind, but efforts have clearly been made to minimise this. Most of the treatments tend towards the physical rather than the chemical side of the subject. For example, about 130 of the book's 468 pages deal with the normal vibrations, force fields and band intensities of simple molecules, whereas only 5 pages are devoted specifically to the interpretation of the spectrum of an unknown organic compound. A fair knowledge of mathematics is assumed in many of the chapters. The book is thus likely to be more useful to professional spectroscopists and physical chemists than to organic and analytical chemists, who use infrared spectroscopy to discover the compositions and structures of their products.

Subject to these limitations, the editor and publishers are to be congratulated on an excellently produced book, that is not unduly expensive and collects together a great deal of valuable information, much of it not previously available in book form.

Following the editor's introduction, there is an excellent description by A. E. Martin of modern infrared spectrometers and accessories. The next chapter on far-infrared spectroscopy by G. R. Wilkinson maintains this high standard. Chapters IV and V by W. Jeremy Jones and I. M. Mills deal with the normal modes of vibration and force constants of simple molecules. Much of this material has been dealt with in other books. The next chapter (rather surprisingly) describes the theory, experimental methods and applications of Raman spectroscopy. Chapter VII by D. Hadzi deals mainly with electronic and steric influences on group characteristics, vibration frequencies and band intensities, and includes a short account of the interpretation of the spectra of unknowns. Solid substances and polymers are discussed, mainly from the viewpoint of a physicist, by S. Krimm, and a chapter on the spectra of inorganic compounds by E. A. V. Ebsworth covers qualitative analysis, determination of molecular symmetry and the assignment of bands to vibrational modes. Quantitative intensity studies and dipole-moment derivatives are fully dealt with by J. Overend. A chapter by J. Fahrenfort sets out the theory of dispersion studies, but makes little concession to the chemist who wishes to use attenuated total reflection to extend his infrared technique. One of the best chapters in the book, by H. E. Hallam, is concerned with hydrogen bonding and solvent effects, and the work concludes with an article by W. C. Price on infrared emission spectra. M. ST. C. FLETT

RECENT ADVANCES IN FOOD SCIENCE—3: BIOCHEMISTRY AND BIOPHYSICS IN FOOD RESEARCH. Edited by JAS. MUIL LEITCH, B.Sc., F.R.I.C., and DOUGLAS N. RHODES, B.Sc., Ph.D. Pp. xiii + 325. London: Butterworth & Co. (Publishers) Ltd. 1963. Price 70s.

The first two volumes of this series appeared as "Food Science: A Symposium," by Bate-Smith and Morris, in 1951, and "Recent Advances in Food Science," by Hawthorn and Muil Leitch, 1962. The present volume is written after the "Third Advanced Study Course in Food Research," organised jointly by the Low Temperature Research Station, Cambridge, and the Department of Food Science, Royal College of Science and Technology, Glasgow, held for advanced students of food technology in 1962. The idea was to present to the student the up-to-date

applications of classical biochemistry and biophysics to food research, and its practical application to world food problems. The course was sponsored by the Scientific Office of N.A.T.O.

There is a section on protein chemistry, and one on the methods used in the laboratory study of protein structure.

The third section is on non-conventional food proteins, and includes an assessment of non-conventional protein sources, by Pirie of Rothamsted, that surveys the possibilities of using leaves, grasses, agae, seaweeds, yeasts, etc., for human protein foods. The section also includes the technological problems of using such proteins, the measurement of nutritive value of these supplements and the nutritional problems arising from such uses. The work in India on a multi-purpose food, in which mainly vegetable proteins are used, is also included.

From the food chemists' point of view, the section on flavour and odour research is probably the most useful portion of the book, since this includes the most up-to-date applications of instrumental analysis to these problems. Obviously the use of gas - liquid chromatography plays an important part in this work, and the methods described include the use of sensitive ionisation detectors as well as mass spectrometry to indicate the products eluted from retention columns. The application of these methods to the odoriferous components of coffee, fruit, potatoes, milks and cheeses is described.

A further section is on water binding in relation to food: this is important to the practical food chemist, since it includes such subjects as hydration of solid proteins, water-imbibing power of foods, enzyme action at low water content, and water activity and the growth of micro-organisms.

The final section is on the structural elements of vegetable foods, with reference to food textures and cell-wall composition and components, such as hemicelluloses, pectins, gums, cellulose, glucans and lignins.

I can recommend this book to all readers of *The Analyst* because: (*a*) we are all interested in food; (*b*) we should all be interested in the problem of feeding the increasing world population; (*c*) there is much for the food technologist; (*d*) in flavour research we are really in the realms of microchemical analysis dealing with concentrations sometimes of the order of $10^{-16}$; (*e*) a review of the application of modern technological advances to research problems is stimulating to us all.

R. F. MILTON

PROGRESS IN NUCLEAR ENERGY. Series IX. ANALYTICAL CHEMISTRY. Volume 3. Part 4. Edited by CARL E. CROUTHAMEL. Pp. vi + 95–162. Oxford, London, New York and Paris: Pergamon Press. 1963. Price 20s.

Part 4 of Volume 3 comprises a general review of the more purely chemical separation methods that have been developed in the decade preceding 1962 for those transition elements that fall triserially into the Periodic Table in the sequence Ti, V, Cr; Zr, Nb, Mo and Hf, Ta, W. Radiochemical, spectrochemical and X-ray fluorescence methods are thus intentionally omitted, but solvent-extraction, ion-exchange, spectrophotometric and polarographic methods are included in the text together with the more general titrimetric and gravimetric techniques. A photonometric method, in which photochemically reduced ferric salt is used, is given for vanadium or chromium determinations.

The review is critical in parts, but it would be of greater use to the analyst if preferred methods were more strongly recommended and the reasons outlined or specifically indicated. An excellent reference bibliography is given.

D. T. LEWIS

PROGRESS IN NUCLEAR ENERGY. Series IX. ANALYTICAL CHEMISTRY. Volume 3. Parts 5–7. Edited by CARL E. CROUTHAMEL. Pp. viii + 163–265. Oxford, London, New York and Paris: Pergamon Press. 1063. Price 30s.

Parts 5, 6 and 7 of Volume 3 deal with three quite separate subjects, all of which are, however, of considerable interest in the nuclear field:

In part 5, L. E. Smythe presents an extremely detailed chapter on the chemical monitoring of heavy-water research reactors and lists what data are known regarding 17 reactors that have been built in 11 countries and have thermal fluxes in the range $10^{12}$ to $10^{14}$ neutrons per sq. cm per second. The general principles of design are discussed, the maintenance of high-purity heavy water, the use of helium or nitrogen blanketing atmospheres, etc., corrosion problems, radiolytic changes, bacterial contamination, all fall into the ambit of this extremely useful survey.

Part 6 is devoted to recent work on the analytical chemistry of beryllium. It is comprehensive and thorough and covers most of the chemical and instrumental studies of more recent years.

In Part 7, D. C. Stewart of the Argonne National Laboratory provides a well illustrated chapter on the construction of laboratory facilities for work with intensively radioactive substances up to megacurie levels. Neutrons and $\gamma$-rays are catered for as well as the less insidious $\alpha$- and $\beta$-particles.

Glass-reinforced polyester resins are much used for glove boxes and duct work and possess a useful resistance to perchloric acid fumes. Concrete is used extensively for walls, and vinyl sheet for floor coverings. Much useful physical data is given, and the chapter will prove of the greatest value to chemists who have to design safe laboratories for nuclear operations of the types described. D.T. LEWIS

ORGANIC ELECTRONIC SPECTRAL DATA. Volume IV: 1958–1959. Edited by J. P. PHILLIPS and F. C. NACHOD. Pp. x + 1179. New York and London: Interscience Publishers; a division of John Wiley & Sons Inc. 1963. Price 50s.

Volume I covers the period 1946 to 1952; Volume II, 1953 to 1955; and Volume III, 1956 to 1957 is in preparation. The present volume follows the pattern of the first two, and a new feature is seen in "occasional reference to page numbers in Volumes I and II that also give data for the indicated compound." If such reference back is to be adopted in future volumes it deserves to be done more thoroughly or, alternatively, it may be used to draw attention to discrepancies. This publication has established itself as a most useful work of reference. R. A. MORTON

INTRODUCTION TO FLUORIMETRY. By A. H. GUNN. Pp. vi + 59. Richmond, Surrey: Electronic Instruments Limited. 1963. Price 35s.

This monograph was compiled by a member of the research laboratories of Electronic Instruments Limited as an introduction and guide to those approaching the subject of fluorimetry for the first time. It is divided into four main chapters, together with a 1-page introduction, a 1-page bibliography and a 2-page subject index. It deals with the elementary principles of fluorescence, the choice of filters in filter-fluorimetry and the preparation of calibration graphs for analytical work. It also includes some hints to help the beginner avoid the more obvious pitfalls, and a chapter giving brief notes on the fluorimetric determination of some forty substances.

The book may be useful to the complete beginner, although it contains some misleading statements. For example, it states that spectrofluorimeters are not suitable for quantitative measurements on extremely dilute solutions, a surprising statement to anyone familiar with the literature on the subject, since modern commercial instruments are extremely sensitive. Apart from this statement, the book does not consider spectrofluorimetry at all, nor does it recommend excitation with wavelengths shorter than the 366 m$\mu$ mercury line, possibly because the instrument concerned may have glass optics.

To sum up, I was left with the impression that this monograph was originally intended as a handbook to be supplied with the commercial instrument. As such, it might well provide a starting point for the study of the current literature on fluorescence. However, at 35s. for 59 pages it is not good value and is not to be recommended. C. A. PARKER

MASS SPECTROMETRY. Edited by CHARLES A. MCDOWELL. Pp. xii + 639. New York, San Francisco, Toronto and London: McGraw-Hill Book Company Inc. 1963. Price 155s.

This book consists of a series of articles, covering all important aspects of mass spectrometry, each of which has been written by a specialist in a particular field. The reason given by the editor for producing a book on mass spectrometry in this form is the perfectly valid one that the subject nowadays comprises such a wide range of topics and techniques that it is beyond the scope of one person to write authoritatively in the whole field. The chief dangers inherent in producing a co-operative work of this nature are repetitiveness and lack of balance, both of which have been fairly successfully avoided.

Only in one instance is the value of an article at all questionable, and that is the one on electronic techniques, which it is felt should be expanded considerably to be of real value.

Although only 2 of the chapters, on chemical analysis and isotope-abundance measurement, are of direct analytical interest, the analyst wishing to learn more of the scope and fundamental basis of mass spectrometry, and to apply this tool in an informed manner to analytical problems, will find much of value in the chapters on types of mass spectrometers, ion sources, vacuum techniques, ionisation and dissociation of molecules and ion - molecule reactions. The chapters

on ion optics, high-resolution mass spectroscopes and free-radicals, whilst they are valuable surveys of these fields, will be of only academic interest.

From the analyst's point of view, the chapter on analysis could well have been rather longer and more detailed, and in particular it is thought that more should have been said about solid-sample mass spectrometry and the factors determining the precision and sensitivity of this application. Hence, one cannot but regret that 6 out of the total of 40 pages should have been devoted to the description, together with 4 half-page photographs, of commercial instruments.

To the beginner in the high-vacuum field, the chapter on vacuum technique will be especially useful because of its eminently practical bias, this being exemplified by the authors occasional practice of giving names and addresses of suppliers of certain items. However, it would have been of value to the analyst if systems for handling gas samples and techniques for opening sealed envelopes *in vacuo* had been dealt with in greater detail, either in this chapter or in that on analysis.

The index of a book of this nature, which will certainly become a reference book for many users, should serve its purpose particularly well, but the few random tests that were made indicated a certain deficiency. For example, "mass discrimination" is not found under this heading but under "ion sources, electron-impact" and "ion sources, thermal-ionisation"; "fringing-field" appears under "ion optics," and "cycloidal mass spectrometer" under "mass spectrometers, static instruments." It must be granted, however, that to obviate this sort of thing might well require an unreasonably large index.

Finally, it should be mentioned that this book provides a rich source of references, extending in most instances to about mid-1962. The book is well produced and, of by no means minor importance in a work on this subject, very clearly illustrated. M. G. CHARLTON

VITAMIN-BESTIMMUNGEN: ERPROBTE METHODEN. By Dr. ROLF STROHECKER and Dr. HIENZ M. HENNING. Pp. 365. Germany: Verlag Chemie GmbH. 1963. Price DM. 42.

This is probably the most comprehensive volume on vitamin analysis yet published in any language, and it is well up to the usual standard of efficiency we are accustomed to see in Merck publications.

There would be little point in enumerating all the vitamins and all the methods included; it should be enough to say that all the vitamins from A to P are covered, and all the various methods for the individual determinations mentioned. These include chemical and physico-chemical methods (colorimetric, fluorimetric, titrimetric, polarographic and chromatographic, including thin-layer techniques). Also included are the microbiological methods in full detail.

In describing techniques, full weight is given to the extraction of the particular vitamin from the assayed material and the cleaning-up procedure before final determination. The authors have a fairly simple literary style that should make the subject matter fairly comprehensible to the average reader with limited experience of the German language. The chapters are well studded with references gathered from the world literature.

The volume can be recommended as a reference book to all those engaged in vitamin assays of any kind. R. F. MILTON

CHEMICAL ANALYSIS. Edited by P. J. ELVING and I. M. KOLTHOFF. Volume XVII. ELECTRON PROBE MICROANALYSIS. By L. S. BIRKS. Pp. x + 253. New York and London: Interscience Publishers, a division of John Wiley & Sons. 1963. Price 70s.

The stated aim of this book is to provide a short, easily readable introduction to electron-probe micro-analysis for those who wish to use the technique rather than study it. Undoubtedly there is a need for such a book, and the volume under review goes some way towards filling this need: but in a book of this sort it is always difficult to judge how much accuracy and detail should be sacrificed for simplicity and ease of presentation, and in the present book the author errs perhaps a little too far on the side of simplicity.

In general terms, the first 2 chapters provide a historical introduction, the next 5 chapters describe practical details of various types of instrument: 2 chapters are devoted to the simplest possible discussion of the theory of quantitative analysis, and the 2 final chapters review current applications and future trends. The book is freely illustrated with line drawings and photographs, and has a useful compendium of appendices containing all the figures and tables necessary for calculating corrected quantitative analyses.

The two brief introductory chapters will be useful to the completely uninitiated, but here, as in much of the book, the brevity and simplicity are achieved mainly by suppressing discussion of theoretical principles. This aversion to discussing precise detail amounts to a serious fault in Chapter 3, which describes the electron optics of micro-analysers: the action of the grid in an electron gun is incorrectly described, the discussion of beam-current intensities is rather misleading, and nowhere is it possible to find a clear statement of all the factors governing beam diameter at the specimen. Chapters 4 and 5 describing specimen observation and preparation are good, but somewhat brief. On the other hand, Chapter 6 is of reasonable length and constitutes an excellent introductory text on X-ray optics, with a nice balance between theoretical and practical detail. The following chapter on X-ray detectors is also good, particularly in its discussion of practical application. In Chapters 8 and 9 the author describes, in some detail, his own approach to quantitative analysis. This approach is not held in high esteeem by the pundits of current theory, but at least Birks' methods do provide a simple, tolerably reliable routine for calculating corrected analyses to within an accuracy of about $\pm 2$ per cent., which is adequate enough for many practical problems. However, the reader must be warned that the values of the intensity function, $F$, used in the worked examples do not always tally exactly with the values given in Appendix 2. Although the chapter on applications is little more than a list of some of the problems that can be tackled by using the electron-probe micro-analyser, it does illustrate the great versatility of this instrument. The final chapter headed "Future Trends, and Related Uses," unfortunately, deals more with the latter than the former: a discussion more closely tied to current developments in electron-probe micro-analysis would have been more to the point.

This slim volume cannot be recommended as sufficient reading for anyone wishing to use a micro-analyser competently, but it could usefully serve as an elementary introduction to some of the practical aspects of electron-probe micro-analysis, and could perhaps be used as an introductory text for undergraduate students of metallurgy, geology and chemistry.

G. W. T. WHITE

## Errata

APRIL (1964) ISSUE, p. 244, equation (2). *For* "$(E_2^0-E_2^0)$" *read* "$(E_2^0-E_1^0)$".

IBID., p. 245, heading to Table II. *For* "$E_{0Ag}$" *read* "$E^0{}_{Ag}$".

IBID., p. 270, 7th line from foot of page. *For* "ratios to calcium" *read* "ratios of calcium".

IBID., facing p. 289, Legend to Fig. 1. *For* "Paper" *read* "Thin-layer".

## Notice to Authors

THE Editor welcomes Papers on all aspects of the theory and practice of analytical chemistry, fundamental and applied, inorganic and organic, including chemical, physical and biological methods. Papers are submitted to the Editorial Committee, who decide on their suitability for publication.

A copy of the current Notice to Authors, last published in full in *The Analyst*, 1964, **89**, 303, can be obtained on application to the Editor, *The Analyst*, 14 Belgrave Square, London, S.W.1. All Papers submitted will be expected to conform to the recommendations there laid down, and any that do not may be returned for amendment.