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Summaries of Papers in this Issue

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H. T. DELVES, G. SHEPHERD and P. VINTER

Department of Chemical Pathology, The Hospital for Sick Children, Great Ormond Street, and the Institute of Child Health, University of London, 30 Guilford Street, London, W.C.1.

Analyst, 1971, **96**, 260–273.

Quantitative Determination of Taurine by an *o*-Phthalaldehyde - Urea Reaction

Taurine is made to react with *o*-phthalaldehyde in the presence of urea and phosphate ions, and on acidifying the mixture with acetic acid a purple product is formed with an extinction maximum at 560 nm. A method based on this reaction is described for the quantitative determination of taurine and it is applied to the determination of this amino-acid in rat brain after passage of the tissue extract through ion-exchange resins. The effect of other amino-acids on the accuracy of the method is discussed.

M. K. GAITONDE and R. A. SHORT

Medical Research Council Neuropsychiatry Unit, Woodmansterne Road, Carshalton, Surrey.

Analyst, 1971, **96**, 274–280.

A Spectrofluorimetric Method for the Determination of Small Amounts of Sulphate Ion

The enhancement of the fluorescence of the binary complex of zirconium with Calcein blue at pH 1·9 is used to determine sulphate ion in the range 0·2 to 12 mg (2 to 12 000 p.p.m.). Excitation and fluorescence maxima occur at 350 and 410 nm, respectively. The fluorescence is stabilised immediately and remains unchanged for over 4 hours. Fluoride present in low concentrations gives rise to high results and must be absent. Other anions that form complexes with or precipitate zirconium, *e.g.*, oxalate, phosphate, tartrate and tungstate, cause low results, but there is a high tolerance towards most cations except iron(III) and cobalt(II).

LAY HAR TAN and T. S. WEST

Chemistry Department, Imperial College, London, S.W.7.

Analyst, 1971, **96**, 281–285.

THE ANALYST

Editorial

The Impact of Instrumentation on the Analytical Laboratory

THAT we live in a time of change is nowhere more evident than in the analytical laboratory. While this has probably been true for most of the 97 years our Society has been in existence, the development of highly specialist instrumentation to supplement, extend or replace "wet" analytical methods of analysis has given the past 30 years a special character. Broadly speaking, the 1940s and the 1950s saw the steady development of hardware incorporating the scientific discoveries of the previous 100 years and the 1960s saw the consolidation of such equipment in our analytical laboratories as well as the advent of high-cost items resulting from the development of better devices to meet the demands for better diagnostic procedures and for faster routine methods. Hence we now have, for example, infrared spectrophotometry, mass spectrometry and nuclear magnetic resonance spectrometry in conjunction, if necessary, with gas or thin-layer chromatography as an extremely powerful set of procedures in the organic field, while in the inorganic area we have been able to add the electron-probe analyser to an already impressive set of diagnostic and measurement techniques.

Great benefits have accrued to the analyst, and thence to the world at large, as a result of these developments. Not only can he give a faster service to his customers, but often a better service in the sense that the information he supplies is better in quality and, not infrequently, he can supply information when hitherto such information would have been impossible to obtain. For example, the studies of many pollution problems have needed the development of suitable analytical methods before they could become meaningful.

These benefits from developments in instrumentation have not been gained cheaply. They are affecting the cost of running our laboratories, the shape of the laboratories themselves, and the staff we need to employ. So although the future shows every promise of further rich gains in new or improved analytical techniques, it is perhaps pertinent to consider the effects of these repercussions and any steps we may need to take to deal with them. The comments that follow have been generated from a necessarily limited environment and are offered more as a basis for discussion rather than any attempt to be dogmatic.

The cost of analytical equipment probably began to be most sharply felt in the late 1950s with the coming of gas chromatographs at about £1000 each. To use gas chromatography was to realise its potential and one soon needed several instruments; it was not uncommon, however, to find laboratories not able to afford such equipment. Infrared and ultraviolet spectrophotometers could be regarded a little differently because, usually, one of each would be enough and they would last for many years; new models of gas chromatographs appeared with the frequency of new cars. Other instruments began to appear with prices in the thousands rather than hundreds of pounds; atomic-absorption spectrometers, polarographs, the do-it-yourself infrared spectrophotometers and X-ray fluorescence spectrometers and, as we moved through the 1960s, £30 000 was becoming commonplace for mass spectrometers, nuclear magnetic resonance equipment and electron-probe analysers. Now, looking ahead, we can visualise a proliferation of improved instruments of all kinds, many of which are becoming virtually automatic in operation (and consequently perhaps a little less versatile!). Inevitably, most laboratories are producing more data and, inevitably, many are turning to computers for help with the difficulties thus created.

It is also inevitable, as we seek to acquire better instrumentation for our laboratories, that management will require some assurance of getting a good return on the capital involved;

fortunately, if the equipment expenditure has been well conceived, there is little difficulty here. Sooner or later, however, the successful use of high-cost equipment is likely to stimulate more work than can be conveniently carried out in the normal working day, and requests for extra equipment are likely to be met with the observation that at 35 to 40 hours per week the existing equipment is hardly overworked. If the laboratory is concerned mainly with process control, a tradition of shift working probably exists already, so that not too much difficulty is experienced in operating the expensive equipment on a shift basis. However, if it is located in a research laboratory, then the possibility of shift working may not be one that commends itself to the staff for whom the normal working day is traditional. It should, of course, be added that not all equipment is amenable to continuous shift working, although up to 12 to 16 hours per day can probably be achieved with most.

There is another vital and inevitably expensive factor to be considered: analytical equipment no longer lasts for ever. The wise laboratory manager must now reckon the life of most of his laboratory instruments as something like 5 years, regarding anything in excess of that as a bonus; he will also keep in mind that an elderly instrument that still produces results may perhaps be more expensive to operate and possibly provides less information than its more modern counterpart. As a result, his annual forecast of capital expenditure must now contain a realistic sum for replacing such equipment as this becomes necessary. We are, of course, always reluctant to throw away instruments, but this is clearly something we shall have to get used to. However, with the increasing cost of equipment, it may eventually prove worthwhile to hire equipment rather than buy it outright; this is a relatively new possibility for analytical chemists, and it remains to be seen how far it becomes feasible.

Yet another factor affecting our laboratory costs is maintenance. With the increasing complexity of our tools, we can no longer allow the local do-it-yourself enthusiast to handle any but the simplest of difficulties, and even in the larger laboratories the resident instrument engineer cannot be expected to cope with the many varieties of equipment now used. Hence, we are having to rely more and more on the expensive service engineer from the instrument manufacturer to keep much of our equipment in operation; of course, the quality of the service thus obtained will play a significant part in our original choice of such equipment.

Many of our laboratories are still much the same shape as they were 20 years ago, except that the benches are now covered with gas chromatographs, atomic-absorption spectrometers, and so on. However, we usually site the larger items of equipment in empty rooms and design the laboratory facilities around them, but we are rarely fortunate enough to be able to plan for any future extension at the same time—and so the seeds of future frustration are sown. When the laboratories are located in the factory, we are finding that a significant part of our process control has been moved out of the laboratory on to the plant, either to be carried out by on-line equipment working continuously or by process operators with semi-automatic devices. Indeed, whenever possible, today's plant designers will be seeking not only to have as much testing as possible on-line, but to have information from such equipment fed back to the control room to play its part in continuous plant control; the days of producing results for process control testing that are studied at leisure are surely numbered.

Finally, the increase in instrumentation has had its effect on the inhabitants of our laboratories. In our quest for accuracy and speed at lowest cost, life can be interesting and often exciting for the research analyst. However, it is becoming more likely that with any particular problem he will now only need to prove the usefulness of a particular technique, leaving the design and construction of the hardware to the instrument manufacturer, who now undertakes much of the development work on the more commonly used techniques. When automatic equipment operates on-line on the plant, responsibility for its continuous operation, its maintenance and the provision of back-up services can involve the analyst in local politics and public-relations exercises for which his analytical training may not have equipped him, and he may need to tread warily. When the equipment sits in the laboratory, especially when routine process control is the main objective, life could become dreadfully dull for the technicians operating it unless some imagination is brought to bear on the arrangement of work schedules. As our laboratories become more highly mechanised, so the staff employed make less and less use of wet-chemical methods and, unless we are very careful, the point might be reached when there is nobody about who can carry out such methods; as the older analysts retire, this is something that needs to be watched carefully. The increase in instru-

mentation is undoubtedly causing changes in the activities of those working in our laboratories and bringing, in turn, changes in the way they need to be trained or re-trained, a subject that is being actively pursued by our new Education and Training Group.

Analytical work has always been interesting but, such has been the impact of modern instrumentation, we are now able to do so much more than was possible in the past. Therefore the future looks bright for the analytical scientist, but it could be fraught with difficulty if he does not learn to use his new tools wisely.

A. G. JONES

Analyst, April, 1971, Vol. 96, *pp.* 260–273

Determination of Eleven Metals in Small Samples of Blood by Sequential Solvent Extraction and Atomic-absorption Spectrophotometry

BY H. T. DELVES, G. SHEPHERD AND P. VINTER

(Department of Chemical Pathology, The Hospital for Sick Children, Great Ormond Street, and the Institute of Child Health, University of London, 30 Guilford Street, London W.C.1)

A method is described for the determination of eleven metals in a 1-ml solution of an oxidised blood sample. The metals iron, copper, bismuth, zinc, cadmium, lead, cobalt, nickel, manganese, strontium and lithium are selectively extracted into small (0·30 to 0·50 ml) volumes of isobutyl methyl ketone as their chelates or ion-association complexes, and are determined in the organic phases by atomic-absorption spectrophotometry. The enhancement effect of the organic solvent combined with the extraction and concentration of the metals results in average sensitivity increases of seven times that obtained by a direct determination on the aqueous solutions.

The recovery of the metals added to blood is quantitative and, with two exceptions (lead and bismuth), a precision of better than 8 per cent. can be achieved at the 0·1 p.p.m. level.

The results are given of an application of the method to a study of the problem of metal ingestion by children who have pica.

TRACE-ELEMENT survey analysis of biological materials has shown that in addition to those metals known to be essential to man a further 20 metals are consistently present in human tissues.[1,2] Many of these "non-essential" metals do not have any known or suspected biochemical function and are thought to be environmental contaminants.[3] The contamination of tissues with metals is a greater problem with children than with adults because normally developing children mouth and chew unfamiliar objects as a way of examining them. For some children the desire to chew such objects is uncontrollable, and these children are said to have pica. Such children have a higher incidence of lead poisoning,[4] because of an increased oral ingestion of lead-containing materials, than children who do not have pica. In view of the wide range of metals used in the manufacture of materials, such as paints, plastics, rubbers and paper, likely to be chewed by children, it is possible that metals other than lead are excessively ingested by children. Multi-metal determination in blood samples taken from one group of children with pica and another group without pica would be of value in establishing whether the excessive ingestion of metals, other than lead, is a problem in young children.

The analytical methods that have been used successfully for multi-metal determination in biological materials are d.c. arc emission spectroscopy,[1] spark-source mass spectrometry[5] and neutron-activation analysis.[6] These techniques are, however, expensive,[5,6] difficult to apply to the routine analysis of a large number of samples[5,6] and require pre-concentration from large sample sizes.[1] Although simultaneous multi-element determination by atomic-absorption spectrophotometry is not yet feasible with commercially available instruments, the technique has good sensitivity for many elements and is easily adapted to routine analysis, and the basic equipment for single-element determinations is relatively cheap. This paper describes a method for the determination of eleven metals in a 1-ml sample solution of oxidised blood by sequential solvent extraction and atomic-absorption spectrophotometry. The metals determined were iron, copper, bismuth, zinc, cadmium, lead, cobalt, nickel, manganese, strontium and lithium.

When investigating biochemical lesions in young children, it is wise to take the minimum amount of blood possible for the determinations required. Any method developed must therefore be capable of providing multi-metal determinations with as little as 1 to 2-ml samples for each of two duplicate determinations.

With the exception of iron, zinc and copper, the normal physiological concentrations of the above metals are so low (0·005 to 0·05 p.p.m.) that a solvent-extraction and concentration stage is essential to obtain atomic-absorption signals that are accurately distinguishable from the background signals. A single extraction and 2-fold concentration from a 1-ml sample solution of oxidised blood would yield only 0·5 ml of solvent for the atomic-absorption determinations. Even by reducing the sample uptake rate of the nebuliser to one half of that recommended by the manufacturer (a Perkin-Elmer 303 instrument was used), this volume would be sufficient for only two determinations (Fig. 1). The determination in duplicate of the above eleven metals by using a single "universal" extractant, if available, would therefore require more than 9 ml of blood. However, a sequential separation of the metals would enable them to be determined by using 1 ml of sample solution and could therefore provide the basis of a method for multi-metal determination on the small samples of blood that can be taken from children.

Experimental

Development of a sequential extraction scheme—

The solvents that can be used for atomic-absorption spectrophotometry with air-supported flames are limited to those which support the combustion processes of the flames. Fortunately, isobutyl methyl ketone, which has been shown by Allan[7] to be an excellent solvent for flame atomic-absorption determinations, has also been proved useful for the extraction of metal chelates.[8,9] This solvent was therefore used for each extraction stage in the separation scheme described.

The extraction and concentration of metals from 1 ml of an aqueous sample solution into 0·5 ml of solvent yielded sufficient solution for one single atomic-absorption determination by using the sample uptake rate of the nebuliser recommended by the manufacturer, *viz.*, 5·6 ml minute^{-1} of isobutyl methyl ketone. When this was reduced to 2·8 ml minute^{-1}, the reduction in sensitivity was 14 per cent. relative, but determinations could be made with as little as 0·20 ml of solvent. It was then possible to make determinations of two different elements with 0·5 ml of extract with little loss in sensitivity or precision (Fig. 1). An extraction scheme was therefore devised to separate the metals to be determined into groups of

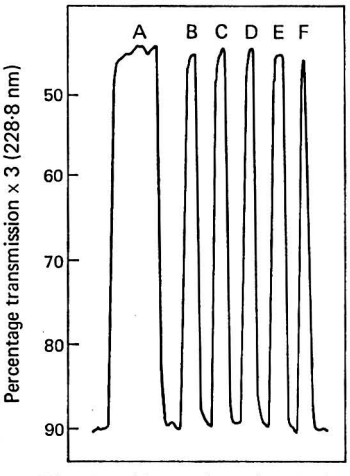


Fig. 1. Absorption signals from a continuous 20-s aspiration of a 0·2 μg ml^{-1} cadmium solution in isobutyl methyl ketone (A), and discrete aspirations (B to F) from 1·0 ml of the same solution until the entire sample was consumed; 1·0 ml of the solution was sufficient for four determinations (B to E) but not for the fifth determination (F)

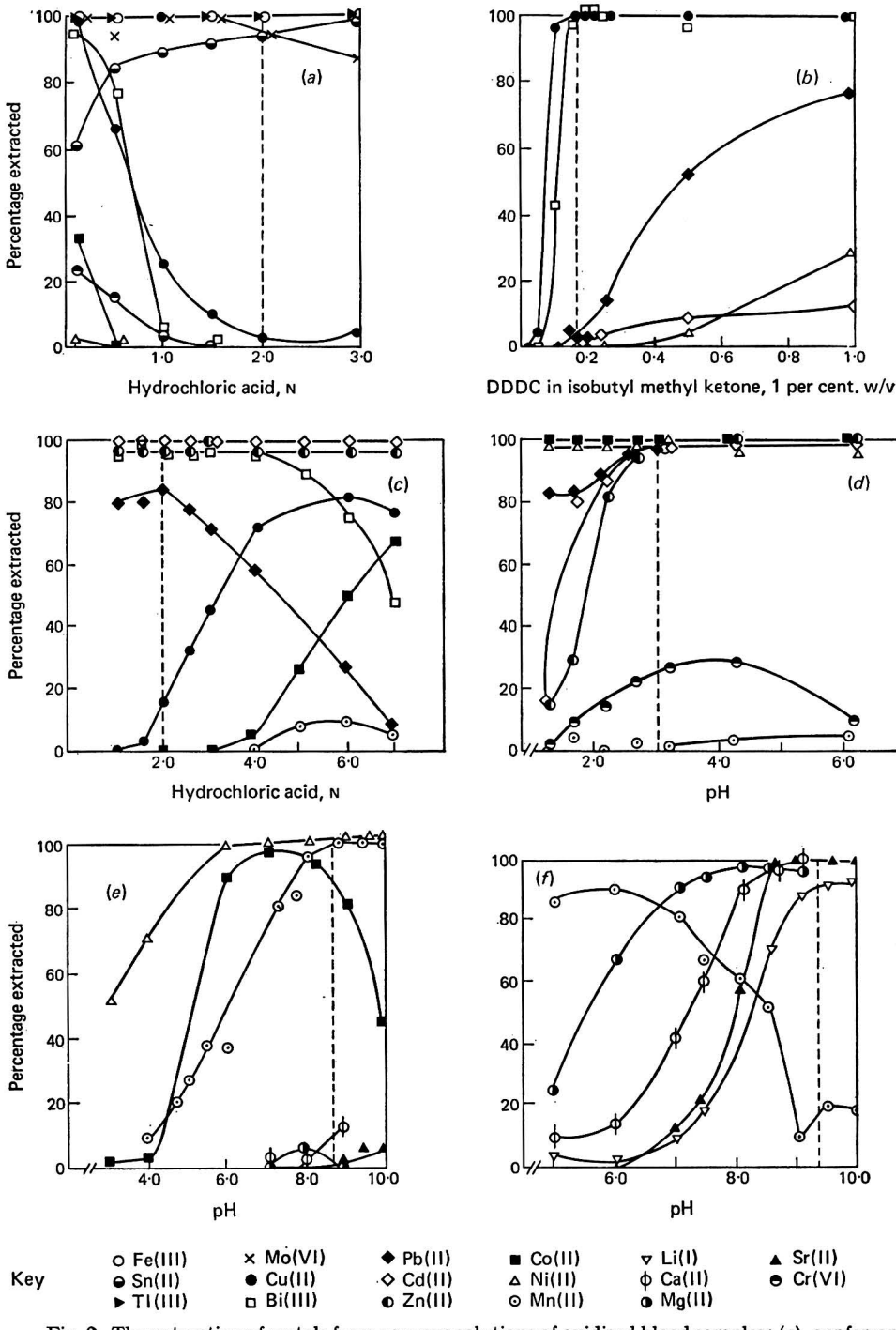
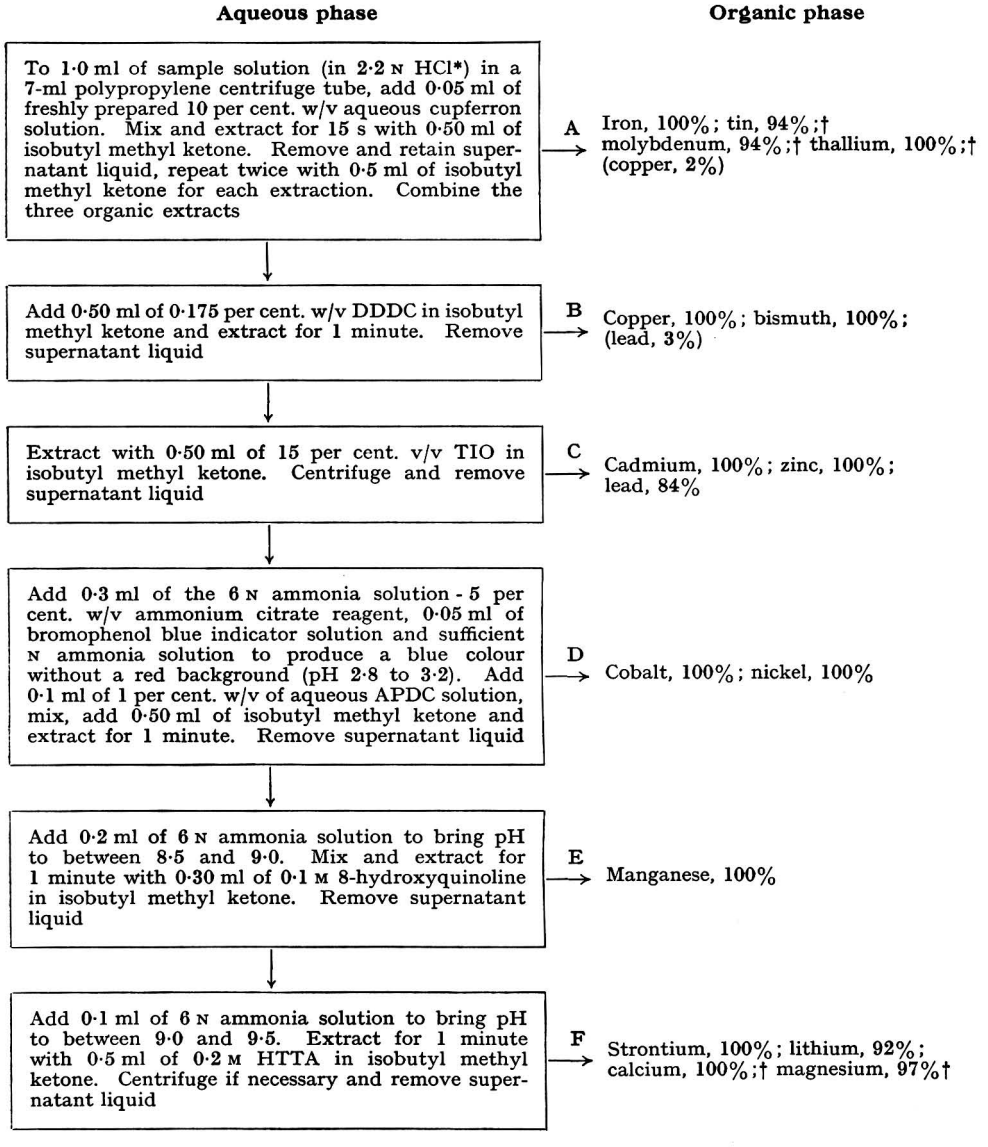


Fig. 2. The extraction of metals from aqueous solutions of oxidised blood samples: (*a*), cupferron; (*b*), diethylammonium diethyldithiocarbamate from 2 N hydrochloric acid; (*c*), triisooctylamine; (*d*), ammonium pyrrolidinedithiocarbamate; (*e*), 8-hydroxyquinoline; and (*f*), 2-thenoyl-3,3,3-trifluoroacetone



* The additions of aqueous cupferron solution reduce the concentration to 2 N hydrochloric acid.

† Metals that are extracted but not determined in the final method.

The precise adjustment of the pH of the aqueous phase for the APDC extraction (pH 2·8 to 3·2) enables the required pH values for the 8-hydroxyquinoline and HTTA extractions to be attained by adding the volumes of 6 N ammonia solution indicated. No further adjustment is necessary.

HTTA undergoes hydrolysis into trifluoroacetate and acetylthiophene at pH values greater than 8·0. The organic extract with this reagent must be removed as quickly as possible after extraction to avoid hydrolysis, which results in precipitation in *both* phases. This precipitate does not contain strontium or lithium but can cause trouble by blocking the nebuliser capillary if allowed to form or to remain in the organic phase.

All the solutions of the organic reagents should be prepared immediately before use.

Fig. 3. Procedure for the sequential separation of metals

two. The number of metals extracted in any one group could be increased to include iron or zinc, or both, as their high concentrations in blood (500 and 10 p.p.m., respectively) would enable determinations of them to be made with a dilution of a small, *e.g.*, 50-μl, portion of the extract.

Seventeen elements that are either essential or toxic to man, or are general environmental contaminants, were investigated and a scheme was developed for the quantitative extraction and separation of 16 of them. Five metals were not included in the final method: molybdenum, tin and thallium, because of their low sensitivity by atomic absorption; and calcium and magnesium, because they were not of interest in the present pica studies. The extraction efficiencies of these metals may be of interest to workers in the field of trace metals and biological systems and are shown in Figs. 2 and 3.

The extraction systems investigated are shown in Fig. 2 (*a* to *f*). In each instance, the aqueous phase was prepared from a hydrochloric acid solution of the inorganic residues from a blood sample that had been wet oxidised with nitric, perchloric and sulphuric acids and evaporated to dryness. The volume of hydrochloric acid solution used to dissolve the inorganic residues was one-fifth of the original volume of blood. This five times concentrated solution was used to prepare the aqueous phases for the extraction studies, so that each aqueous phase contained all of the inorganic constituents of blood at their normal physiological concentrations. This took into account any effects that these constituents, *e.g.*, phosphate ions, may have had on the extraction equilibria. Ammonium citrate solution (to give a final concentration of 1 per cent. w/v of ammonium citrate) was added to those aqueous phases the pH values of which were adjusted to greater than 1·0 for the extraction studies. This gave some buffering action and prevented the precipitation of metal hydroxides at high pH values.

A 2:1 ratio of the volumes of the aqueous to organic phases was used to ensure that a concentration stage was obtained. A single 60-s extraction was used for all systems except that with cupferron (Fig. 3 A) for which three successive 15-s extractions were necessary to remove completely the high concentration of iron present in blood.

The metals to be investigated were added to the aqueous phases at concentrations that were sufficiently high (2 to 10 μg ml^{-1}) to permit their accurate determination, before and after extraction, by atomic-absorption spectrophotometry.

The experimental conditions giving the optimum separation of the metals are indicated by the dotted lines intercepting the axis of abscissae in Fig. 2 (*a* to *f*). The procedure for the sequential separation of the metals prior to their determination is given in Fig. 3.

Iron, which is present at high concentrations in blood (450 μg ml^{-1}), was separated in the first extraction stage (Fig. 2 *a*) by extracting with cupferron and isobutyl methyl ketone. This extraction stage was used to remove iron from the aqueous solution of oxidised blood prior to investigating the other extraction systems (Fig. 2, *b* to *f*). This eliminated any interference from the high concentrations of iron in these solutions that could react with, and thus remove, most of the added reagent or form chelates that are insoluble in isobutyl methyl ketone, such as the iron(III) - ammonium pyrrolidinedithiocarbamate (APDC) chelate and iron(III) 8-hydroxyquinolinate.

Group A—Three successive extractions with isobutyl methyl ketone from an aqueous phase that was 2 N in hydrochloric acid and contained 0·05 per cent. w/v of cupferron gave a quantitative extraction of iron(III), molybdenum(VI), thallium(III) and tin(II), with only 2 per cent. extraction of copper(II).

Group B—Quantitative extraction of copper and bismuth was easily achieved during preliminary tests with diethylammonium diethyldithiocarbamate (DDDC) in isobutyl methyl ketone, but it was not possible to separate bismuth from lead, or bismuth and lead from the other metals. This extraction system was therefore investigated by using a 6 × 4 factorial arrangement of six different reagent concentrations in isobutyl methyl ketone, and four different hydrochloric acid concentrations (0·5, 1·0, 2·0 and 4·0 N) in the aqueous phases. The extraction from 2 N hydrochloric acid solution into a 0·175 per cent. w/v solution of the reagent in isobutyl methyl ketone gave the best separation of copper(II) and bismuth(III), both of which were completely extracted, from lead(II), 3 per cent. of which was extracted. Cadmium(II) and all of the other metals studied were not extracted under these conditions.

Group C—Triisooctylamine (TIO) forms a quaternary salt in hydrochloric acid solutions, the chloride ion of which can undergo anion exchange with chloride ion-association complexes

of metals to yield extractable species. Zinc(II), cadmium(II) and lead(II) were separated with this reagent from those metals which survived the first two extraction stages.

Group D—Ammonium pyrrolidinedithiocarbamate has poor selectivity but was used to separate quantitatively cobalt(II) and nickel(II) from manganese(II) and the other metals remaining after the extractions for groups A, B and C.

Group E—Manganese(II) was quantitatively extracted and separated from the alkali metals and the alkaline earths at pH 8·5 to 9·0 with 0·1 M 8-hydroxyquinoline in isobutyl methyl ketone.

Group F—Lithium(I), strontium(II), calcium(II) and magnesium(II) were all extracted with 0·2 M thenoyltrifluoroacetone (HTTA) in isobutyl methyl ketone (IBMK) at pH 9 to 9·5. The quantitative extraction of lithium(I) was unexpected but has been reported by Healy[10]

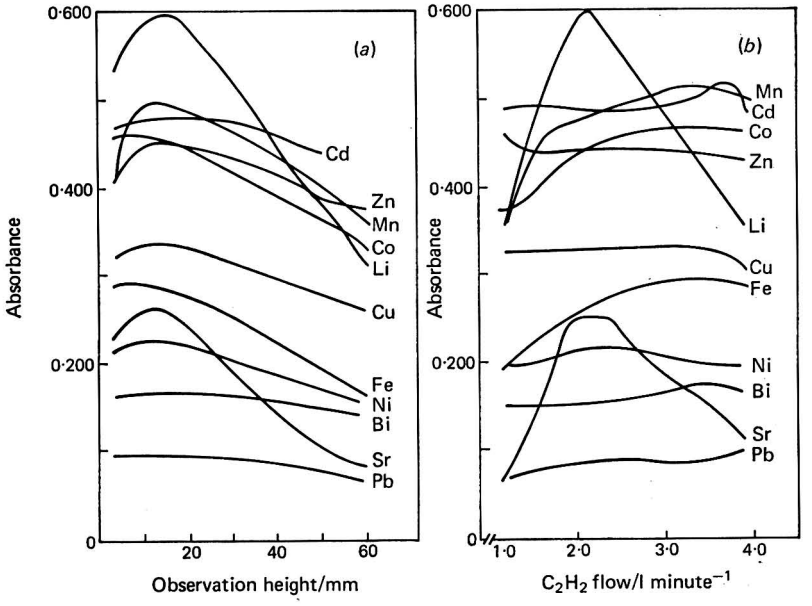


Fig. 4. Effect of (*a*) observation height and (*b*) acetylene flow-rate on flame absorbance for organic solutions of metals

TABLE I

EXPERIMENTAL CONDITIONS FOR ATOMIC ABSORPTION

Metal	λ/nm	Slit width/mm	Band pass/nm	Range expansion	Observation height/mm	Acetylene flow/l minute^{-1}
Fe	302·0	0·3	0·2	1	10·0	2·9
Cu	324·7	1·0	0·7	1	10·0	2·9
Bi	223·1	0·3	0·2	3	7·5	2·6
Cd	228·8	1·0	0·7	3	10·0	2·6
Pb	283·3	1·0	0·7	3	10·0	2·9
Zn	213·9	1·0	0·7	1	12·0	2·6
Co	240·7	0·3	0·2	3	7·5	2·7
Ni	232·0	0·3	0·2	3	10·0	2·7
Mn	280*	1·0	0·7	3	10·0	2·9
Sr	460·7	1·0	1·3	10	10·0	2·1
Li	670·8	1·0	1·3	10	15·0	2·1

*Unresolved triplet 279·5, 279·8 and 280·1 nm.
Sample uptake rate 2·8 ml minute^{-1} of isobutyl methyl ketone.
Air flow 22·8 l minute^{-1}, 30 p.s.i.; acetylene pressure, 5 p.s.i.
Noise suppression 2, *i.e.*, 2-s time constant in amplifier output circuit.

to be the result of adduct formation, with isobutyl methyl ketone acting as a neutral donor ligand according to the reaction

$$Li^{+}_{(aq)} + HTTA_{(org)} + 2IBMK_{(org} \rightleftharpoons LiTTA(IBMK)_{2\,(org)} + H^{+}_{(aq)}$$

OPTIMUM FLAME CONDITIONS FOR THE ATOMIC-ABSORPTION DETERMINATION OF METALS IN ISOBUTYL METHYL KETONE SOLUTIONS—

The optimum observation height, *i.e.*, the vertical distance between the optical axis of the monochromator and the top of the burner head, and the optimum fuel-to-oxidant flow ratio were determined for the eleven metals under investigation.

An air - acetylene flame and a triple-slot (Boling) burner were used for the investigations. The acetylene and air flow meters of the Perkin-Elmer 303 instrument were calibrated in free litres per minute at 15 °C in accordance with the recommendations of Mansfield and Winefordner[11] and Kirkbright and Sargent,[12] who have criticised the publication of arbitrary and meaningless flow-rates of gases in atomic-absorption methods.

The results are given in Fig. 4 (*a* and *b*). The optimum flame conditions established from these observations and other instrumental data for the atomic-absorption determination of the metals are given in Table I.

Strontium and lithium were the only metals that showed any marked dependence of free-atom concentration in the flame on observation height and acetylene-to-air ratio. Both metals form hydroxide and oxide species in air-supported flames and the reduction of these species depends on the atomic hydrogen concentration in the flame. The maximum absorption signals were obtained with observation heights just above the primary reaction zone, where the atomic hydrogen concentration is known to be greatest.[13] The response of the other metals (Fig. 4, *a* and *b*) indicated varying degrees of oxidation in the higher regions of the flame and increased reduction to free metal atoms with fuel-rich flames.

METHOD

APPARATUS—

A Perkin-Elmer 303 atomic-absorption spectrophotometer fitted with a 10-cm long triple-slot air - acetylene burner head (Boling) was used. Hollow-cathode lamps were used as radiation sources for each of the metals determined. The absorption signals were recorded on a 10-mV chart recorder, Rikadenki B-24X. The instrumental settings for the atomic-absorption determinations are given in Table I.

Silica conical flasks—25-ml capacity.

Stoppered glass tubes—5-ml capacity, graduated in 0·1-ml divisions.

Glass tubes—10-ml capacity, 125 × 15 mm i.d.

Polypropylene tubes—7-ml capacity, 100 × 100 mm i.d., and 3-ml capacity, 75 × 7 mm i.d.

Pasteur pipettes.

Glass syringes—2-ml capacity.

Rotamixer—Made by Hook and Tucker.

Sand-bath—Thermostatically controlled up to 300 °C.

REAGENTS—

The concentrated acids and ammonia solution used were Aristar grade reagents. All the other reagents used were of analytical-reagent grade unless otherwise specified.

Nitric acid, concentrated, sp.gr. 1·42.

Perchloric acid, sp.gr. 1·54—This substance should be handled cautiously, and the wet ashing should be carried out in suitably designed fume cupboards.

Sulphuric acid, concentrated, sp.gr. 1·84.

Dilute sulphuric acid, (1 + 4 *v*/*v*).

Hydrochloric acid, concentrated, sp.gr. 1·18.

Dilute hydrochloric acid solutions—Prepare 10 and 4·5 N hydrochloric acid solutions by diluting the concentrated acid with de-ionised water, and standardise by titration. Prepare a 1 + 10 v/v dilute hydrochloric acid solution.

Ammonia solution, concentrated, sp.gr. 0·88.

Dilute ammonia solutions, 6 N and 1 N (approximately)—Prepare by diluting 42 and 7 ml, respectively, of the concentrated reagent solution to 100 ml with de-ionised water.

Phenol red indicator solution, 0·02 *per cent. w/v.*

Bromophenol blue indicator solution, 0·04 *per cent. w/v.*

Ammonia - ammonium citrate solution, 5 per cent. w/v ammonium citrate in 6 N *ammonia solution*—Dissolve 40 g of citric acid in about 200 ml of de-ionised water. Add 2 drops of phenol red indicator solution and sufficient concentrated ammonia solution (about 100 ml) to produce a red colour. Remove trace metals from this solution by extraction with a 0·1 per cent. w/v solution of dithizone in chloroform as previously described,[14] add 5 ml of concentrated nitric acid solution and dilute to 500 ml with de-ionised water. Dilute 50 ml of this 10 per cent. w/v ammonium citrate solution to 100 ml with 42 ml of ammonia solution (sp.gr. 0·88) and de-ionised water.

Isobutyl methyl ketone saturated with water.

Cupferron, 10 per cent. w/v solution in de-ionised water—Dissolve 1·0 g of the reagent in 10 ml of water.

Diethylammonium diethyldithiocarbamate, 0·176 per cent. w/v solution in isobutyl methyl ketone—Dissolve 0·044 g of the reagent in 25 ml of isobutyl methyl ketone.

Triisooctylamine, 15 per cent. v/v solution in isobutyl methyl ketone—Dilute 4·5 ml of the reagent to 30 ml with isobutyl methyl ketone.

Ammonium pyrrolidinedithiocarbamate, 1·0 per cent. w/v aqueous solution—Dissolve 0·1 g of the reagent in 10 ml of de-ionised water.

8-*Hydroxyquinoline, 0·1* M *solution in isobutyl methyl ketone*—Dissolve 0·36 g of the reagent in 25 ml of isobutyl methyl ketone.

Standard solutions of the metals—Prepare from the pure metal, or a suitable salt, standard solutions of each metal, containing 5 mg ml^{-1} for iron and 1 mg ml^{-1} for the other metals, in 1 per cent. w/v hydrochloric acid.

Mixed standard solution of metals—For each of the metals except iron, prepare dilute standard solutions containing 100 μg ml^{-1} of metal in 1 per cent. v/v hydrochloric acid solution. Place the following volumes of the 100 μg ml^{-1} standard solutions of the metals indicated into a 100-ml calibrated flask and dilute to volume with de-ionised water: lithium, 0·10 ml; cadmium, cobalt, nickel, manganese and strontium, 0·50 ml; lead and bismuth, 3·00 ml; copper, 5·00 ml; and zinc, 25·0 ml.

Working standard solutions—To a series of six 10-ml calibrated flasks, add 0, 0·5, 1·0, 2·0, 3·0 and 4·0 ml of the mixed standard solution of metals. Add, in the same order, 0, 0·2, 0·4, 0·6, 0·8 and 1·0 ml of the 5 mg ml^{-1} standard iron solution. Add 2·2 ml of 10 N hydrochloric acid solution to each flask and dilute to volume with de-ionised water. These solutions are 2·2 N with respect to hydrochloric acid and contain:

		Solution					
Metal		1	2	3	4	5	6
Li	μg per 100 ml	0	0·5	1·0	2·0	3·0	4·0
Cd, Co, Ni, Mn and Sr	μg per 100 ml	0	2·5	5·0	10·0	15·0	20·0
Bi and Pb	μg per 100 ml	0	15	30	60	90	120
Cu	μg per 100 ml	0	25	50	100	150	200
Zn	μg per 100 ml	0	125	250	500	750	1000
Fe	μg ml^{-1}	0	100	200	300	400	500

PROCEDURE—

With a pipette introduce 2·0 ml of haemolysed whole blood into a 25-ml silica conical flask. Rinse down the sides of the flask with water (about 2 ml) and add 5 ml of concentrated nitric acid, 1·0 ml of perchloric acid (sp.gr. 1·54) and 0·1 ml of the dilute sulphuric acid (1 + 4) solution. Mix and allow to stand for 15 minutes.

Place the flask on a sand-bath (or hot-plate) at a temperature of 150 °C. Watch carefully for any signs of vigorous reaction and frothing and remove the flask if the frothing becomes excessive. When the initial reaction has subsided, leave the flask on the sand-bath until a clear yellow - brown solution is obtained. Increase the temperature to 200 °C and evaporate to fumes of perchloric acid, then increase the temperature to about 300 °C and evaporate to dryness. For convenience this can be done overnight.

Remove the flask from the sand-bath. When cool add 0·5 ml of the 4·5 N hydrochloric acid solution and return it to the sand-bath at a temperature of 150 °C. Evaporate the solution just to dryness and remove the flask from the sand-bath. When cool, the residue may become yellow as a result of residual hydrochloric acid vapour in the flask, but no

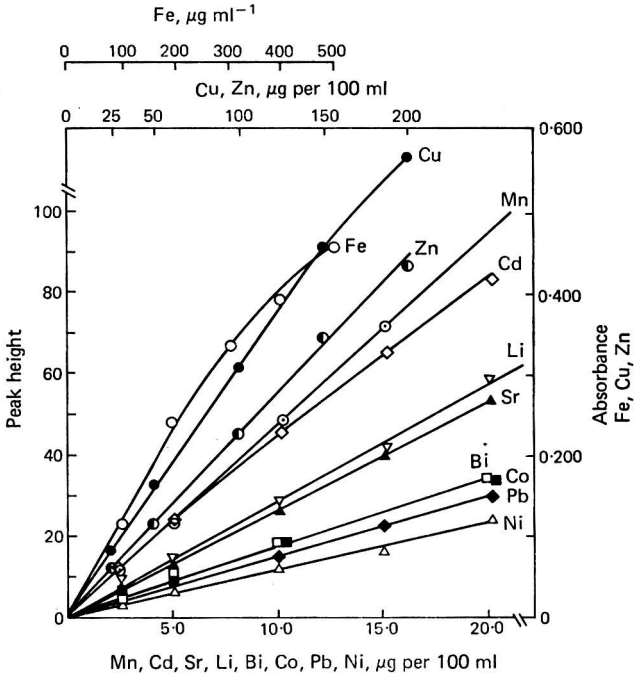


Fig. 5. Calibration graphs (lithium concentration was one-fifth of that shown; zinc concentration was 5 times that shown; and lead and bismuth concentrations were 6 times those shown)

liquid should remain. Add 1·00 ml of 4·5 N hydrochloric acid solution to the flask and place it on a sand-bath at a temperature of about 150 °C for about 2 minutes. Remove the flask from the sand-bath and allow it to cool. A clear yellow solution should be obtained. Transfer it to a 5-ml graduated tube, rinsing the flask three times with 0·2 to 0·3 ml of water, dilute to 2·00 ml with de-ionised water and mix.

With a pipette place a 1·00-ml aliquot of the solution in a 7-ml polypropylene centrifuge test-tube for the sequential extraction and separation of the metals by the procedure given in Fig. 3. Mix the phases by holding the tubes against the rubber pad of a vibrator - mixer. When the phases have separated, transfer the upper organic phase into a 3-ml polypropylene tube with a syringe-controlled Pasteur pipette.

After each extraction stage, determine the concentrations of the metals in the organic phases by using the experimental conditions given in Table I. For the determination of iron and zinc dilute 0·05 ml of the appropriate organic phase with 2·00 ml of isobutyl methyl ketone in a 7-ml polypropylene tube. Determine all other metals directly in the organic phases.

Determine duplicate reagent blanks with each batch of samples by using the method as described, except that water is substituted for haemolysed whole blood.

Establish calibration graphs by transferring with a pipette 2·00 ml of the mixed working standard solutions into 10-ml glass tubes and carry out the separation scheme as described but with twice the given volumes of the reagents. This provides enough solution for the atomic-absorption determinations before and during a run of samples.

To obtain the best results with the method described it is necessary to exercise a little more care than would normally be required for atomic-absorption determinations with larger volumes of solution. The nebulisation of 0·2 ml from a 0·5-ml fraction is judged on a time basis (about 4 s) and some practice is needed to enable a new operator to become skilled at making (at least) four determinations with 1 ml of solvent. Losses of isobutyl methyl ketone by volatilisation from the extraction tubes are negligible during the time required for analysis (about 0·7 per cent. loss after 30 minutes).

Results

Calibration graphs and the results of recovery tests are given in Fig. 5 and Table II.

Recovery tests were carried out for both synthetic aqueous standards and for solutions of metals added to blood samples. With the former, the solution of the inorganic residues from the oxidation stage was divided into two portions. A 1-ml portion was subjected to

Table II

Recovery of metals added to blood analysed by the described procedure

Concentration of metal added, μg per 100 ml	Mean per cent. recovered							Number of tests
	Bi	Cd	Co	Ni	Mn	Sr	Li*	
5	104	79	100	94	112	88	88	4
10	118	98	96	89	113	103	105	4
20	120	108	104	91	111	109	110	4
30	107	102	98	89	106	104	101	5
40	120	101	107	90	106	105	103	3
50	108	91	98	89	103	98	103	5

	Mean per cent. recovered				
	Pb	Cu	Zn†	Fe‡	
20	110	73	95	105	4
40	90	98	101	105	4
60	97	104	97	110	4
80	105	108	101	101	5
100	110	106	99	103	3
120	108	102	100	103	5

* Li added at one-fifth of concentration shown.
† Zn added at 10 times concentration shown.
‡ Fe added at 500 times concentration shown.

the separation scheme and the metals determined in the organic extracts (Table III); the remaining 4-ml portion was analysed directly by atomic-absorption spectrophotometry for the same metals. The correlations between the concentrations found by both techniques are given in Table IV.

Table III

Recovery of synthetic standard solutions of metals after oxidation and extraction

Results are expressed as regression equations of the form: concentration found = (concentration added) $m + c$. Ideally m should approach 1·000 and c should approach zero

Metal*	Constants in regression equation (μg per 100 ml)		Concentration range studied, μg per 100 ml
	m	c	
Cu	0·919	−2·2	10 to 400
Bi	1·075	−4·399	2·5 to 100
Cd	1·009	+0·806	2·5 to 100
Zn	1·001	−0·218	25 to 1000
Pb	0·945	+2·086	2·5 to 100
Co	0·992	+0·115	2·5 to 100
Ni	0·889	+3·17	2·5 to 100
Sr	0·996	−0·138	2·5 to 100
Li	1·037	+0·515	0·5 to 20

* Only one result was obtained for manganese because of mechanical loss of sample: added 100 μg per 100 ml; found 102 μg per 100 ml. Iron was not included in these studies.

Results for precision are given in Table V.

The detection limits given in Table VI are twice the standard deviation of the results of replicate blank determinations. Iron and zinc are not included in this table because they are determined on dilutions of the organic extracts.

TABLE IV

CORRELATION COEFFICIENTS FOR CONCENTRATIONS FOUND AFTER EXTRACTION AND BY DIRECT ANALYSIS OF AQUEOUS SOLUTIONS

Metal	Cu	Bi	Cd	Zn	Pb	Co	Ni	Sr	Li
Correlation coefficient	0·998	*	0·998	0·992	*	0·994	0·990	0·994	0·993

* No results for direct determinations because of insensitivity of atomic-absorption spectrophotometry for these metals in aqueous solutions.

The average increase in sensitivity resulting from solvent extraction and a 2-fold concentration was 6·7 times (range 5·9 to 7·1) that obtained by direct analysis of the corresponding aqueous solution. The increased sensitivity factor was 9·1 for manganese, which resulted from a 3-fold concentration stage.

APPLICATION—

The method described was applied to the analysis of small samples of blood taken from two groups of children. One group had a history of pica whereas the other group did not. For brevity they are referred to as the "pica group" and the "control group," respectively. The results are given in Tables VII and VIII.

TABLE V

PRECISION OF THE METHOD

Metal	Sample	Mean concentration found, μg per 100 ml	Standard deviation	Coefficient of variation, per cent.	Number of tests
Fe*	Blood	343·3	16·49	4·8	19
Cu	Blood	81	2·73	3·3	10
	Blood *plus* 100 μg of Cu per 100 ml	181	7·42	4·1	9
Bi	Blood	2·1	1·63	78	10
	Blood *plus* 60 μg of Bi per 100 ml	66·0	2·63	4·0	9
Cd	Blood	2·4	0·22	9·2	10
	Blood *plus* 10 μg of Cd per 100 ml	12·3	0·22	1·8	10
Pb	Blood	10·0	3·1	31	10
	Blood *plus* 60 μg of Pb per 100 ml	71·4	1·8	2·5	10
Zn	Blood	382	27·4	7·2	10
	Blood *plus* 500 μg of Zn per 100 ml	821	52·0	6·3	10
Co	Blood	0·61	0·28	46	10
	Blood *plus* 10 μg of Co per 100 ml	10·1	0·61	6·0	9
Ni	Blood	10·0	0·78	7·8	10
	Blood *plus* 10 μg of Ni per 100 ml	19·8	1·84	9·3	9
Mn	Blood	1·37	0·17	12·4	10
	Blood *plus* 10 μg of Mn per 100 ml	11·6	0·60	5·2	10
Sr	Blood	4·7	0·37	7·9	10
	Blood *plus* 10 μg of Sr per 100 ml	15·6	0·36	2·3	9
Li	Blood	0·45	0·09	20	6
	Blood *plus* 2·0 μg of Li per 100 ml	2·06	0·15	7·3	10

* Fe concentrations are in micrograms per millilitre.

TABLE VI

LIMITS OF DETECTION

Metal	Cu	Bi	Cd	Pb	Co	Ni	Mn	Sr	Li
Detection limit, μg per 100 ml	0·58	3·2	0·74	4·0*	0·44	4·2	0·2	0·56	0·1*

* The blank signals were not distinguishable from the flame background signals. These values were twice the standard deviation of the flame background signals.

TABLE VII

CONCENTRATIONS OF METALS FOUND IN BLOOD SAMPLES FROM THE CONTROL GROUP COMPARED WITH OTHER REPORTED VALUES

Metal	From literature Mean,* μg per 100 ml	Found in this study: Mean, μg per 100 ml	Standard deviation	Number of tests
Fe	475 × 10²	381 × 10²	47·2	88
Cu	107	97	21·8	82
Bi	1·2	0·9	1·7	44
Zn	650	509	140	83
Cd	0·7	0·5	0·5	88
Pb	20	11	6·5	37
Co	0·03	0·4	0·6	65
Ni	4·6	2·2	2·2	76
Mn	2·6	1·2	0·9	90
Sr	0·95	2·9	2·5	75
Li	2·0	0·3	0·4	70

* Data from Bowen.[3]

TABLE VIII

RAISED CONCENTRATIONS* OF METALS FOUND IN BLOOD SAMPLES

	Pb	Zn	Mn	Fe	Sr	Cd	Cu	Bi	Li	Ni	Co
Upper concentration limit, μg per 100 ml	36	930	3·8	240 × 10²†	10·5	1·9	160	6·1	1·6	8·7	2·3
Number of children with raised concentrations of metals in:											
Control group ..	0	1	1	0	2	0	0	1	3	1	1
Pica group ..	60	22	19	10	9	7	1	3	3	0	0

* Greater than the mean *plus* three times the standard deviation of the mean of the control group.
† Abnormal concentrations of iron in whole blood will be lowered, *e.g.*, in anaemia. This lower concentration limit is the mean *less* three times the standard deviation of the mean of the control group. Samples listed are those below 240 μg ml^{-1} of iron.
There were 194 children in the "pica" group.

DISCUSSION

No investigations were made into the effects of the hollow-cathode lamp current or slit width on the intensity of the absorption signals. The primary aim in these analyses was to obtain both a sufficiently intense and stable emission of radiation from the hollow-cathode lamp, which would permit a range expansion of up to ten times and at the same time keep the photomultiplier gain to a minimum. This enabled a small time constant to be used in the smoothing circuit of the recorder amplifier, which allowed determinations to be made satisfactorily with 0·20 to 0·30 ml of the organic extract (Fig. 1). The lamp currents and slit widths used fulfilled these requirements, and were usually those recommended by the manufacturer.

No study of potential interferences of sample concomitants on the determinations was made because the extraction equilibria were established in the presence of the inorganic constituents of blood *plus* large numbers of added metal ions at concentrations up to about 1 000 times those normally present in blood. Further, the high specificity of atomic-absorption spectrophotometry would be complemented by the selective solvent extraction of the metals to be determined. A check for spectral interference between metals extracted together was negative, as expected.

The number of operations in the extraction scheme was kept to a minimum, so that the method could easily be applied to large numbers of samples. For example, the hydrochloric acid concentration of the aqueous solutions of oxidised blood was fixed at 2·2 N, and this enabled the first three extractions to be carried out without any direct adjustments of the acidity of the aqueous phases. Only one accurate adjustment of the pH was necessary. By adjusting the pH of the aqueous phase for the fourth extraction stage to 3·0 ± 0·2 the pH values of the aqueous phases for the subsequent stages were obtained simply by adding the stated volumes of 6 N ammonia solution.

For routine application to large numbers of samples the extractions were carried out batchwise over a period of 3 days. In this way two people could in 1 week analyse fifteen samples in duplicate for each of the eleven metals. This amounted to about 440 determinations including reagent blanks and standards.

The recovery tests for synthetic aqueous standard solutions of metals were quantitative (Table III) and good correlations were observed between the concentrations found by direct analysis of these solutions and of the extracted organic phases (Table IV). The latter results showed that the extraction procedure, which was established for solutions of oxidised blood samples, was also quantitative for synthetic aqueous standards. The recoveries of metals added to whole blood (Table V) were quantitative for all of the metals determined. The coefficients of variation were better than ± 8 per cent. relative at a concentration of 10 μg per 100 ml for nine of the metals (Table V). With bismuth and lead, however, the precision was poor at this low concentration, which approached the detection limits for these metals. At higher concentrations of about 60 μg per 100 ml the precision was better, being 4·0 per cent. for bismuth and 2·5 per cent. for lead. The precision for lithium was good, being 7·3 per cent. even at the low concentration of 2·00 μg per 100 ml (*i.e.*, 0·02 p.p.m.).

The extraction - concentration stages resulted in increased atomic-absorption sensitivities of about seven times that obtained by direct analysis of aqueous solutions, and eliminated interferences from any non-specific absorption signals from the constituents of oxidised blood solutions, and from the effect of phosphate ions on strontium. The increase in detection limits was of the same magnitude as the increased sensitivity because the variations in the flame background signals were about the same when aqueous or organic solvents were burned. It would have been possible to improve on the detection limits obtained (Table VI) by using increased range expansion and noise suppression. However, this would have required larger volumes of solution and hence larger sample sizes to avoid dilution.

The method described recommends that 2 ml of blood be taken for analysis but only 1 ml is used for the extraction - atomic absorption determinations. In our study of children with pica the remaining 1 ml was used for the determination of chromium, which is not reported here. The sensitivity of the method could be improved by using the whole of the 2 ml of oxidised blood solution for the determinations.

On the other hand, smaller volumes of blood, *i.e.*, 1 ml or less, could be used for the preparation of the oxidised blood solution, if only a small sample is available.

The results for the "control group" (Table VII) agreed with published results for normal persons where these were available. It must be pointed out that the children from this group were those undergoing surgery and were assumed to be biochemically normal. A study of their clinical histories is necessary to confirm this. The abnormally high concentrations given in Table VIII showed that, in addition to lead, the metals zinc, manganese, strontium, cadmium and lithium could be excessively ingested by children with pica. A detailed survey of all of the results is being carried out, and the clinical histories of the children concerned are being evaluated by medically qualified colleagues.

Conclusion

The method described provides the means of trace-metal survey analysis of large numbers of blood samples at a capital cost far below that of other available techniques such as spark-source mass spectrometry, neutron-activation analysis, etc. The sequential separation of metals from a 1-ml portion of oxidised blood solution, as described in this paper, enables eleven metals to be determined in 2 ml or less of blood, which is of value when the samples are taken from a child who is young or sick, or both.

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References

1. Tipton, I. H., *in* Seven, M. J., and Johnson, L. A., *Editors*, "Metal Binding in Medicine," J. B. Lipincott Co., Philadelphia, Montreal, 1960, p. 27.
2. Butt, E. M., Nusbaum, R. E., Gilmour, T. C., Didio, S. L., and Sister Mariano, *Archs. Envir. Hlth*, 1964, **8**, 60.
3. Bowen, H. J. M., "Trace Elements in Biochemistry," Academic Press, London and New York, p. 159.

4. Bicknell, J., Clayton, B. E., and Delves, H. T., *J. Ment. Defic. Res.*, 1968, **12**, 282.
5. Evans, C. A., jun., and Morrison, G. H., *Analyt. Chem.*, 1968, **40**, 869.
6. Samsahl, K., Brune, D., and Wester, P. O., *Int. J. Appl. Radiat. Isotopes*, 1965, **16**, 273.
7. Allan, J. E., *Spectrochim. Acta*, 1961, **17**, 467.
8. Starý, J., "The Solvent Extraction of Metal Chelates," Pergamon Press, Oxford, 1964, p. 76.
9. Sandell, E. B., "Colorimetric Determination of Traces of Metals," Third Edition, Interscience Publishers Inc., New York, 1959, p. 60.
10. Healy, T. V., *J. Inorg. Nucl. Chem.*, 1968, **30**, 1025.
11. Mansfield, J. M., and Winefordner, J. D., *Analytica Chim. Acta*, 1968, **40**, 357.
12. Kirkbright, G. F., and Sargent, M., *Analyst*, 1968, **93**, 552.
13. Hermann, R., and Alkemade, C. Th. J., "Chemical Analysis by Flame Photometry," Second Edition, Interscience Publishers Inc., New York and London, 1963, p. 32.
14. Delves, H. T., and Vinter, P., *J. Clin. Path.*, 1966, **19**, 504.

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Quantitative Determination of Taurine by an *o*-Phthalaldehyde-Urea Reaction

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Taurine is made to react with *o*-phthalaldehyde in the presence of urea and phosphate ions, and on acidifying the mixture with acetic acid a purple product is formed with an extinction maximum at 560 nm. A method based on this reaction is described for the quantitative determination of taurine and it is applied to the determination of this amino-acid in rat brain after passage of the tissue extract through ion-exchange resins. The effect of other amino-acids on the accuracy of the method is discussed.

Amines react with *o*-phthalaldehyde[1,2] to give coloured products and this reaction has also been used for their detection on paper chromatograms. Curzon and Giltrow[3] used the reaction for identifying spots of taurine and other amino-acids and they also noted that different products resulted if the reaction was performed in the presence of urea. These observations have been made the basis of a specific quantitative method for the determination of taurine in tissue extracts.

Method

Materials and reagents—

Amino compounds were obtained from Koch-Light Laboratories Limited (Colnbrook, Buckinghamshire) or from California Corporation for Biochemical Research (Los Angeles, California). A sample of homotaurine (3-aminopropane-1-sulphonic acid) was kindly supplied by Dr. J. C. Watkins. *o*-Phthalaldehyde, urea, hydrochloric acid (sp.gr. 1·18), acetic acid (99·6 per cent.), phosphoric acid (sp.gr. 1·75) and other reagents were products of B.D.H. (Chemicals) Limited, Poole, Dorset.

Urea solution—A 50 per cent. urea solution was prepared by dissolving 50 g of urea in water and diluting to 100 ml at room temperature (21 °C).

o-Phthalaldehyde reagent—A saturated solution of *o*-phthalaldehyde (0·6 per cent.) was prepared by suspending 2 g of *o*-phthalaldehyde in 100 ml of water in a loosely stoppered conical flask and warming it in a boiling water bath for 2 to 3 minutes; the flask was then removed from the bath and stoppered tightly, and the contents were shaken vigorously. By repeated warming and mixing most of the residue in the flask dissolved in the water, although a small amount of yellow oily material remained adhered to the surface of the flask. The contents were then mixed on a mechanical mixer at room temperature for 1 to 2 hours and filtered through Whatman glass-fibre paper (GF/C). The clear filtrate was used directly or stored in a brown bottle for several weeks in the cold room at about 8 to 10 °C until required.

Quantitative determination of taurine with *o*-phthalaldehyde—

To 1-ml aqueous samples (containing 0·1 to 1·0 μmole of taurine) in stoppered tubes, 1 ml of 0·02 M sodium phosphate buffer (pH 6·8) was added and the contents were mixed. The tubes were then placed in an ice-bath for 5 to 10 minutes, 1 ml of freshly prepared 50 per cent. urea solution was added consecutively to all tubes and the contents were mixed. The saturated (0·6 per cent. w/v) ice-cold solution of *o*-phthalaldehyde was then added in 1-ml amounts to all tubes. After mixing of the contents, the tubes were stoppered and placed in the ice-bath.

After 5 minutes, 0·5 ml of 99·6 per cent. acetic acid was added, the contents were mixed immediately by repeatedly inverting the stoppered tubes, and the tubes were again placed in the ice-bath. The solution in the tubes was transferred to a 1-cm cell and the extinction at 560 nm measured against water until it reached a maximum value, which was then recorded. All optical measurements were made within 40 minutes of the addition of the acetic acid,

and the time required for the extinction to reach the maximum value was between 2 and 3 minutes for those samples taken from the ice-bath 30 to 40 minutes after the addition of acetic acid. For rapid measurement of the highest extinction given by each sample, a limited number of tubes were removed from the ice-bath and left at room temperature, while the previous set of samples were investigated with the spectrophotometer to find their maximum extinctions.

The amount of taurine in the sample was determined by reference to a calibration curve. The extinction at 560 nm was proportional to the amount of taurine in the range 0·2 to 1·0 μmole, and that at the isosbestic point at 490 nm to the range of taurine from 0·05 to 0·7 μmole (Fig. 1).

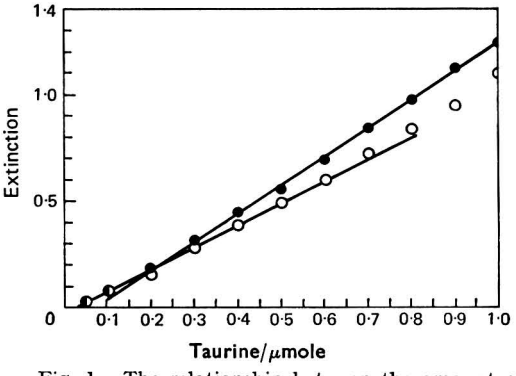


Fig. 1. The relationship between the amount of taurine with *o*-phthalaldehyde when using 50 per cent. urea solution and 0·02 M phosphate buffer (pH 6·8), and the extinction at 560 nm (–●–●–) and at the isosbestic point, 490 nm, (–○–○–) of the purple product formed after the addition of acetic acid

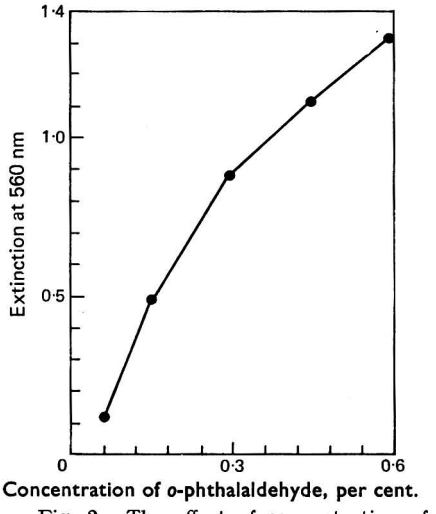


Fig. 2. The effect of concentration of *o*-phthalaldehyde solution on the extinction at 560 nm of the product formed on reaction with taurine (0·5 μmole) when using 0·02 M phosphate buffer (pH 6·8) and 50 per cent. urea solution

Results

Factors affecting the reaction of taurine with *o*-phthalaldehyde—

Concentration of o-phthalaldehyde—The saturated 0·6 per cent. solution of *o*-phthalaldehyde was diluted with water. The diluted reagent solutions gave lower extinction values than the saturated solution of the reagent when allowed to react with taurine in the presence of urea and phosphate (Fig. 2).

Concentration of urea—The typical purple product was formed from taurine after its reaction with *o*-phthalaldehyde only in the presence of urea. The extinction at 560 nm of the purple product formed on reaction of taurine (0·5 μmole) with *o*-phthalaldehyde increased with increasing concentration of urea, readings being 0·60, 0·63, 0·64, 0·65, 0·65 and 0·67 when using 10, 20, 25, 30, 40 and 50 per cent. urea solution, respectively. However, the stability of the purple product decreased with increasing concentration of urea solution (Fig. 3).

The acid reagent for development of the colour—The intensity of the colour was greater on acidifying the reaction mixture with acetic acid than with hydrochloric, phosphoric or sulphuric acids. The purple product was formed at pH values between 2·0 and 2·9.

Temperature of incubation—The product formed on acidification of the reaction mixture was purple, provided that the temperature of the reaction mixture was between 0 and 39 °C. At higher temperatures the reaction product was yellow.

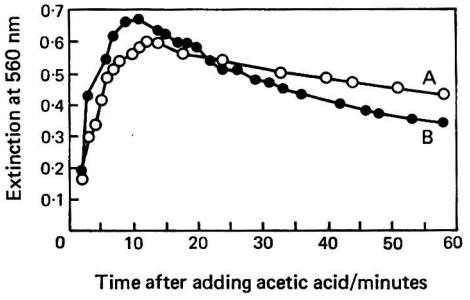


Fig. 3. The effect of concentration of added urea solution on the extinction at 560 nm of the product formed on reaction of taurine (0·5 μmole) with *o*-phthalaldehyde in the presence of 0·06 M phosphate buffer (pH 6·6). A, 10 per cent. urea solution and B, 50 per cent. urea solution

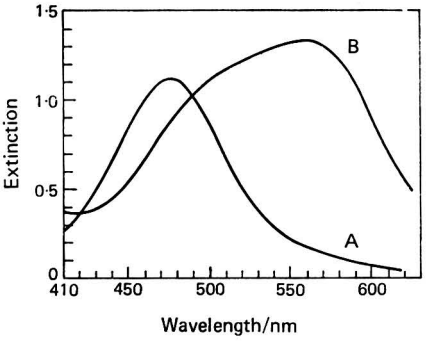


Fig. 4. The optical spectra of the reaction products of taurine (1 μmole) with *o*-phthalaldehyde when using 50 per cent. urea solution and 0·02 M phosphate buffer (pH 6·8) at 0 °C. The spectra were recorded using a Unicam SP800 spectrophotometer at 0·5 minute (A) and at 8·5 minutes (B) after the addition of acetic acid

Samples pre-incubated at 0 °C and subsequently acidified gave an orange product, with an extinction peak at 470 nm, which gradually changed into a purple product with maximum extinction at 560 nm (Fig. 4). The development of the purple product was almost complete in 40 to 50 minutes at 0 °C and could be accelerated on warming to between 16 and 21 °C (Fig. 5). The purple colour was stable for 2 to 3 minutes on reaching its maximum extinction at 560 nm (see Fig. 3) and was stable for 10 minutes at the isosbestic point between 490 and 500 nm.

Samples pre-incubated at higher temperatures (21 to 36 °C) gave the purple product with maximum extinction at 560 nm immediately on acidification, its extinction increasing during the first 2 minutes and then showing a slow decrease.

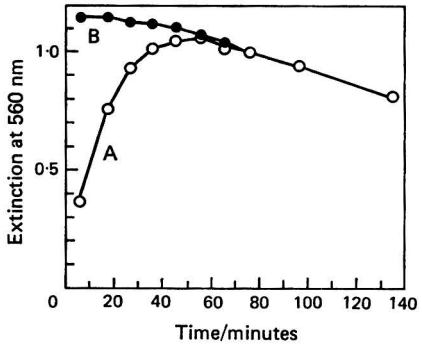


Fig. 5. Rate of development of purple colour after reaction of taurine (1 μmole) with *o*-phthalaldehyde when using 0·06 M sodium phosphate (pH 6·4) and 50 per cent. urea solution. Curve A gives the extinction at 560 nm recorded directly after withdrawal of the reaction mixture from the ice-bath at different times after the addition of acetic acid. Curve B gives the extinction at 560 nm of the same samples on allowing the development of the purple colour up to its maximum value in the spectrophotometer

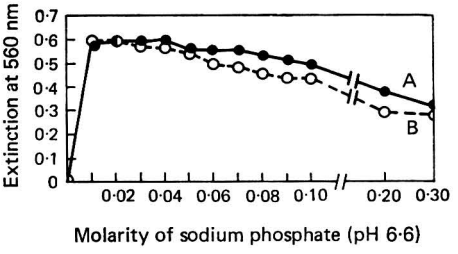


Fig. 6. The effect of phosphate ions (pH 6·6) on the extinction at 560 nm of the product formed on reaction of taurine (0·5 μmole) with *o*-phthalaldehyde when using 50 per cent. urea solution (A), and 25 per cent. urea solution (B)

TABLE I

EXTINCTION OF THE PRODUCTS OF AMINO COMPOUNDS ON TREATMENT WITH *o*-PHTHALALDEHYDE

Amino compound	λ_{max}	Extinction at 560 nm	
		at 0 °C	at 36 °C
Taurine	560	1·24	1·13
Homotaurine	560	0·75	0·66
Hypotaurine	500	0·32	0·23
Glycine	560	0·97	0·22
Lysine	560	1·05	0·72
δ-Hydroxylysine	560	1·00	—
γ-Aminobutyric acid	560	0·32	0·28
β-Alanine	560	0·58	—
Ethanolamine	560	0·40	0·12
Ornithine	560	0·26	0·24
Cysteamine	560	0·27	0·14
Cystamine	560	0·46	0·30
Tyramine	520 to 570	0·27	0·65
3-Hydroxytyramine (dopamine)	530 to 630	0·57	0·69
Phenylethylamine	510 to 560	0·41	0·25
Tryptamine	480 to 495	0·17	—
5-Hydroxytryptamine	480	0·33	0·34
Tryptophan	450	0·07	0·16
5-Hydroxytryptophan	475	0·15	—
Histamine	560	0·12	0·11
N-ε-Methyllysine	—	0·08	—
α-Alanine	—	0·05	0·26
Arginine	—	0·06	0·16
Aspartic acid	—	0·04	0·28
Asparagine	—	0·05	0·21
Citrulline	—	0·05	0·33
Cysteine hydrochloride	—	0·04	0·17
S-Methylcysteine	—	0·09	—
Penicillamine	—	0·05	0·11
Cysteic acid	—	0·04	0·27
Cysteine sulphinic acid	—	0·02	0·14
Cystine	—	0·04	0·09
Cystine disulphoxide	—	—	0·30
Homocystine	—	0·11	0·34
Homocysteic acid	—	0·04	0·27
Glutathione, GSH	—	0·04	0·27
,, GSSG	—	—	0·29
L-Cystathionine	—	0·10	0·20
Methionine	—	0·11	0·36
Methionine sulphoxide	—	0·04	0·17
Methionine sulphone	—	0·05	0·20
Glutamic acid	—	0·04	0·21
Glutamine	—	0·04	0·31
Histidine	—	0·04	0·12
1-Methylhistidine	—	0·02	—
3-Methylhistidine	—	0·02	—
Proline	—	0·02	—
Serine	—	0·05	0·12
Homoserine	—	0·06	1·19
Threonine	—	0·04	0·13
Leucine	—	0·04	—
Isoleucine	—	0·03	—
Valine	—	0·06	—
Diaminopimelic acid	—	0·06	—
Phenylalanine	—	0·04	0·26
Tyrosine	—	0·04	0·34
Ammonium chloride	—	0·03	0·05
Ergothioneine	—	0·02	0·02
Glucosamine	—	0·02	—

Each compound (1 μmole) was treated with 0·6 per cent. of *o*-phthalaldehyde solution while using 50 per cent. urea solution and 0·06 M sodium phosphate solution (pH 6·4) at 0 °C for 5 minutes or at 36 °C for 30 minutes. The reaction mixture was acidified with acetic acid and the maximum extinction value was recorded.

Molarity and pH of the phosphate buffer—The presence of small amounts of phosphate ions was necessary for the formation of the purple product but phosphate buffer solutions of higher molarity (greater than 0·1 M phosphate) gave a considerable decrease in the extinction of the purple solution (Fig. 6). The extinction was highest if the reaction mixture was incubated at a pH between 6·5 and 7·2 (Fig. 7). A reagent of 0·02 M phosphate buffer (pH 6·8) was chosen for use in the quantitative determination of taurine.

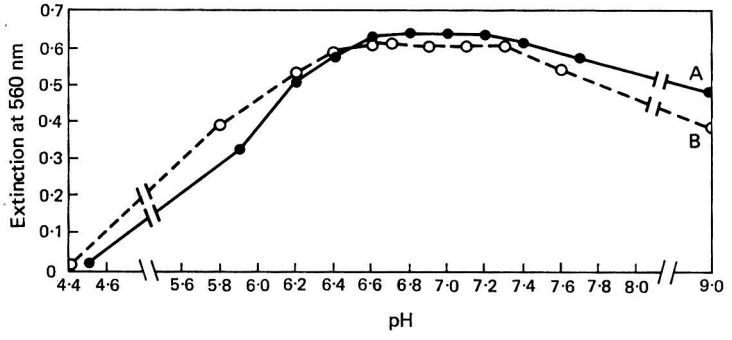


Fig. 7. The effect of 0·02 M and 0·06 M sodium phosphate buffers on the extinction at 560 nm of the product formed on reaction of taurine (0·5 μmole) with *o*-phthalaldehyde when using 50 per cent. urea solution. A, 0·02 M sodium phosphate buffer and B, 0·06 M sodium phosphate buffer

The presence of chloride ions—Samples of taurine solutions up to 0·05 M in sodium chloride did not show any appreciable interference effect. However, higher concentrations of the chloride ions gave a decrease in the extinction of the purple product.

REACTION OF AMINO COMPOUNDS WITH *o*-PHTHALALDEHYDE IN THE PRESENCE OF UREA—

Several amino compounds were treated with *o*-phthalaldehyde and 50 per cent. urea solution at 0 or 36 °C. After 5 minutes, the reaction mixture was acidified with acetic acid, and the extinction maximum of the product and its extinction at 560 nm were recorded (Table I). The first twelve amino compounds, except hypotaurine, listed in Table I gave products with extinction maxima at 560 nm. Hypotaurine gave an extinction maximum at 500 nm, with an extinction of 1·26 under the conditions described in Table I. The products of other amino compounds had extinction maxima below 490 nm or had a wide absorption band between 510 and 630 nm.

For the quantitative determination of taurine, the reaction with *o*-phthalaldehyde at 0 °C was preferred to that at 36 °C because (*i*) under the conditions used several α-aminocarboxylic acids gave a yellow product ($\epsilon_{450} = 2\,250$) at 36 °C but no coloured product at 0° C. The extinction of the products at 560 nm was less when these α-aminocarboxylic acids were reacted at 0 °C than at 36 °C; (*ii*) the calibration curve was a straight line; and (*iii*) the procedure is applicable under certain conditions to the determination of lysine, hydroxylysine and glycine (*e.g.*, in protein hydrolysates). Lysine gave a brownish-black product (λ_{max} 450) and glycine a yellowish green product (λ_{max} 450) on treatment with *o*-phthalaldehyde.

EXTRACTION OF THE REACTION PRODUCTS WITH CHLOROFORM—

After optical measurement the reaction mixture was equilibrated with 4 ml of chloroform.[1] The purple products formed from glycine, cysteamine and cystamine were found to be extractable with chloroform, in which the products of the two sulphur-containing amino-acids were stable for 16 to 20 hours. This property can be utilised in the detection of these acids in the presence of other sulphur-containing amino-acids. The reaction products of tryptamine and phenylethylamine were extractable into the chloroform phase but those given by 5-hydroxytryptamine, tyramine and 3-hydroxytyramine remained predominantly in the aqueous phase.

MECHANISM OF REACTION OF TAURINE WITH *o*-PHTHALALDEHYDE IN THE PRESENCE OF UREA—

Taurine also gave rise to a purple product if urea solution in the reaction mixture was replaced by thiourea solution (10 per cent. w/v), acetamide solution (50 per cent. w/v) or glutamine solution (5 mM), but not if urea solution was replaced by acetone or ethyl methyl ketone.

It was found that under conditions in which a mixture of urea and phosphate was pre-incubated for 5 minutes with taurine and *o*-phthalaldehyde, or with taurine and subsequently *o*-phthalaldehyde, or with *o*-phthalaldehyde and subsequently taurine, the reaction product formed on the addition of acetic acid was yellow-orange, changing with time into a purple product. The purple product was not formed if phosphate was omitted from the incubation mixture, or if taurine was omitted from the incubation mixture but added to the acidified reaction mixture.

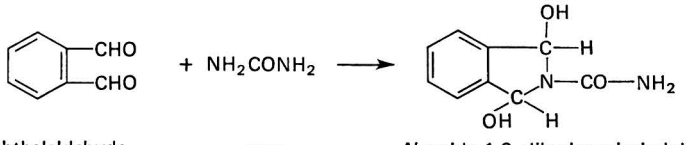
The following mechanism, which is consistent with the experimental findings, is proposed:

(*i*) urea + phosphate + *o*-phthalaldehyde ⟶ [A]

(*ii*) [A] + taurine ⟶ [B]

(*iii*) [B] + acid ⟶ [C] ⟶ [D] ⟶ [E]
orange purple yellow

It may be assumed that urea, thiourea and glutamine form addition products [A] similar to those reported for acetamide.[4]



o-phthalaldehyde urea *N*-amido-1,3-dihydroxyisoindoline

The compound [A] is then converted into the purple product according to the mechanism postulated in reactions (*ii*) and (*iii*) above. Phosphate probably acts as a catalyst in the formation and stabilisation of the reaction product [A]. In view of the fact that glutamine can replace urea in the reaction it appears that only one amido group of urea is reacting with *o*-phthalaldehyde. In general, several amino compounds of the type NH_2–CH_2–R, where R is –COOH or an aliphatic carbon chain (*e.g.*, taurine, homotaurine, lysine, glycine, cystamine, cysteamine, γ-aminobutyric acid, β-alanine, ornithine, ethanolamine or butylamine), gave purple products on treatment with *o*-phthalaldehyde at 0 °C; hyptotaurine gave a pink product. An intense purple product was also given by isopropylamine. α-Aminocarboxylic acids, except those mentioned above, gave no purple products on acidification of their reaction products with *o*-phthalaldehyde.

In experiments in which urea was not present during the incubation but was added at the end of 5 minutes of incubation and then allowed to react for 1 to 5 minutes, no purple product resulted on subsequent acidification. This observation, which showed that taurine also reacts with *o*-phthalaldehyde in the absence of urea, was also found to be dependent on the presence of phosphate ions in the incubation mixture. The reaction mechanism may be similar to the one postulated above for urea. The addition compound so formed is probably responsible for fluorescent products reported for several amines (and α-aminocarboxylic acids) by earlier workers.[5 to 10]

APPLICATION OF THE METHOD TO TISSUE EXTRACTS

The method can be applied directly to the determination of taurine (and homotaurine) in the *in vitro* assay of enzymes involved in metabolism with methionine and other sulphur-containing amino-acids. Taurine is present in large amounts in rat brain and certain other tissues. Its concentration in tissue extracts was determined by the present method after removal of γ-aminobutyric acid, glycine and lysine, which interfere to some extent, depending on their concentration in the tissue extract. Rat brain was homogenised in ice-cold 5 per cent. w/v perchloric acid or 10 per cent. w/v trichloroacetic acid. The suspension was centrifuged and the clear extract decanted, after which the tissue residue was washed once with the same acid. The washing was combined with the main extract, which was then neutralised to precipitate perchlorate ions or washed three times with ether to remove tri-

chloroacetic acid. The aqueous extract was filtered into a calibrated cylinder and made up to a suitable volume (*e.g.*, 25 ml). A fraction containing taurine that was free from interfering amino-acids was obtained from the extract by one of the following two procedures.

In the first, a portion of the aqueous extract (10 to 20 ml) was passed through a two-column assembly consisting of Zeo-Karb 225 (H^+ form, 10 cm) fitted on top of Dowex 1 X-10 (carbonate form, 12 cm). After the passage of the sample, the column assembly was washed with 50 ml of water. The Dowex 1 column was disconnected from the assembly; taurine adsorbed on this resin was eluted with 0·1 N acetic acid under pressure of nitrogen gas until all the carbonate was exchanged with acetate and free acid emerged from the column. The neutral eluate was evaporated to dryness and the residue dissolved in 5 ml of water.

In the alternative procedure a portion of the aqueous tissue extract (10 to 20 ml) was passed through a two-column assembly consisting of Zeo-Karb 225 (H^+ form, 10 cm) fitted on top of Dowex 1 X-10 (acetate form, 12 cm). The effluent emerging from the column assembly contained taurine and neutral compounds, which were collected quantitatively by washing the column assembly with water (50 ml). The effluent (*plus* washings) was evaporated to dryness and the residue dissolved in 5 ml of water. Because chloride ions inhibit the *o*-phthalaldehyde - urea reaction, the substitution of Dowex 1 in the acetate form with a Dowex 1 column in the chloride form should be avoided. If it nevertheless occurs, the chloride ions (washed out as hydrochloric acid) should be removed by repeated evaporation of the effluent fraction.

Homotaurine, when present in the tissue extracts, was found in the taurine fraction on ion-exchange chromatography. The taurine fraction obtained by these two procedures contained phosphoethanolamine and, probably, glycerylphosphorylethanolamine. They were resolved on paper chromatograms developed with acetone - ethyl methyl ketone - water (2 + 2 + 1), which gave relative R_F values, with respect to that of taurine, of 0·32 for phosphoethanolamine and 0·55 for glycerylphosphorylethanolamine. Moreover, the fraction obtained by the second procedure also contained free sugars. None of these compounds interfered in the determination of taurine in the tissue extracts.

A mean value of 6·76 ± 0·09 (s.e.m.) μmole of taurine per gram of brain was obtained for Wistar albino rats of 100-g body weight. From the same tissue extracts taurine was isolated by combined ion-exchange and paper chromatography. Taurine was eluted from paper chromatograms and the eluate, after removal of interfering volatile material[11] and reaction with ninhydrin,[12] gave a mean value of 6·88 ± 0·16 (s.e.m.) μmole of taurine per gram of brain. The present value may be compared with a value of 6·94 μmole per gram of brain for rats of 73-g body weight.[13] An inspection of the values reported for taurine content of brains of rats of different body weights shows a wide scatter (this has been reviewed[14]). This suggests that, besides age, other factors such as strain and nutritional state of the animal may also affect the taurine content of the brain.

As all the interfering amino compounds are adsorbed on the cation-exchange resin (Zeo-Karb 225, H^+ form), the use of a second column of anion-exchange resin is not necessary in enzyme assay systems. The effluent emerging from the cation-exchange resin is either assayed for taurine directly, or after concentration of the material by evaporation.

References

1. Klein, G., and Linser, H., *Hoppe-Seyler's Z. physiol. Chem.*, **1932**, **205**, 251.
2. Patton, A. R., *J. Biol. Chem.*, **1935**, **108**, 267.
3. Curzon, G., and Giltrow, J., *Nature*, **1954**, **173**, 314.
4. Reynolds, R. D., and Conboy, R. J., *J. Org. Chem.*, **1965**, **30**, 2251.
5. Shore, P. A., Burkhalter, A., and Cohn, V. H., jun., *J. Pharmac. Exp. Ther.*, **1959**, **127**, 182.
6. Juhlin, L., and Shelley, W. B., *J. Histochem. Cytochem.*, **1966**, **14**, 525.
7. Cohn, V. H., jun., and Shore, P. A., *Analyt. Biochem.*, **1961**, **2**, 237.
8. Kremzner, L. T., *Ibid.*, **1966**, **15**, 270.
9. Rogers, C. J., Chambers, C. W., and Clarke, N. A., *Ibid.*, **1967**, **20**, 321.
10. Sen, N. P., Somers, E., and O'Brien, R. C., *Ibid.*, **1968**, **26**, 457.
11. Gaitonde, M. K., Dahl, D. R., and Elliott, K. A. C., *Biochem. J.*, **1965**, **94**, 345.
12. Sörbo, B., *Clinica Chim. Acta*, **1961**, **6**, 87.
13. Garvin, J. E., *Archs. Biochem. Biophys.*, **1960**, **91**, 219.
14. Gaitonde, M. K., *in* Lajtha, A., *Editor*, "Handbook of Neurochemistry," Volume III, Plenum Press, New York, **1970**, p. 225.

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A Spectrofluorimetric Method for the Determination of Small Amounts of Sulphate Ion

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The enhancement of the fluorescence of the binary complex of zirconium with Calcein blue at pH 1·9 is used to determine sulphate ion in the range 0·2 to 12 mg (2 to 12 000 p.p.m.). Excitation and fluorescence maxima occur at 350 and 410 nm, respectively. The fluorescence is stabilised immediately and remains unchanged for over 4 hours. Fluoride present in low concentrations gives rise to high results and must be absent. Other anions that form complexes with or precipitate zirconium, *e.g.*, oxalate, phosphate, tartrate and tungstate, cause low results, but there is a high tolerance towards most cations except iron(III) and cobalt(II).

THE most widely used methods for the determination of moderately large amounts of sulphate involve precipitation of barium sulphate followed by a gravimetric or titrimetric procedure. Smaller amounts can be measured turbidimetrically or nephelometrically after precipitation. Several indirect spectrophotometric[1] and fluorimetric[2] procedures have also been described, based on the bleaching or quenching action of sulphate ion on some highly coloured or fluorescent metal - dyestuff complexes. Jones and Letham[3] have described another novel indirect method based on the use of chloroaminobiphenyl. Recently a kinetochromic procedure has been used to provide a direct absorptiometric method based on the catalytic effect of sulphate ion on the reaction between methylthymol blue and a partially polymerised solution of zirconyl ions.[4] A similar fluorimetric procedure has been devised for sulphate ion in which the fluorescent reagent morin is used instead of methylthymol blue.[5]

Although the last two methods mentioned above are very sensitive they are kinetically controlled and require to be applied under rigidly maintained conditions. In this paper, we describe a direct fluorimetric determination that depends on the enhancement of fluorescence produced by allowing sulphate ion to react with an unpolymerised zirconium solution and the fluorescent reagent Calcein blue. We have recently described a similar reaction for fluoride ion[6] based on the zirconium - Calcein blue system and have described the probable mechanism of the reaction resulting from the formation of a ternary complex between the three reactants. The sulphate reaction is much weaker than the fluoride reaction and it was found necessary to use a considerable (100 to 1000 mole ratio) amount of sulphate ion to obtain a linear calibration graph. Nevertheless, although this requirement completely vitiated our efforts to establish the existence of a similar ternary complex involving sulphate ion, zirconium and Calcein blue, we were easily able to establish linear relationships between the increase of fluorescence and the amount of added sulphate.

Fig. 1 shows the excitation and emission spectra of a 10^{-6} M solution of Calcein blue (curves A and A′, respectively), an exactly formulated 1:1 zirconium - Calcein

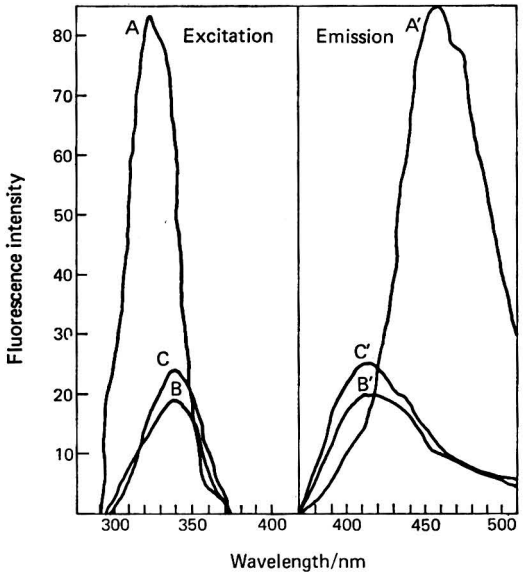

Fig. 1. Excitation and emission spectra
Curves A and A′: 10^{-6} M Calcein blue
Curves B and B′: 10^{-6} M 1 : 1 Calcein blue - zirconium
Curves C and C′: 10^{-6} M 1 : 1 Calcein blue - zirconium + 200-fold excess of sulphate

Excitation spectrum	Emission spectrum
A: emission at 450 nm	A′: excitation at 325 nm
B: emission at 410 nm	B′: excitation at 350 nm
C: emission at 410 nm	C′: excitation at 350 nm

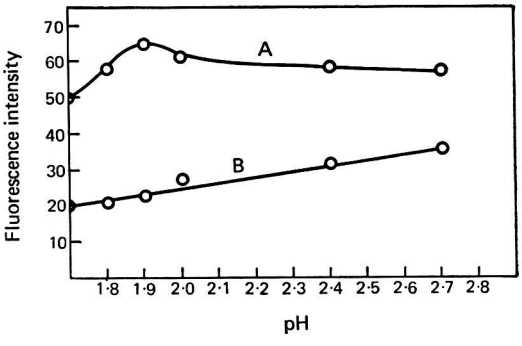

Fig. 2. Effect of pH on sensitivity: curve A, zirconium - Calcein blue - sulphate [2 ml of 10^{-4} M Calcein blue + 2 ml of 10^{-4} M zirconium(IV) in 3 M hydrochloric acid + 4 ml of 10^{-2} M potassium sulphate in 100 ml); and curve B, zirconium - Calcein blue (as A but without potassium sulphate)

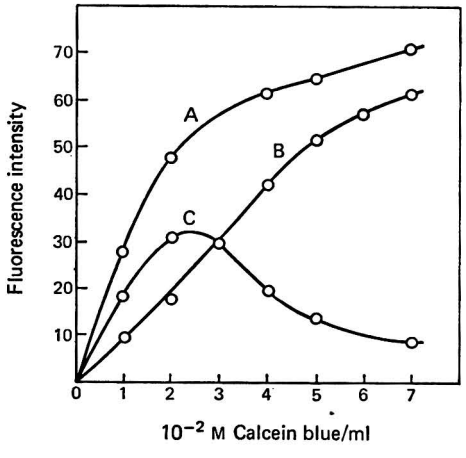

Fig. 3. Effect of concentration of Calcein blue: curve A, zirconium - Calcium blue - sulphate [2 ml of 10^{-4} M zirconium(IV) in 3 M hydrochloric acid + 4 ml of 10^{-2} M potassium sulphate]; curve B, zirconium - Calcein blue (as A but without potassium sulphate); and curve C, effect caused by sulphate ion (B—A)

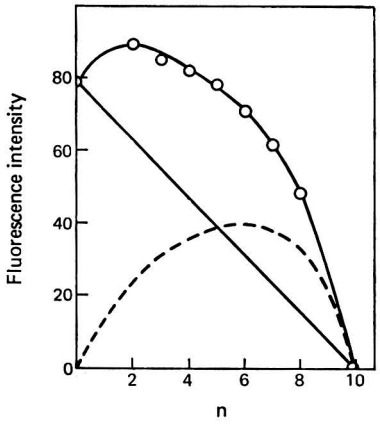

Fig. 4. Attempted continuous variation graph: broken line, net effect, *i.e.*, corrected curve; n = millilitres of 10^{-2} M potassium sulphate added to (10 − n) ml of 10^{-2} M zirconium - Calcein blue

blue solution (curves B and B′, respectively) and an exactly formulated 1:1 zirconium - Calcein blue solution with a 200-fold molar excess of sulphate ion (curves C and C′, respectively), all at pH 1·9. These spectra are not corrected for the spectral response characteristics of the gratings of the monochromators or of the photomultiplier, nor do they account for changes in the lamp emission with wavelength.

As in the previously reported reaction with fluoride, sulphate ions enhance the fluorescence of the zirconium - Calcein blue complex, but produce no changes in the wavelengths of excitation or fluorescence maxima.

Fig. 2 shows the effects of varying the pH of the reaction of a solution containing Calcein blue, zirconium and potassium sulphate (curve A). Curve B is for an exactly similar solution but without sulphate ions. The fluorescence of the sulphate-enhanced system reaches a maximum at pH 1·9. The pH adjustments were made by the addition of small amounts of concentrated ammonia solution. The fluorescence intensities of solutions with pH values greater than 3 are not given because their fluorescence decreases rapidly on standing. It may, however, be relevant to observe that the fluorescence intensities of the zirconium - Calcein blue solution and the sulphate-enhanced solution increases with increasing pH above 3 when measurements are made immediately.

The effect of varying the concentration of Calcein blue is shown in Fig. 3. Curve A is for zirconium and potassium sulphate, curve B is for a similar solution without sulphate and curve C represents the difference between the two solutions, *i.e.*, the net effect of the sulphate ion. It is apparent that the optimum ratio of Calcein blue to zirconium is about 1·3. This is the same ratio as that previously found for the reaction with fluoride[6] and can be interpreted as evidence for a true ratio of 1:1 because of the low assay of the Calcein blue, which could not be obtained or purified to any greater extent in these studies.

A continuous variation graph was made (Fig. 4) to investigate the probable nature of a ternary complex formed between sulphate, zirconium and Calcein blue. This was done by varying a 10^{-2} M sulphate solution against an exactly formulated 10^{-2} M solution of 1:1 zirconium - Calcein blue in the usual way. Such a graph requires the use of approximately equimolar solutions of zirconium - Calcein blue and sulphate but, as explained previously, the analytical procedure requires the presence of about a 100-fold molar excess of sulphate ion to obtain reasonable sensitivity. Consequently it was not possible to obtain a sharp maximum. The broad maximum obtained suggests the probable existence of a 1:1:1 ternary complex, but cannot be interpreted with any confidence as definite evidence.

TABLE I

INTERFERENCE EFFECTS

Molar excess in brackets

Ion added		Percentage change	Ion added		Percentage change
Chloride	(100)	0	Sulphite	(100)	+100
Nitrate	(100)	0		(10)	+52
Phosphate	(10)	−58		(1)	+13
	(1)	−46	Fluoride	(0·1)	+100
Borate	(100)	0		(0·01)	+14
Molybdate	(100)	Quenched	Aluminium	(100)	0
	(10)	−19	Beryllium	(100)	0
	(1)	0	Arsenic(V)	(100)	−42
Tungstate	(100)	Quenched		(10)	−7
	(10)	−75		(1)	0
	(1)	−56	Nickel	(100)	0
Vanadate	(100)	−16	Copper(II)	(100)	0
	(10)	0	Lead	(100)	0
Acetate	(100)	0	Calcium	(100)	0
Oxalate	(100)	−77	Cobalt	(100)	−76
	(10)	−63		(10)	−49
	(1)	−46		(1)	0
Tartrate	(100)	−45	Zinc	(100)	0
	(10)	−37	Cadmium	(100)	0
	(1)	−8	Magnesium	(100)	0
Sulphide	(100)	+67	Iron(III)	(100)	−72
	(10)	0		(1)	−50
Thiosulphate	(100)	+33			
	(1)	0			

The effects of foreign ions on the determination of sulphate ion by this procedure are shown in Table I. Each solution contained 4 mg of sulphate ion in a final volume of 100 ml. The solutions were prepared by the recommended procedure and the foreign ions were added in 100-fold, 10-fold or equivalent amounts to the sulphate solution before the addition of the other reagents. It will be seen that the only serious cationic interference observed in the range of metals studied is that caused by iron(III). None of the common anions interferes, except those which form more stable complexes than sulphate with the zirconium ion, *e.g.*, oxalate and tartrate, or which tend to form insoluble compounds, *e.g.*, tungstate and phosphate. These anions cause low results whereas fluoride, exceptionally, gave rise to a positive result. Large amounts of fluoride, however, break down the zirconium - Calcein blue complex by formation of ZrF_6^{2-}, etc. The positive interference caused by sulphite, and to a lesser extent thiosulphate, can almost certainly be attributed to oxidation to sulphate in these dilute solutions and are not regarded as reaction of these ions *per se*.

Experimental

Reagents—

Potassium sulphate solution, 10^{-2} M.

Zirconium oxychloride solution, 10^{-4} M—Prepare by dissolving 0·032 2 g of $ZrOCl_2.8H_2O$ in 100 ml of 3 M hydrochloric acid. Prepare a 10^{-5} M solution by 10-fold dilution of the 10^{-4} M solution with 3 M hydrochloric acid.

Calcein blue solution, 10^{-4} M—Dissolve 0·016 g of Calcein blue in a few drops of 0·1 M potassium hydroxide and dilute to 500 ml with distilled water. The solution must be discarded after 2 to 3 days.

Ammonia solution, 8 *per cent.*

Apparatus—

Fluorescence measurements were made with a double monochromating spectrofluorimeter (Farrand Optical Co. Catalogue No. 104244) fitted with a 150-W Xenon arc lamp (Hanovia Division Catalogue No. 901 C-1) and an RCA IP28 photomultiplier. A Honeywell Brown recorder was used in conjunction with the spectrofluorimeter. Fused quartz cells (10 × 20 × 50 mm) were used throughout and a pH meter was used to adjust the pH.

Procedure—

Calibration graph (2 *to* 12 *mg or* 20 *to* 12 000 *p.p.m.*)—Transfer 2 to 12 ml of 10^{-2} M potassium sulphate solution at suitable volume intervals into a series of 100-ml calibrated flasks and add, in the following order, 2 ml of 10^{-4} M Calcein blue, 3 to 4 ml of 8 per cent. ammonia solution and 2 ml of 10^{-4} M zirconium oxychloride in 3 M hydrochloric acid. Make up to the mark. Measure the fluorescence of the solutions at 410 nm with an excitation wavelength of 350 nm. Deduct the fluorescence of the blank solution containing all of the reagents except the sulphate solution. These measurements can be made immediately or within 4 hours of preparation.

As the volume of reagents added is less than 10 ml, unknown test solutions containing down to 20 p.p.m. of sulphate can be measured if 90 ml of sample are available, or a solution as concentrated as 12 000 p.p.m., if the test aliquot is restricted to 1 ml.

Calibration graph (200 *to* 1 000 *μg or* 2 *to* 1 000 *p.p.m.*)—Repeat the above procedure by using 2 to 10 ml of 10^{-3} M potassium sulphate solution, 2 ml of 10^{-5} M Calcein blue, 3 to 4 ml of 8 per cent. ammonia solution and 2 ml of 10^{-5} M zirconium oxychloride in 3 M hydrochloric acid.

In this instance solutions as dilute as 2 p.p.m. can be analysed if 90 ml of test solution are available.

Conclusions

A fluorimetric method has been established for the determination of sulphate ion in the range 200 μg to 12 mg, or concentration range 2 to 12 000 p.p.m., assuming a sample solution availability of 90 and 1 ml, respectively. The method is rapid and simple and has a reproducibility equal to or better than 5 per cent. Phosphate, oxalate and tartrate cause low recoveries and should be absent. Tungstate also interferes. Fluoride in small amounts produces a much greater sensitisation,[6] gives rise to high results and must be absent. Large

amounts of fluoride destroy the fluorescence of the zirconium - Calcein blue complex completely. Sulphur species, which are easily oxidised in aqueous solution, interfere by sulphite formation, but the procedure shows a high tolerance towards the cations examined, except cobalt(II) and iron(III).

The mechanism whereby the increase in fluorescence is produced is not apparent from these experiments, and although ternary complex formation may possibly be involved, the complex is too weak to allow unequivocal evidence to be adduced. The analytical method is moderately sensitive (lower limit of 2 p.p.m.) and it has the fairly wide range characteristic of many fluorimetric procedures. It furnishes a potentially useful spectroscopic analytical procedure in addition to those very few which are currently available for the determination of sulphate ion.

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References

1. Bertolacini, R. J., and Barney, J. E., *Analyt. Chem.*, 1957, **29**, 281.
2. Guyon, J. C., and Lorah, E. J., *Ibid.*, 1966, **38**, 155.
3. Jones, A. S., and Letham, D. S., *Analyst*, 1956, **81**, 15.
4. Hems, R. V., Kirkbright, G. F., and West, T. S., *Talanta*, 1969, **16**, 789.
5. Hems, R. V., Ph.D. Thesis, Imperial College, 1969.
6. Tan, Lay Har, and West, T. S., *Analyt. Chem.*, in the press.

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Potentiometric Method for the Determination of Aromatic Monothiosemicarbazones

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A method has been developed for the quantitative determination of aromatic monothiosemicarbazones, in which addition of silver nitrate to a solution of the organic compound leads to complex formation. The hydrogen ion liberated on complex formation is determined by potentiometric titration.

Thiosemicarbazones are compounds of great importance because of their pharmacological activity against tuberculosis,[1] viruses,[2] protozoa,[3] smallpox[4] and certain kinds of tumours.[5] During our investigation (unpublished work) of metal complexes of these compounds we observed that no reliable method for quantitative determination of these organic ligands was available. Koshkin's method,[6] which involves precipitation, filtration and back-titration, is time consuming and suffers from the disadvantage that it does not give a sharp end-point colour change; this change is, moreover, affected by the pH of the solution.

In this paper we outline a simple and rapid method for the quantitative determination of monothiosemicarbazones by potentiometric titration of the hydrogen ion liberated on complex formation when silver ion is added to a solution of the organic compounds.

Experimental

Reagents—

All reagents used were of analytical-reagent grade.

Hydrochloric acid, 0·1 N—Prepared from standard volumetric acid.

Sodium hydrogen carbonate solution, 0·1 N—About 8·4 g of sodium hydrogen carbonate were dissolved in 1 litre of distilled water. The solution was standardised with the above acid, the end-point being determined potentiometrically.

Silver nitrate solution, 5 *per cent.*

Apparatus—

A Vibret Laboratory pH meter, Model 46A, was used in conjunction with a glass - calomel combined electrode, the results being plotted manually from the meter readings. Measurements were also carried out on the automatic instrument, Metrohm potentiograph (Series E436), fitted with a 10-ml automatic burette and a glass - calomel electrode.

General procedure—

Weigh accurately about 150 mg of benzaldehyde thiosemicarbazone and dissolve it in 35 ml of dioxan. To this solution add 80 ml of water and 5 ml of silver nitrate solution. Stir the mixture by using a magnetic stirrer and titrate with the standardised sodium hydrogen carbonate solution (Note). If the manual method is adopted, add 0·1-ml portions of the titrant near the end-point. A typical set of titration figures obtained by using the Vibret pH meter with manual plotting is given below.

Titrant added/ml ..	0·0	6·4	6·6	6·7	6·8	6·9	7·0	7·1	7·2	7·3	7·4	7·6	7·8
pH of solution ..	2·01	2·57	2·68	2·78	2·90	3·03	3·18	3·40	4·16	4·46	4·60	4·72	4·80

Determine a blank under the same experimental conditions. The value found was about 0·02 ml of titrant.

The percentage purity is easily calculated from the equation—

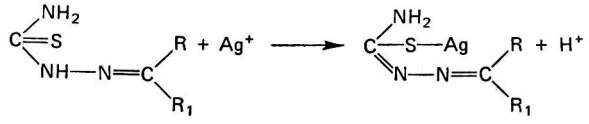
$$\text{Percentage purity} = \frac{V \times N \times E}{W \times 10}$$

where V is the volume of titrant in millilitres, corrected for the blank titre; N is the normality of titrant; E is the equivalent weight of organic compound in grams; and W is the weight taken in grams.

NOTE—We found that the use of other basic reagents such as sodium hydroxide or sodium carbonate causes precipitation of silver salts before the end-point is reached.

RESULTS AND DISCUSSION

Addition of silver nitrate to the thiosemicarbazone results in complex formation, in which an equivalent amount of hydrogen ion is released into the solution.



The nature of the complexes formed is under investigation and the results will be reported at a later date.

The determinations were carried out on ten different samples for each thiosemicarbazone studied and the results obtained were within the narrow range of 100 ± 0·5 per cent. The method was also successful with thiosemicarbazones of the following carbonyl compounds: *p*-isopropylbenzaldehyde (cutizone),[2] salicylaldehyde, isatin and furfuraldehyde. With the thiosemicarbazones of alkyl ketones and acetophenone reduction of silver ion to silver took place, while with benzophenone no complex formation occurred.

A satisfactory end-point could be obtained even for amounts as small as 20 mg; however, the scatter of results increased to ±2 per cent.

Erroneous results can be caused by the decomposition of complex on the electrode if the concentration of the solid in the mixture is too great. With the automatic titrator, concentrations of 150 mg of ligand in 25 ml of dioxan and 25 ml of water can be used, but greater dilution had to be used in the manual method.

The potentiometric method has the advantage over the Koshkin procedure that it is rapid, even with the manual apparatus, and the end-point is easier to determine. However, Koshkin obtained good results even with alkyl thiosemicarbazones, such as acetone thiosemicarbazone, that cannot be determined by the potentiometric method.

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REFERENCES

1. Domagk, G., Behenish, R., Mietzsch, F., and Schmidt, H., *Naturwissenschaften*, 1946, **33**, 315.
2. Orlova, N. N., Aksensova, V. A., Selidovkin, D. A., Bogdanova, N. S., and Pershin, G. N., Farmakologiya i Toksikologiya, 1968, **31**, 725; translated as *Russ. Pharm. Toxic.*, 1968, 348.
3. Butler, K., U.S. Patent No. 3 382 266, 1968.
4. Bauer, D. J., St. Vincent, L., Kempe, C. H., and Dowine, A. W., *Lancet*, 1963, **ii**, 494.
5. Petering, H. G., Buskirk, H. H., and Underwood, G. E., *Cancer Res.*, 1964, **64**, 367.
6. Koshkin, N. V., *Zh. Analit. Khim.*, 1963, **18**, 1492.

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The Determination of Glycerol by the I.U.P.A.C. Form of the Malaprade Method*

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The accepted periodate method for the determination of glycerol has come under review by the International Organisation for Standardisation (Sub-Committee ISO/TC47/GT2) and certain modifications have been suggested with regard to the different pH end-points for the sample and blank and the possible loss of formic acid from the system by volatilisation. The present paper summarises the view of the U.K. delegates to ISO/TC47/GT2, which are:

(*i*) The choice of pH about 8·0 for the sample is justified on the ground that it contains formic acid in addition to strong acids. The blank, which contains only strong acids, should, on general grounds and by calculation, be titrated to pH 7·0. The use of pH 6·5 instead of 7·0 for the blank is an empirical correction designed to compensate for some lack of stoicheiometry or other bias in the procedure and to bring the results into agreement with those obtained by independent moisture and specific gravity determinations. This "correction" amounts to 0·03 per cent.

(*ii*) There is a potential loss of formic acid by volatilisation from the system (estimated variously to be equivalent to 0·01 to 0·04 per cent. of glycerol), which partly explains the apparent lack of stoicheiometry.

(*iii*) Modifications have been proposed to minimise this loss of formic acid and (by adding formate ions to both solutions) to unify the end-points at or about pH 8·0. Unless these modifications lead to a pronounced improvement in reliability, it seems doubtful whether further extensive trials on an international basis would be justified.

THE accepted method of determining glycerol is based on oxidation by sodium periodate to formic acid and measurement of the latter by titration with standard alkali, which is a modification of that outlined by Malaprade.[1] It involves the use of an end-point of pH 6·5 for the blank and of pH 8·1 for the sample. This procedure was originally put foward by the American Oil Chemists' Society and was substantiated by the results of a series of international collaborative trials conducted under its auspices in 1956–57. It was subsequently adopted by the International Union of Pure and Applied Chemistry (I.U.P.A.C.)[2] and also by the British Standards Institution (B.S.I.) and other national bodies, and as such it has formed a satisfactory basis for commercial transactions in glycerine in all parts of the world.

More recently, the method has come under review by the International Organisation for Standardisation (Sub-Committee ISO/TC47/GT2), when it was stressed that the choice of pH values for the dual end-points has not been adequately clarified. The belief was expressed that this discrepancy existed to compensate for some consistent error or bias elsewhere in the test, which should be further examined before adoption. As a result of experimental work by members of the Sub-Committee, the following suggestions have been made: there

* This paper was written at the suggestion of the British Standards Institution Committee CIC/6-Glycerol by two of its members who are also U.K. delegates to the Working Group on glycerol of the International Organisation for Standardisation (ISO/TC47/GT2). It therefore represents the current U.K. attitude on the problem of the determination of glycerol, which is at present engaging the attention of ISO.

is a slight loss of formic acid from the test sample by volatilisation, which could be a source of consistent error[3,4]; and a unified end-point could be achieved by addition of formate ions to both sample and blank.[4]

It is appropriate, therefore, to record the views of the U.K. delegates to the committee, which are summarised as follows.

pH OF END-POINTS CURRENTLY USED—

In the standard I.U.P.A.C. procedure, excess of periodic acid (a weak acid) is reduced before titration to iodic acid (a strong acid) so that its buffering action is eliminated. The acids being titrated are, therefore, for sample: sulphuric, iodic and formic acids, and for blank: sulphuric and iodic acids. Nominally, therefore, the end-points used for the titrations (pH 8·1 and 6·5) should accord with the equivalence points of formic acid and of the sulphuric acid - iodic acid mixture, respectively.

It is conceded that it is unusual to subject a blank to a different procedure from that used for the sample and for this reason, although we retain the term here, it might be better referred to as a control.

In the 1956 deliberations of the B.S.I. Sub-Committee on Glycerine it was suggested that the result of carrying the pH of the sample to 8·1 was to over-titrate the strong acids contained therein, and that the blank should therefore also be titrated to pH 8·1 so that the errors in each instance should cancel out in the subsequent calculation. This suggestion was rejected on theoretical grounds and was not supported by practical tests in which solutions of formic acid and of a mixture of formic, sulphuric and iodic acids in concentrations equivalent to those of an actual glycerol determination were titrated potentiometrically under nitrogen with 0·1 N carbonate-free sodium hydroxide solution from pH 6·5 to 8·1. There was no significant difference in the amounts of alkali required by the two solutions unless there was contamination by atmospheric carbon dioxide (Lazarus, W., private communication), thereby indicating that in the mixture the alkali required to cover this pH range is determined effectively by the formic acid alone. No additional alkali is required by the presence of sulphuric and iodic acids, which are therefore not over-titrated. It is appropriate to consider what end-points for sample and blank might be predicted by strictly theoretical means. These are recorded in Appendix I, which gives estimates of the pH at the stoicheiometric end-points for solutions containing, for sample: sodium formate, iodate and sulphate, and for blank: sodium iodate and sulphate.

The calculated pH values are 7·96 for the sample and 7·05 for the blank, respectively. This confirms the need for two different pH end-points for sample and blank and reasonably supports the choice of pH 8·1 for the sample titration, but not pH 6·5 instead of about 7 for the blank. Examination of the original correspondence in the period 1952 to 1955 between the United Kingdom Glycerine Producers' Association and the American Oil Chemists' Society reveals that the A.O.C.S. Glycerine Analysis Committee tried various pH values between 6·5 and 7·5 for the blank on a largely empirical basis; of these, pH 6·5 was selected as giving the best results, *i.e.*, those most in conformity with the independent assay of the sample by specific gravity and moisture determinations. This latter method of assay was based on the belief that the sample of glycerol for assay was pure and conformed with that used for establishing the standard Bosart and Snoddy tables of specific gravity for glycerol - water solutions.[5] The pH 7·0, which was the inflexion point actually found for the titration curve,* was discarded, and as late as 1957 pH 6·5 was being erroneously quoted as the equivalence point.

It is clear, therefore, that the pH of about 7·0 as the equivalence point for the blank is borne out by both theory and practice and that the selection of pH 6·5 in its place represents an empirical correction designed to allow for some slight imperfection in the over-all analysis, whether of stoicheiometry or otherwise.

The effects of such pH differences as have been quoted are indicated by the pH - titration curves presented in Appendix B of the Minutes of the October 1965 Meeting of ISO/TC47/GT2, from which the following figures are taken.

* It is recognised that inflexion point and equivalence point are not identical, but with strong electrolytes, as in the blank, the difference is negligible.[6]

Blank—A pH of 6·5 to 7·0 requires 0·012 ml of 0·125 N alkali solution equivalent to 0·03 per cent. of glycerol (at 0·41 g sample weight), and a pH of 6·5 to 8·1 requires 0·036 ml of alkali solution equivalent to 0·10 per cent. of glycerol.

Sample—A pH of 6·5 to 7·0 requires 0·08 ml of alkali solution equivalent to 0·22 per cent. of glycerol, and a pH of 6·5 to 8·1 requires 0·16 ml of alkali solution equivalent to 0·45 per cent. of glycerol.

Thus, titration of the blank to pH 6·5 instead of 7·0 has the effect of raising the glycerol result by about 0·03 per cent. However, titration of both sample and blank to pH 7·0 introduces an error of about −0·26 per cent. of glycerol,* while titration of both to pH 8·1 introduces an error of about −0·10 per cent. of glycerol. In this consideration it is important that no significant amounts of other buffers should be present, *e.g.*, carbon dioxide from the atmosphere or impurities in the sample.

USE OF MODIFIED END-POINTS—

As the presence of formic acid in the sample titration has the effect of raising the stoicheiometric or "equivalence" end-point to about pH 8, it was suggested that formic acid (or sodium formate) should be added to both blank and sample, which should be then titrated to pH 7·9 ± 0·2.[4] This addition would eliminate the need for different end-points and yield titration curves of similar character. Tests carried out in three laboratories, in which the results obtained in this way are compared with those by the normal I.U.P.A.C. method, are recorded in Appendix II. They confirm that, within experimental error, the modification yields the same results as the normal blank to pH 6·5. This seems to remove the apparent undesirability of using different end-points for sample and "blank." However, errors can arise in the procedure of adding the formate required to unify the end-points and more extensive tests would be necessary to assess the effect on reproducibility.

VOLATILITY OF FORMIC ACID—

Mormont and Gillet[3] have pointed out that because of the exothermic reaction between glycerol and periodate there is a slight rise in the temperature of the solution and consequently some evaporation: this effect is made obvious by the slight condensation that occurs on the under surface of the clock-glass with which the beaker is closed. The condensate from the sample is acidic in contrast to that from the blank. This acidity led to the suggestion that there is a loss of formic acid from the vessel by volatilisation and that this loss is the source of the systematic error that is otherwise accounted for by the use of the empirically chosen value of pH 6·5 for the blank titration. In a model solution of formic and sulphuric acids similar in composition to that in a glycerol determination, a loss of 0·2 per cent. of its strength was demonstrated at ambient temperature within 30 minutes in the open air ("à l'air libre").

This problem was examined in the U.K. in 1953 (Lazarus, W., Walley, G., and Wilkie, A. L., private communication) when an equivalent solution of formic acid was titrated, with and without a stream of nitrogen being bubbled through the solution for periods of up to 20 minutes prior to and during the titration: no significant loss of formic acid was detected. In a series of tests, variations observed in carrying out the glycerol determination at 0, 20 and 45 °C with the procedure then in use were not regarded as significant, and variations for a slight rise above room temperature certainly could not be regarded as significant.

We have re-examined this problem in the light of Mormont and Gillet's observation, taking into account the finer differences now being looked for, with the results given in Appendix III. We agree that there is a rise of 4 to 5 °C in the temperature of the solution and have shown that the presence of formic acid can be specifically demonstrated by a colorimetric test in the condensate on the clock-glass from a single determination.[7] While there is no doubt about the potential volatilisation of formic acid, the extent of the loss from the system in the average analytical procedure is more difficult to assess. Our recent tests indicate losses varying from zero up to 0·04 per cent. of glycerol, although some uncertainty must attach to those tests in which the solutions were left open to the atmosphere to exaggerate the effect; the subsequent loss would be compensated for to some extent by absorption

* This is close to the figure of "about 0·3%" given in B.S. 2621–5 : 1964 as the correction that results from using the pH end-points 6·5 and 8·1. In our view the term "correction" should more properly be applied to the 0·03 per cent. difference that results from using pH 6·5 instead of 7·0 in the blank.

of atmospheric carbon dioxide. This tendency towards compensation must, of course, operate whatever the imperfection in the closure of the beaker may be.

The potential volatilisation of formic acid has been re-examined also by Mormont, Gillet and Heinerth.[4] The condensates from several tests were collected and tested by infrared spectroscopy, which again demonstrated the presence of formic acid. The condensates on the clock-glasses from thirty-eight tests were also collected and combined for titration; the result obtained was equivalent to a mean recovery per test of 0·01 per cent. of glycerol on the clock-glass. The actual *loss* by leakage from the system was thought to be in excess of this value, by a factor approaching 10, and the difference between the loss of formic acid and the gain in carbon dioxide, for a model mixture of formic and sulphuric acids, has been put at the mean figure of 0·2 per cent. per hour (Mormont, R., private communication). However, some doubt exists on whether this refers to open air conditions ("à l'air libre") or conditions with a closed beaker ("en becher couvert") since both these forms are used.

The loss of formic acid from the system, found in terms of glycerol by two sets of investigators therefore varies from zero to upwards of 0·04 per cent. with one set, and approaches 0·2 per cent. with the other. The mean effect in our own tests is of the same order as the 0·03 per cent. of glycerol, which is equivalent to the gain accounted for by titrating the blank to pH 6·5 instead of 7·0. However, the following considerations make it inadvisable to equate the two effects at present: (*a*) it cannot be said whether it is normal practice to wash down the clock-glass before titration; (*b*) the formic acid in the condensate is not a measure of that lost from the system by volatilisation; and (*c*) the effective loss by volatilisation depends on: (*i*) the type of beaker (600 ml, tall, in I.U.P.A.C. directions; 600 ml, squat, in B.S. 2621–5: 1964), (*ii*) fit of the clock-glass, (*iii*) size of the beaker lip and (*iv*) absolute reaction temperature reached, which will depend partly on the initial ambient temperatures of the laboratory and of the reagents.

It may be thought that there is reason to reconsider the equipment used, because originally the use of a beaker was presumably conditioned by the necessity of inserting separate electrodes. Present-day electrodes can be inserted as a single piece through a small ground-glass joint so that a suitable standard flask could be used with effective stoppering during the reaction period. Alternatively, a beaker without a lip and the top surface ground to take a ground-glass plate could be used. Even with the desire to retain as simple equipment as possible, the failure to close the vessel, in the light of errors ascribed to the passage of volatile components, appears to be a retrograde step.

This still leaves a minor problem as to how much formic acid remains, after cooling for half an hour, in the vapour phase, some of which might disperse on opening the beaker to the atmosphere to conduct the titration. This amount should not be significant, especially if the simple expedient of immersing the reaction vessel in a bath of cold water is adopted.* The alternative approach chosen by Mormont *et al.*[4] is to damp down the exothermic effect, not by external cooling, but by adding the dilution water before the periodate reagent instead of at the end of the reaction. This is claimed to halve the error caused by the combined loss of formic acid and gain of carbon dioxide. However, it appears to be generally held by analysts familiar with the collaborative work in the U.K. in the 1950s that over-dilution affects the determination unfavourably, possibly because of the difficulty of excluding carbon dioxide, which affects the sample and blank to different extents.

Conclusions

The following aspects have been clarified by recent work on the standard procedure for the periodate determination of glycerol:

(*i*) The use of two different pH end-points for the blank and sample is necessitated by the different nature of the acids being titrated, but whereas a pH of 8·1 for the sample is in reasonable conformity with the equivalence point of the formic acid, which is present only in the sample, the pH required for the blank, both as determined and for theoretical reasons, is about 7·0. The use of a pH of 6·5 in its place is not warranted except as a correction to account for an apparent lack of complete stoicheiometry in the reaction. This form of

* Our calculation suggests a total vapour content equivalent to less than 0·02 per cent. of glycerol, under present standard conditions.

correction does not commend itself to all analysts; others accept it on the grounds that it is equivalent to only 0·03 per cent. of the glycerol present and that a correction of this magnitude hardly warrants modification of an established method that has served industry well.

(*ii*) There is a potential loss of formic acid from the system by volatilisation which, in principle, could account for the apparent lack of stoicheiometry. On the basis of the restricted amount of evidence available it is not yet possible to equate these two factors.

(*iii*) Modifications to the standard procedure have been suggested[4] to overcome these difficulties. They entail (*a*) adding the dilution water before instead of after the reaction, and (*b*) adding equal amounts of sodium formate to blank and sample, both of which are then titrated to the equivalence point of the formic ions, which is considered to be pH 7·9 $\pm$ 0·2. The few tests carried out on item (*b*) give the same results as the standard I.U.P.A.C. procedure, which it is designed to supplant as being based on sounder theoretical principles. Further extensive trials are envisaged to determine which of these procedures is preferable on grounds of accuracy and reproducibility.

(*iv*) It is questionable whether further work to detect a bias of 0·03 per cent. of glycerol in a test with a standard deviation between laboratories that we estimate to be of the order of 0·22 per cent. is justifiable, as a very large number of tests would be required. But if this was thought desirable we suggest that an alternative approach, which should be considered, would be prevention of any formic acid loss by slight modification of the apparatus. This would only entail effectively sealing it and placing it in a bath of cold tap water during the reaction. It would then be appropriate to use the true equivalence points of pH 7·0 and about 8·0 for the respective titrations. This would have the advantage of placing the method on a sound theoretical basis without altering the nature of the solutions to be titrated and possibly introducing errors in the addition of sodium formate.

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References

1. Malaprade, L., *Bull. Soc. Chim. Fr.*, 1928, **43**, 683.
2. Standard Methods of the Oils and Fats Section of the I.U.P.A.C., Fifth Edition. Butterworths, London, 1966.
3. Mormont, R., and Gillet, A. C., jun., XXXVIe Congrès Int. Chim. Ind., Bruxelles, September 1966, Gr. X. S26, 691.
4. Mormont, R., Gillet, A. C., jun., and Heinerth, E., *Talanta*, 1969, **16**, 701.
5. Bosart, L. W., and Snoddy, A. O., *Ind. Engng Chem.*, 1927, **19**, 506.
6. Marinenko, G., and Champion, C. E., *Analyt. Chem.*, 1969, **41**, 1208.
7. Feigl, F., "Spot Tests in Organic Analysis," Fifth Edition, Elsevier Publishing Company, Amsterdam, London, New York and Princeton, 1956, p. 340.

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Appendix I

Calculation of end-points for sample and blank

Solution volume = 300 ml
Periodate added = 3·0 g
Molar concentration of iodate formed therefrom

$$= \frac{3{\cdot}0 \times 1\,000}{214 \times 300} = 0{\cdot}047 \text{ mole l}^{-1}$$

Glycerol taken = 0·41 g (at 100 per cent. glycerol)
Molar concentration of formic acid (and formate) produced

$$= \frac{0{\cdot}41 \times 1\,000}{92 \times 300} = 0{\cdot}015 \text{ mole l}^{-1}$$

Dissociation constants at 25 °C, taken from the "Handbook of Chemistry and Physics," Forty-fifth Edition, 1964 (Chemical Rubber Co.), are formic acid $1{\cdot}77 \times 10^{-4} = K_a$; iodic acid $1{\cdot}69 \times 10^{-1} = K_b$; and water $10^{-14} = K_w$.

With sodium formate and iodate present the equilibrium may be represented as—

$$\begin{array}{c} HB \rightleftharpoons B^- + H^+ + A^- \rightleftharpoons HA \\ + \\ OH^- \\ \Updownarrow \\ H_2O \end{array}$$

where A^- and B^- are formate and iodate ions, respectively. Where C_a and C_b are total molar concentrations of (formate ions + formic acid) and (iodate ions + iodic acid), respectively, this equilibrium gives—

$$\begin{array}{l} [H^+] \times [OH^-] = K_w \\ [H^+] \times [A^-] \ = K_a\,[HA] \\ [H^+] \times [B^-] \ = K_b\,[HB] \\ [A^-] + [HA] \ = C_a = 0{\cdot}015 \\ [B^-] + [HB] \ = C_b = 0{\cdot}047 \end{array}$$

$$[H^+] + [HA] + [HB] + [H_2O] = [OH^-] + [H_2O].$$

(In these dilute solutions the activity coefficients are taken as close to unity.) This last relationship is based on the fact that when only pure sodium formate and iodate are present, without added acid or alkali, the "free" *plus* "combined" H^+ ionic concentration must equal the "free" *plus* "combined" OH^- ionic concentration, which leads to the following relationship—

$$[H^+]^4 + [H^+]^3\,(K_a + K_b + C_a + C_b) + [H^+]^2\,(K_aK_b + K_aC_b + K_bC_a - K_w) - [H^+]\,(K_aK_w + K_bK_w) - K_aK_bK_w = 0 \quad .. \quad .. \quad .. \quad .. \quad (1)$$

or, applying the above numerical values

$$[H^+]^4 + 0{\cdot}23\,[H^+]^3 + 2{\cdot}6 \times 10^{-3}\,[H^+]^2 - 1{\cdot}7 \times 10^{-15}\,[H^+] - 3{\cdot}0 \times 10^{-19} = 0 \quad .. \quad (2)$$

A Sturm analysis indicates only one positive root; and this may be approximated, iteratively, by initially ignoring the 3rd and 4th degree terms, solving the remaining 2nd degree relation for the positive root, inserting this in the 3rd and 4th degree terms, absorbing their values in the constant term, re-solving, re-inserting and proceeding to convergence.

Thus, the 2nd degree solution is

$$[H^+] = \frac{1}{2\,(K_aK_b + K_bC_a + K_aC_b - K_w)}\left[K_w\,(K_a + K_b) + \sqrt{\begin{array}{l} K_w^2\,(K_a + K_b)^2 \\ + 4\,K_aK_bK_w\,(K_aK_b + K_bC_a \\ + K_aC_b - K_w) \end{array}}\right]$$

In this instance K_b is of the order of $1\,000 \times K_a$; and hence in the above bracketed *sums* the only significant terms are those in K_b and K_bC_a. The above thus reduces to

$$[H^+] = \frac{1}{2\,C_a}\left[K_w + \sqrt{K_w^2 + 4\,K_wK_aC_a}\right]$$

$= 1{\cdot}1 \times 10^{-8}$, applying the above numerical values.

This gives $pH = -\log [1{\cdot}1 \times 10^{-8}] = 7{\cdot}96$.

Inserting the above value in the 3rd and 4th degree terms yields a value of the order 10^{-25}, while the constant term is of the order 10^{-19}. Thus the 3rd and 4th degree terms have negligible effect on the estimate, which remains at pH 7·96; and this indicates that a suitable pH end-point for the sample titration would be expected to be about 8.

In the absence of iodate ions, $C_b = 0$, and $[H^+] + K_b$ becomes a factor in equation (1), thereby cancelling and reducing this equation to—

$$[H^+]^3 + [H^+]^2\,(K_a + C_a) - [H^+]\,K_w - K_aK_w = 0$$

As before, ignoring the 3rd degree term, this yields—

$$[H^+] = \frac{1}{2\,(K_a + C_a)}\left[K_w + \sqrt{K_w^2 + 4\,K_aK_w\,(K_a + C_a)}\right]$$

$= 1{\cdot}1 \times 10^{-8}$, applying the above numerical values.

As before, the 3rd degree term remains negligible. This gives pH = 7·96, which is effectively the same as that when the iodate is present with the formate, as is to be expected from the form of the two expressions, where K_a is approximately 100 times smaller than C_a. Thus the iodate has practically no effect on the pH produced by the formate and, for the same reason, the effect of the sulphate can be ignored in the sample and blank titrations.

In the blank titration formate is absent and, as above, the relationship becomes with $C_a = 0$,

$$[H^+] = \frac{1}{2\,(K_b + C_b)}\left[K_w + \sqrt{K_w^2 + 4\,K_b K_w\,(K_b + C_b)}\right]$$

$$= 0{\cdot}89 \times 10^{-7}, \text{ applying the above numerical values.}$$

This gives pH = $-\log\,(0{\cdot}89 \times 10^{-7})$ = 7·05.

Thus a suitable pH end-point would be expected to be about 7.

Appendix II

COMPARISON OF I.U.P.A.C. PROCEDURE WITH THE "UNIFIED END-POINT" PROCEDURE

The data are taken from document ISO/TC47/GT2-No. 87; a record of tests performed in Brussels, Newcastle and London is given as follows.

The method of test consisted in performing the normal I.U.P.A.C. glycerol determination to the usual end-points, *viz.*, sample pH 8·1 and blank pH 6·5, at which stage equal amounts of formic acid solution were added to both sample and blank, and the titrations were continued, both to pH 8·1. For this second stage the sample and blank titrations are taken as the sums of the titrations before and after formic acid addition, respectively.

	Titration/ml			
	(I) I.U.P.A.C.		(II) With added formic acid	
*Brussels**—				
Sample: to pH 8·1	29·90	29·91	45·99	46·00
Blank: to pH 6·5	4·89	4·89	—	—
to pH 8·1	—	—	20·97	20·98
(Sample *less* blank)	25·01	25·02	25·02	25·02
(Sample *less* blank) (Mean)	25·015		25·02	
(Sample *less* blank) (Mean) (I − II)	−0·005			

For the amounts of sample taken this is equivalent to −0·018 per cent. of glycerol.

* Reported by Mr. A. C. Gillet, jun., of Solvay et Cie, S.A., to whom our thanks are given.

	Titration/ml	
	(I) I.U.P.A.C.	(II) With added formic acid
London— (with distilled glycerine)		
Sample: to pH 8·1	42·65	62·37
Blank: to pH 6·5	4·84	—
to pH 8·1	—	24·56
(Sample *less* blank)	37·81	37·81
(Sample *less* blank) (I − II)	0·00	

	Titration/ml							
	(I) I.U.P.A.C.				(II) With added formic acid			
Newcastle— (three crude glycerines were used)								
Sample: to pH 8·1	36·64	38·06	37·54		62·01	63·45	62·96	
Blank: to pH 6·5	4·78	4·78	4·76	4·78	—	—	—	—
to pH 8·1	—	—	—	—	30·14	30·20	30·18	30·20
Mean blank	4·775				30·18			
(Sample *less* mean blank)	31·865	33·285	32·765		31·83	33·27	32·78	
(Sample *less* mean blank) (I − II)	+0·035		+0·015		−0·015			
(Sample *less* mean blank) (I − II) (Mean)	+0·012							

For the amounts of sample taken this is equivalent to +0·028 per cent of glycerol.

SUMMARY—

	Sample *less* blank (I — II) (Mean)/ml
Brussels	−0·005
London	0·00
Newcastle	+0·012
Mean	+0·002

This indicates that, within experimental error of measurement, titration of the blank to pH 6·5 gives effectively the same result as addition of formic acid and titration of the blank to pH 8·1. This does not, however, measure the resulting reproducibility.

Appendix III

VOLATILISATION OF FORMIC ACID DURING THE DETERMINATION OF GLYCEROL

(*i*) The condensate on the watch-glass from a single determination was treated with magnesium powder and dilute hydrochloric acid and then with sulphuric acid and chromotropic acid according to Feigl.[7] Production of a violet - pink colour indicated the presence of formic acid in the original sample.*

The following data are taken from Document ISO/TC 47/GT2-No. 66:

(*ii*) The I.U.P.A.C. glycerol determination was carried out and the traces of condensate on the clock-glasses were collected separately and titrated. Two such tests showed an average acidity equivalent to 0·02 ml of 0·125 N sodium hydroxide, *i.e.*, equivalent to about 0·04 per cent. of glycerol.

(*iii*) The loss of formic acid to atmosphere from the test-reaction liquor was measured over 72 hours, and two such tests showed an average loss per hour (*i.e.*, approximate duration of normal test) equivalent to 0·025 per cent. of glycerol. The loss during an actual determination would probably be slightly higher, because during the first 0·5 to 1 hour the liquor is slightly warmer than it is later.

(*iv*) Three tests similar to (*ii*) above, but with the condensate washings combined, showed a total equivalent to 0·02 ml of 0·125 N sodium hydroxide. This is equivalent to about 0·015 per cent. of glycerol per test.

(*v*) A stream of nitrogen was passed through an aqueous solution of formic acid, of concentration similar to that in a glycerol determination, and the acid was subsequently titrated. No significant loss of formic acid was found.

The above tests indicate that during the glycerol determination there could be an actual or potential loss of formic acid equivalent to 0·015 to 0·04 per cent. of glycerol.

* We are indebted for this test to Mr. S. M. Farrer of Imperial Chemical Industries Ltd., Nobel Division, Stevenston, Ayrshire.

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The Separation and Determination of Pentachlorophenol in Treated Softwoods and Preservative Solutions

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A method is described for the extraction, separation and determination of pentachlorophenol or its sodium salt in softwoods and preservative solutions. Pentachlorophenol is separated from lower chlorophenols and wood extractives by adsorption on to Bio-Rad AG 2-x8 anion-exchange resin, eluted with glacial acetic acid, extracted into chloroform, and determined by spectrophotometric measurement of the blue 4-aminophenazone - pentachlorophenol complex.

The effect of time and temperature on the oxidation - condensation reaction of 4-aminophenazone with pentachlorophenol has been investigated.

The procedure is particularly useful for the study of the distribution of pentachlorophenol-containing preservatives in wood.

Pentachlorophenol and its sodium salt have excellent fungicidal properties and are used extensively in wood preserving solutions. Some preservative solutions contain pentachlorophenol alone, but in others the compound may be present together with γ-benzene hexachloride, dieldrin, tri-n-butyltin oxide, α-monochloronaphthalene, zinc naphthenate, copper naphthenate or disodium octaborate. Often it is necessary to be able to determine pentachlorophenol in preserved wood in the presence of lower chlorophenols, phenols and chlorinated hydrocarbons. The commercial product usually contains between 10 and 15 per cent. of lower chlorophenols, the principal impurity being 2,3,4,6-tetrachlorophenol. The lower chlorophenols interfere in the determination of pentachlorophenol when the existing standard methods[1,2] are being used and chlorinated hydrocarbons will also cause interference in techniques that rely on the determination of the total chlorine content.[2]

Methods based on neutron-activation analysis,[3] X-ray fluorescence spectrometry,[4] combustion methods,[5,6,7,8] colorimetric techniques[9,10,11] and titration procedures[12,13,14] have also been described for the determination of pentachlorophenol. However, none of these methods is entirely satisfactory, as they are either insensitive or not specific for pentachlorophenol, or require expensive instrumentation. Often they are slow, tedious and unsuitable for batch analysis, while some give inaccurate results. Methods based on gas - liquid chromatography[15,16,17] that are very sensitive and specific are available for pentachlorophenol, but gas - liquid chromatographs are not always accessible and wood extractives interfere in the determination of pentachlorophenol by this technique. In the present work it has been shown that pentachlorophenol can be readily determined by ion-exchange separation and colorimetric analysis with a relatively inexpensive spectrophotometer.

Chemical analysis of pentachlorophenol is needed for the study of the loading, permanence and distribution of preservatives; because of the variable nature of wood, research projects often require a large number of determinations to ensure significant results. For these reasons, the developed analytical technique should require minimum operator time, permit batch analysis, and give results that are both accurate and relate specifically to pentachlorophenol. The method[1] usually employed at present for separating the pentachlorophenol from wood involves leaching out the preservative by reflux distillation with sodium hydroxide solution followed by steam distillation of the solution after acidification. This procedure is slow, requires constant attention (which makes it unsuitable when large batches of samples need to be analysed), and does not separate pentachlorophenol from lower chlorophenols or phenols that occur naturally in wood. Recent work in this laboratory has shown that preservation chemicals can be rapidly and quantitatively leached from thin sections of wood up to 0·3 mm in thickness[18,19,20] or from sawdust.[21] It has now been found that

pentachlorophenol or its sodium salt can be rapidly leached from thin sections of wood with acetic acid - methanol and separated from the lower chlorophenols, phenols and wood extractives by anion-exchange chromatography with AG 2-x8 resin in the acetate form, 200 to 400 mesh.

The most sensitive reagent for the colorimetric determination of phenols is 4-aminophenazone. This reagent reacts with pentachlorophenol in the presence of an oxidising agent between pH 7·0 and 7·5 to form a blue 4-aminophenazone - pentachlorophenol oxidation condensate that can be extracted into chloroform. 2,3,5,6-Tetrachlorophenol also reacts at pH 7·0 to 7·5 to form a chloroform-soluble complex, but this complex shows maximum absorbance at wavelength 480 nm and would cause a small but significant interference in the measurement of the optical density of solutions containing the 4-aminophenazone - pentachlorophenol condensate, which shows a maximum absorbance at wavelength 585 nm. 2,3,5,6-Tetrachlorophenol is separated from pentachlorophenol during the anion-exchange stage. Ammonium persulphate was used in the oxidation - condensation reaction, as Bencze[22] observed that this reagent is much more stable than potassium ferricyanide. During work on the colour formation it was observed that the oxidation - condensation reaction depends on time and temperature.

Gas - liquid chromatography was used to check the purity of the pentachlorophenol used for calibration purposes and to examine anion-exchange eluates. Wood extractives in leach solutions examined by gas - liquid chromatography seriously contaminated the column material. The proposed procedure has been successfully applied to the determination of pentachlorophenol in treated samples of Corsican pine, Douglas fir, Japanese larch, Norway spruce, Scots pine, Sitka spruce, Western hemlock and Western red cedar.

Experimental

Colour formation—

To bring about the oxidation - condensation of 4-aminophenazone with pentachlorophenol the pH of the solution must be in the range 7·0 to 7·5. A borax - boric acid buffer solution was used to achieve the pH range for the maximum colour intensity. To obtain the correct mixture, solutions of 0·05 M borax and 0·2 M boric acid were mixed together and added to 50-μg quantities of pentachlorophenol in 1 ml of methanol. Volumes of 0·5 ml of 0·3 per cent. w/v 4-aminophenazone and 0·5 ml of 8 per cent. w/v ammonium persulphate solution were added. After 3·5 minutes the blue complex was extracted into 5 ml of chloroform and its optical density was measured at wavelength 585 nm. The results below showed that the

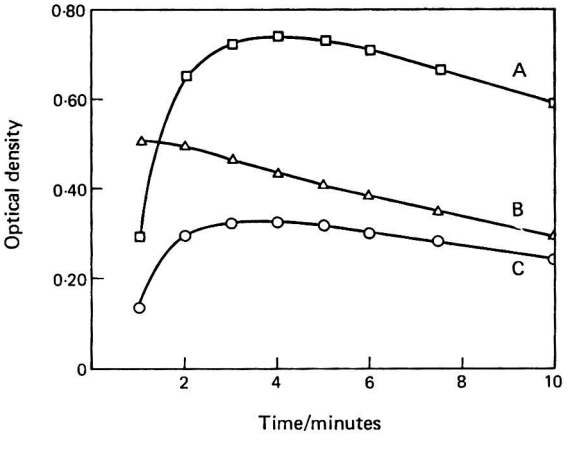

Fig. 1. Change of colour intensity with time at 20 °C: A, solution containing 90 μg of pentachlorophenol *plus* ammonium persulphate; B, solution containing 50μg of pentachlorophenol *plus* potassium ferricyanide; and C, solution containing 40 μg of pentachlorophenol *plus* ammonium persulphate

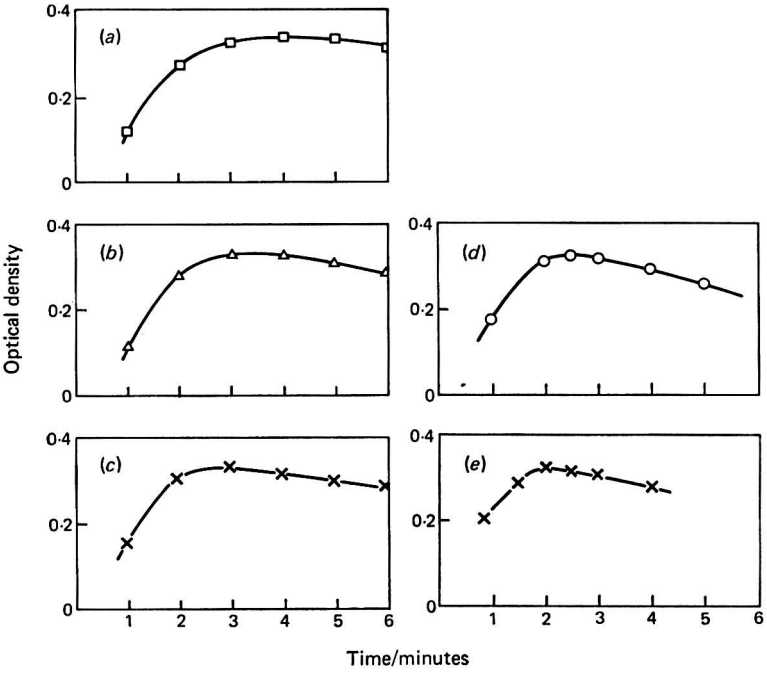

Fig. 2. Change of colour intensity with time at various temperatures: (*a*), 18 °C; (*b*), 20 °C; (*c*), 22 °C; (*d*), 24 °C; and (*e*), 26 °C

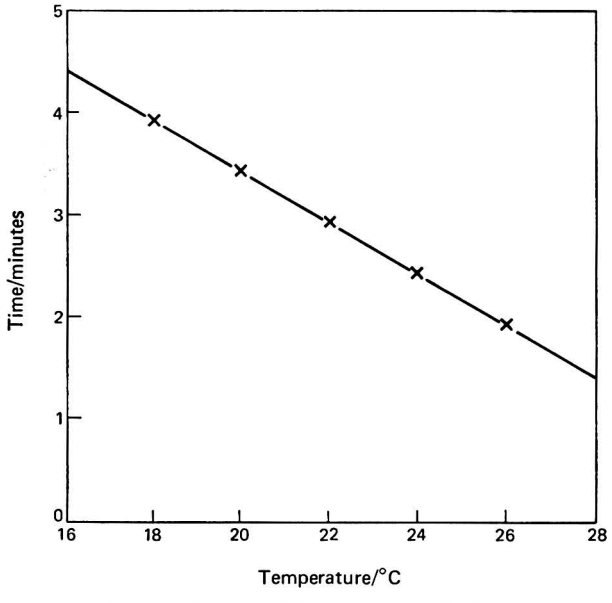

Fig. 3. Time and temperature relationship for development of maximum colour intensity

maximum colour intensity was produced in a mixture of 0·15 ml of 0·05 M borax solution and 9·85 ml of 0·2 M boric acid solution. A buffer solution of this composition was used in all subsequent work.

Volume of 0·05 M borax solution/ml	0·05	0·10	0·15	0·20	0·25
Volume of 0·2 M boric acid solution/ml	9·95	9·90	9·85	9·80	9·75
Optical density	0·243	0·390	0·392	0·366	0·345

During the investigation of buffer solution mixtures it was found that the optical densities of the chloroform extracts were dependent on the length of time between adding the ammonium persulphate solution and extracting the blue complex into chloroform. Two series of solutions of 40 and 90 μg of pentachlorophenol in 1-ml volumes of methanol were diluted with 10 ml of buffer solution and 0·5 ml of 0·3 per cent. w/v 4-aminophenazone solution. Then 0·5 ml of 8 per cent. w/v ammonium persulphate solution was added to each solution and the reactions were allowed to proceed for timed periods (1 to 10 minutes) at 20 °C. Twenty seconds before the end of the period 5 ml of chloroform were added. At the end of the period the solution was shaken for 1·5 minutes, the phases were separated, and the optical density of the chloroform extract was measured at wavelength 585 nm. The optical densities were plotted against the timed reaction period. The curves plotted from the results are shown in Fig. 1, from which it is obvious that the colour-forming reaction depends on time. The experiment was repeated by using solutions containing 50 μg of pentachlorophenol and with 2 per cent. w/v potassium ferricyanide solution instead of ammonium persulphate solution, but maximum colour intensity occurred much earlier (Fig. 1) and the reaction was too quick to allow time for mixing and preparation for the next stage of the procedure. Therefore, ammonium persulphate was retained as the more suitable oxidising reagent.

It was observed that the time for development of maximum colour intensity also varied with different temperatures, so this effect was investigated. Curves similar to that for 40 μg of pentachlorophenol shown in Fig. 1 were plotted from the results of experiments carried out at constant temperatures of 18, 20, 22, 24 and 26 °C in the time range from 1 to 6 minutes. The results (Fig. 2) show that the oxidation - condensation reaction depends on both time and temperature. To obtain a working relationship the time to reach maximum colour intensity was plotted against temperature. A linear relationship was obtained (Fig. 3). This time *versus* temperature relationship was used during subsequent work to select the

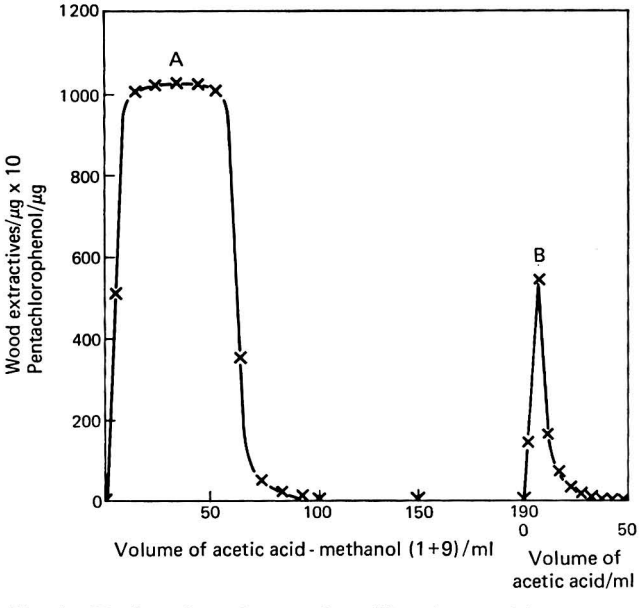

Fig. 4. Elution of wood extractives (A) and pentachlorophenol (B)

period between adding ammonium persulphate and extracting the coloured complex into chloroform for the temperature concerned.

SEPARATION OF PENTACHLOROPHENOL—

Skelly[23] has shown that pentachlorophenol and lower chlorophenols can be adsorbed on to anion-exchange resin and selectively eluted with acetic acid - methanol. This technique was adapted and applied to leach solutions containing commercial pentachlorophenol and wood extractives. It was found that pentachlorophenol or its sodium salt can be rapidly and quantitatively leached from thin sections of wood or sawdust with acetic acid - methanol (1 + 9 v/v). Pentachlorophenol can then be separated from lower chlorophenols and wood extractives by passing the leach solutions through chromatographic columns containing anion-exchange resin Bio-Rad AG 2-x8 in the acetate form, 200 to 400 mesh.

To determine the volume of solution needed to elute wood extractives, 1 g of Scots pine heartwood was leached with acetic acid - methanol (1 + 9) and the leach solution was passed through the resin column. The eluate was collected in fractions and evaporated to dryness, and the residues were weighed. The weights of the residues were plotted against the volumes of the fractions of eluate and the results (Fig. 4, curve A) show that the bulk of the wood extractives does not remain adsorbed on the resin. Traces do remain on the resin, however, and contaminate the eluate containing pentachlorophenol to give a blank value equivalent to 13 μg of pentachlorophenol. Next, the elution of commercial pentachlorophenol was examined. A volume of 25 ml of acetic acid - methanol (1 + 9) containing 1 mg of pentachlorophenol, as the sodium salt, was passed through the resin column. Elution was continued with the same solvent, and the collected fractions were analysed for pentachlorophenol by the colorimetric procedure and by gas - liquid chromatography. The results are given in Fig. 5, curves A and B. Lower chlorophenols were detected in the early fractions (curve A) but not in the later fractions (curve B), which contained only pentachlorophenol.

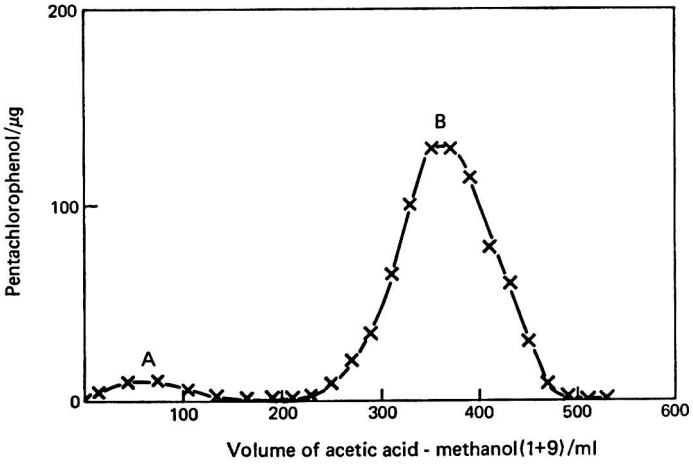

Fig. 5. Elution of lower phenols (A) and pentachlorophenol (B)

It can be seen from Fig. 5 that a large volume of acetic acid - methanol (1 + 9) is needed to remove pentachlorophenol from the resin; hence, glacial acetic acid was used to speed up the elution of the compound. The rapid elution of 1 mg of pentachlorophenol with glacial acetic acid, after elution with 190 ml of acetic acid - methanol (1 + 9), is shown by Fig. 4, curve B. Pentachlorophenol in the acetic acid eluate was concentrated by extraction into 10 ml of chloroform. The eluate was diluted with water and the pentachlorophenol was extracted, serially, with 3 volumes of chloroform. The pentachlorophenol was quantitatively transferred into the chloroform extracts together with small amounts of acetic acid. A portion or all, if necessary, of the chloroform extract was evaporated to dryness in a cylindrical separating funnel, held in the horizontal position, by gently sucking or blowing air over the

solution at room temperature. At temperatures up to 21 °C no pentachlorophenol was lost by volatilisation, but slight losses occurred above 21 °C. These losses were predictable and therefore, instead of maintaining the laboratory temperature at or below 21 °C, calibration graphs were constructed at the various temperatures encountered up to 28 °C. The residue from the evaporation stage was dissolved in methanol, then borax - boric acid buffer solution and 4-aminophenazone solution were added. Ammonium persulphate solution was added and the reaction was timed according to the atmospheric temperature by using the graph given in Fig. 3. A 5-ml volume of chloroform was added so that completion of the delivery coincided with the end of the timed reaction period. The blue complex was extracted into the chloroform and its optical density was measured at wavelength 585 nm.

ELUTION RATE—

Quickfit semimicro chromatographic columns fitted with reservoirs and Buchner flask receivers for use under vacuum were used. The elution rate was adjusted to approximately 1 drop per second by applying gentle suction to the eluate receiver. This allowed the determination to be completed within 4 hours.

When the suction technique is being used to assist elution, care must be taken not to reduce the pressure to such an extent that the resin bed is compacted.

PREPARATION OF STANDARD SAMPLES—

To examine techniques for determining pentachlorophenol in wood it was necessary to prepare standard samples of different species of wood containing a known amount of preservative. This was achieved by impregnating wood with aqueous solutions of sodium pentachlorophenate of known different concentrations and using the full-cell process[24] and freeze-drying[25] to prevent redistribution of preservative.

Weighed flat-sawn blocks of wood of known moisture content, with over-all dimensions of $3 \times 2 \times 1$ cm, cross-section 2×1 cm, radial face 3×1 cm and tangential face 3×2 cm, were subjected to a vacuum of 71 cm of Hg for 3 hours to evacuate the air from the wood cells. While still under vacuum, sodium pentachlorophenate solution was run into the treating vessel until the wood was submerged, and the vacuum was released. A pressure of 10·5 kg cm^{-2} was applied for 1 hour to the solution containing the wood. The pressure was released and the specimens were removed from the solution and weighed after their surfaces had been superficially dried on filter-paper to remove excess of solution. The sample blocks were freeze-dried to a moisture content of about 6 per cent. From the observed weight of treating solution retained in the blocks the percentage of sodium pentachlorophenate, expressed as the pentachlorophenol equivalent and based on oven-dry wood, was found by calculation to range from 0·001 8 to 0·353 per cent.

Owing to the anatomical structure of the wood the distribution of pentachlorophenol in the treated blocks will not be uniform. Concentration gradients of deposited sodium pentachlorophenate can occur across the annual rings; more will be found in the spring or early wood, as the void space is greater, than in the summer or late wood. Therefore, for development work on the procedure, it was decided to use radial sections, cut across the annual rings, because they are more representative of the bulk of the wood. The standard samples were sectioned on a microtome, thin sections being taken at intervals through the block and combined to make one sample. Adjacent thin sections were taken to make up another matched sample for the determination of moisture content of the wood. The weights of the samples taken for analysis were in the range of 0·1 to 1·0 g.

EFFECT OF INSECTICIDES AND OTHER FUNGICIDES—

Commercial formulations of pentachlorophenol wood-preservative solutions may also contain lindane (γ-isomer of 1,2,3,4,5,6-hexachlorocyclohexane), dieldrin, tri-n-butyltin oxide, α-monochloronaphthalene, copper naphthenate or zinc naphthenate. Aqueous solutions of disodium octaborate, used for the diffusion treatment of timber, frequently include sodium pentachlorophenate. The effect of the presence of these compounds on the determination of pentachlorophenol was examined.

Solutions containing 250 μg of pentachlorophenol and 10 mg each of lindane, dieldrin, tri-n-butyltin oxide and disodium octaborate were analysed by the proposed procedure. In each case no interference occurred and complete recovery of pentachlorophenol was attained.

Lindane, dieldrin, tri-n-butyltin oxide and disodium octaborate were recovered in the first 65 ml of acetic acid - methanol eluate.

To investigate the effect of α-monochloronaphthalene, copper naphthenate and zinc naphthenate on the determination of pentachlorophenol by the proposed procedure, commercial solutions containing these chemicals were examined. Samples of the solutions were dissolved in acetic acid - methanol (1 + 9) and put through the procedure. The results given below are in good agreement with those obtained by gas - liquid chromatographic analysis of the preservative solutions. No interference was caused by the organic solvent white spirit, solution A, or the presence of the anti-blooming agent di-n-butylphthalate, solution B. Solution C contained zinc naphthenate and solution D copper naphthenate and α-monochloronaphthalene.

Preservative solution	Pentachlorophenol content, per cent. w/v	
	by proposed procedure	by gas - liquid chromatography
A	3·17	3·11
B	4·67	4·71
C	1·47	1·54
D	3·54	3·45

Results

The procedure described was used to determine the loading of pentachlorophenol in standard samples of Corsican pine, Douglas fir, Japanese larch, Norway spruce, Scots pine, Sitka spruce, Western hemlock and Western red cedar. The results (Table I) were in good agreement with the calculated pentachlorophenol content. The standard deviation, based on six determinations at the 0·02 per cent. of pentachlorophenol level, was ±0·000 5 per cent.

To demonstrate the usefulness of the proposed method, the distribution and depth of penetration of pentachlorophenol in Scots pine sapwood that had been dip-treated for 3 minutes in a commercial wood-preservative solution were investigated. A specimen, surface dimensions 25 × 25 mm and depth 20 mm, was sawn from the bulk of the treated wood. Starting at the surface, 10 thin sections, 0·1 mm in thickness, were cut on a microtome to form one

Table I

Loading of pentachlorophenol in standard samples compared with loading calculated from solution retentions

Species and sample number	Calculated pentachlorophenol content based on oven-dry weight, per cent.	Pentachlorophenol found, per cent.
Corsican pine heartwood 1	0·117	0·113
2	0·122	0·124
Scots pine sapwood 1	0·227	0·227
2	0·215	0·222
Sitka spruce 1	0·346	0·342
2	0·353	0·347
Western hemlock 1	0·268	0·276
2	0·262	0·268
Douglas fir heartwood 1	0·019	0·019
2	0·019	0·019
Japanese larch heartwood 1	0·014	0·014
2	0·015	0·016
Norway spruce 1	0·026	0·026
2	0·033	0·034
Western red cedar heartwood 1	0·033	0·035
2	0·033	0·034
Douglas fir heartwood 1	0·001 8	0·001 8
2	0·001 9	0·001 9
Scots pine sapwood 1	0·002 0	0·002 1
2	0·002 1	0·002 2
Western hemlock 1	0·002 1	0·002 0
2	0·002 3	0·002 3
Western red cedar heartwood 1	0·003 3	0·003 3
2	0·003 2	0·003 2

sample for analysis. The sampling process was repeated down to a depth of 10 mm from the surface of the specimen. The curve obtained by plotting the pentachlorophenol content of each of the ten samples against depth is shown in Fig. 6.

It can be seen from Fig. 6 that it is possible to evaluate rapidly the distribution of pentachlorophenol-containing preservatives over very small areas. If necessary, it is practicable to analyse each 0·1-mm section of wood separately to obtain a more close distribution pattern. This is not feasible by previously available methods of chemical analysis.

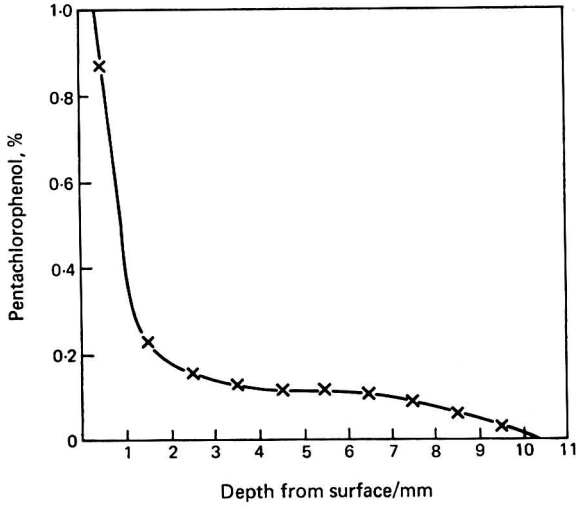

Fig. 6. Distribution of pentachlorophenol in dip-treated Scots pine sapwood. The pentachlorophenol content is based on oven-dry wood

Method

Apparatus—

Chromatographic column—Quickfit semimicro, 10-cm effective length, 1-cm bore, with taps. Reservoirs, 50-ml capacity. Buchner flask receivers, 100-ml capacity.

Separating funnels—50-ml cylindrical, 250-ml pear-shaped.

Spectrophotometer—Unicam SP600.

Reagents—

Use analytical-grade reagents when possible.

Acetic acid - methanol, (1 + 9 *v/v*)—Dilute 100 ml of glacial acetic acid to 1 litre with methanol.

Buffer solution—Dissolve 0·133 8 g of sodium borate, $Na_2B_4O_7.10H_2O$, and 6·113 2 g of boric acid in water and dilute to 500 ml with water.

4-*Aminophenazone solution*, 0·3 *per cent. w/v*—Dissolve 0·15 g of 4-aminophenazone in water, dilute to 50 ml with water and mix.

Ammonium persulphate solution, 8 *per cent. w/v*—Dissolve 4 g of ammonium persulphate in water, dilute to 50 ml with water and mix. Prepare freshly each day and store at 0 °C.

Anion-exchange resin—Bio-Rad AG 2-x8, 200 to 400 mesh, chloride form.

Preparation of chromatographic column—

Soak the anion-exchange resin in water for 24 hours. Slurry sufficient resin into the chromatographic column to form a bed 8-cm deep when the solids settle down. Elute with 10 per cent. w/v sodium acetate solution until chloride cannot be detected in the eluate with silver nitrate solution acidified with nitric acid. Elute, sequentially, with 100 ml of water,

50 ml of glacial acetic acid and 100 ml of methanol. The resin column is now ready for use. At no stage should the column be allowed to run dry; maintain the liquid level at approximately 0·5 cm above the top of the resin bed.

To regenerate the resin after each run elute successively with 50 ml of glacial acetic acid and 50 ml of methanol.

PROCEDURE—

Weigh the sample and transfer to a 100-ml covered beaker. Add 25 ml of acetic acid - methanol and warm in a water-bath for 10 minutes at 55 °C. Remove the beaker from the water-bath, cool to room temperature, and decant the solution into the reservoir of the chromatographic column. Open the column tap and apply gentle suction to the Buchner flask receiver so that the eluate flows at the rate of approximately 1 drop per second. Wash the beaker wall and cover with 20 ml of acetic acid - methanol, warm the sample and washings at 55 °C for 10 minutes, cool to room temperature, and decant the solution into the column reservoir when the first leach solution has eluted. Repeat once more with another 20 ml of acetic acid - methanol. When this solution has run through the resin, elute with 125 ml of acetic acid - methanol. Discard the acetic acid - methanol eluates. Elute with 50 ml of glacial acetic acid. Transfer the acetic acid eluate to a 250-ml pear-shaped separating funnel, dilute to 230 ml with water, and mix. Extract the solution serially with 5, 4 and 3 ml of chloroform, run off the organic phases into a 10-ml calibrated flask, make up to the mark with chloroform, and mix again. Transfer a suitable aliquot of the chloroform solution to a 50-ml cylindrical separating funnel. Carefully clamp the separating funnel in the horizontal position and evaporate the solution to dryness by sucking or blowing air over the solution.

Dissolve the residue in 1 ml of methanol, add 10 ml of buffer solution, mix, add 0·5 ml of 0·3 per cent. w/v 4-aminophenazone solution, mix, add 0·5 ml of 8 per cent. w/v ammonium persulphate solution, and mix. Time the reaction period according to the atmospheric temperature by using the graph shown in Fig. 3. Twenty seconds before the end of the timed period add 5 ml of chloroform. At the end of the timed period shake the solution for 1·5 minutes, allow the phases to separate, and run off the organic layer through a dry 7·0-cm Whatman No. 1 filter-paper into a 10-mm cell. Measure the optical density of the blue complex against chloroform at wavelength 585 nm. To obtain the pentachlorophenol content of the test solution compare the spectrophotometer reading with a calibration graph. Subtract the blank value obtained in a similar way to that of the test solution.

PRESERVATIVE SOLUTIONS—

Transfer 1 ml of preservative solution to a 100-ml calibrated flask, dilute to the mark with acetic acid - methanol, and mix. Dilute 1 ml of solution to 25 ml with acetic acid - methanol, transfer to the reservoir of the chromatographic column, and continue as described in the second paragraph of the Procedure.

CALIBRATION—

Preparation of standard solution A—Dissolve 0·100 0 g of pure pentachlorophenol in chloroform, transfer to a 100-ml calibrated flask, dilute to the mark with chloroform, and mix.

1 ml of standard solution A $\equiv$ 1 000 μg of pentachlorophenol.

Preparation of standard solution B—Transfer, by pipette, with suitable precautions, 10 ml of standard solution A into a 100-ml calibrated flask, dilute to the mark with chloroform, and mix.

1 ml of standard solution B $\equiv$ 100 μg of pentachlorophenol.

Transfer 0·1, 0·2, 0·4, 0·6, 0·8, 1·0, 1·2 and 1·4 ml of standard solution B into separate cylindrical separating funnels. The volumes taken contain 10, 20, 40, 60, 80, 100, 120 and 140 μg of pentachlorophenol, respectively. Evaporate the solutions to dryness as described under Procedure. Dissolve each residue in 1 ml of methanol and continue as described under Procedure, paragraph 2.

Unless the laboratory temperature is controlled, prepare calibration graphs at the atmospheric temperatures normally encountered.

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References

1. British Wood Preserving Association, London, Standard 104, 10.
2. British Wood Preserving Association, London, Standard 104, 12.
3. Gheorge, M., Catrina, I., and Constantinescu, O., *Drevar Vyskum*, **1964**, **3**, 113.
4. Wright, J. P., and Storks, K. H., American Wood-Preservers Association Annual Convention, Chicago, 1957, p. 57.
5. Parr, S. W., *J. Amer. Chem. Soc.*, **1943**, **30**, 764.
6. Bonstein, T. E., Frauenfelder, L. J., and Lohr, L. J., *Analyt. Chem.*, **1953**, **25**, 1115.
7. Agazzi, E. J., Brooks, F. R., and Parks, T. D., *Ibid.*, 1951, **23**, 1011.
8. Safford, H. W., and Stragand, G. L., *Ibid.*, 1951, **23**, 520.
9. Wallin, G. R., *Ibid.*, 1950, **22**, 1208.
10. Haskins, W. T., *Ibid.*, 1951, **23**, 1672.
11. Erkama, J., and Loamanen, A., *Acta Chem. Fenn.*, 1956, **13**, 37.
12. Fritz, J. S., and Keen, R. T., *Analyt. Chem.*, 1953, **25**, 179.
13. McKinney, R. W., and Reynolds, C. A., *Talanta*, 1958, **1**, 46.
14. Bruss, D. B., and Harlow, G. A., *Analyt. Chem.*, 1958, **30**, 1836.
15. Barthel, W. F., Curley, A., Thrasher, C. L., Sedlak, V. A., and Armstrong, R., *J. Ass. Off. Agric. Chem.*, 1969, **52**, 294.
16. Bevenue, A., Emerson, M. L., Casarett, L. J., and Yauger, W. L., *J. Chromat.*, 1968, **38**, 467.
17. Argauer, P. J., *Analyt. Chem.*, 1968, **40**, 122.
18. Williams, A. I., *Analyst*, 1968, **93**, 111.
19. ——, *Ibid.*, 1968, **93**, 611.
20. ——, *Ibid.*, 1969, **94**, 30.
21. ——, *Ibid.*, 1970, **95**, 498.
22. Bencze, K., *Ibid.*, 1963, **88**, 622.
23. Skelly, N. E., *Analyt. Chem.*, 1961, **33**, 271.
24. Cartwright, K. St. G., and Findlay, W. P. K., "Decay of Timber and its Prevention," Second Edition, H.M. Stationery Office, London, 1958, p. 286.
25. Smith, D. N. R., and Cockcroft, R., *Nature*, 1961, **189**, 163.

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The Determination of Ethanol in Paints, Inks and Adhesives by Gas Chromatography

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A gas-chromatographic method for the determination of ethanol in paints, inks, adhesives and similar composite products has been developed by which the time required for the analysis of samples has been greatly reduced. The ethanol is distilled azeotropically from the sample in the presence of toluene, and the distillate is examined directly by gas chromatography without any further treatment other than mixing with an internal standard. The problem of the longer retention time of toluene, compared with those of the lower aliphatic alcohols, has been overcome by the use of back-flushing. From the results obtained by the analysis in duplicate of a series of samples, including a number prepared in the laboratory containing known amounts of ethanol, the accuracy and precision of the method have been established.

For many years, the most widely used method for the determination of ethanol in an extensive variety of products has been that of obtaining, by distillation, an aqueous mixture containing all of the ethanol, measuring its specific gravity, and finding out the ethanol content by reference to the appropriate Spirit Tables.[1] For essences, tinctures and toilet preparations, Methods I, II and III of the British Pharmacopoeia[2] and Method IV of the British Pharmaceutical Codex[3] are generally used.

Paints, inks and adhesives, however, often contain a variety of organic solvents such as alcohols, esters, hydrocarbons and ketones, and for this reason more elaborate and lengthy methods for the determination of ethanol are necessary. By means of an azeotropic distillation with toluene as the entraining agent, the ethanol, together with certain other low boiling-point solvents, can be separated. The distillate thus obtained can then be treated by one of the extraction procedures of the British Pharmacopoeia or of the British Pharmaceutical Codex and an aqueous distillate containing the ethanol ultimately results. In spite of the clean-up procedures used, the distillate may still contain substantial amounts of methanol, propan-2-ol, low boiling-point ketones, etc. The determination of ethanol in these circumstances is particularly time-consuming and relies to a large extent on the identification of the interfering substances and the selection of suitable procedures to effect their removal. If removal is not possible, the ethanol must be determined by a chemical method, the one most frequently used being that of Boorman,[4] in which the ethanol is oxidised to acetic acid.

Gas chromatography, having been successfully applied to the determination of ethanol in tinctures, etc.,[5] appeared to offer an alternative method which could lead to a considerable reduction in the time required for analysis. Its use in the determination of the ethanol content of such composite products as paints has attracted less attention than in the case of toilet goods or tinctures. Jones, Ritchie and Newburger[6] described a method for the analysis of the solvents used in nail lacquers, but were unable to separate ethanol and propan-2-ol; Hoover[7] and Haken[8] have both reviewed the application of gas chromatography in the analysis of coating materials. However, no method for the routine determination of ethanol in these products appears to have been published.

By their nature paints, inks and similar products are not amenable to direct injection into a gas chromatograph, although Jones *et al.* and Hoover described procedures in which this was done. Haken states that the solvents must be separated from the resins and pigments, and this principle was adhered to in devising the procedure described in this paper. Distillation offered the simplest method for the preliminary separation of the ethanol, but it was essential for the distillate to be homogeneous for subsequent examination by gas

chromatography. For this reason the distillation was carried out in the presence of toluene, which is relatively non-toxic and forms an ethanol-rich azeotrope. As the final separation of the ethanol was by gas chromatography, the distillate needed no additional clean-up. Furthermore, it was not necessary to perform a controlled azeotropic distillation; a fairly rapid distillation, with minimal loss of ethanol, was all that was required and this was achieved by using a flask fitted with a still-head and Revenue condenser.

The lower aliphatic alcohols were adequately resolved at 160 °C on a 2-m column filled with Chromosorb 102, mesh size 80 to 100; typical retention times were 4 minutes for ethanol and 8 minutes for propan-1-ol. Toluene, however, was found to have a retention time of 50 to 60 minutes, so that injections of the toluene distillate could be made only at hourly intervals, thereby lessening the advantages of the rapid distillation and gas-chromatographic determination of ethanol. To overcome this problem of long retention, the use of both back-flushing and temperature programming was investigated. The former technique was preferred. By reversing the gas flow through the first column after 2 to 3 minutes (from the time of injection) most of the unwanted components were removed from the system and injections could be made at intervals of approximately 15 minutes. As a result of using a two-column system the retention times of the ethanol and propan-1-ol were slightly increased, to 5·5 and 11·5 minutes, respectively. Temperature programming permitted injections to be made at intervals of approximately 30 minutes and gave highly satisfactory results, but it was not adopted in view of the superiority of back-flushing.

Method

Apparatus—

A Perkin-Elmer F11 gas chromatograph was used, equipped with a flame-ionisation detector. By the use of suitable pressure gauges and valves, the existing single-column arrangement was modified for back-flushing in accordance with the principles of Deans,[9] whose system avoids the use of taps in the sample path. The columns used were both of stainless steel, with internal diameter 2·2 mm and lengths 0·38 and 2·0 m. The layout was such that the sample passed through the shorter column first, and unwanted substances were back-flushed from this column by reversing the gas flow through it. Nitrogen was used as the carrier gas, and the applied gas pressures were chosen so that no change in pressure occurred at the junction of the two columns when the direction of the gas flow through the first column was reversed. A pressure gauge temporarily connected at this point facilitated the selection of the correct gas pressures. The carrier gas flow-rate was approximately 10 ml minute^{-1} and that of the hydrogen 40 ml minute^{-1}, the temperatures of the oven and injection block being 160 °C and approximately 200 °C, respectively. The flame-ionisation amplifier with the output set at 10 mV was connected to a potentiometric recorder and the peak areas were measured as integrator pulses by a Kent Chromalog 3 integrator set at 100-mV input and connected in parallel with the recorder. The amplifier attenuation was normally set at 20×10^2 to give peaks of adequate size.

Procedure—

Standard ethanol solutions—Prepare a series of ethanol - water mixtures covering the range from 0·2 to 20 per cent. v/v of ethanol. Determine the specific gravities of these mixtures and obtain the actual ethanol contents by reference to the Spirit Tables.[1] Prepare the standards for the gas chromatograph by adding 3·0 ml of propan-1-ol to 25·0 ml of each of the ethanol solutions.

Distillation of the sample—Weigh out into a 30-ml specimen tube an amount of sample expected to contain not more than 3 ml of ethanol. Transfer the tube and its contents to a wide-necked distillation flask and add 30 ml of toluene, or 20 ml of toluene and 10 ml of benzyl alcohol if the sample contains nitrocellulose. Fit the flask with a still-head and Revenue condenser and distil the contents of the flask, collecting 20 ml of distillate, preferably in a 25-ml stoppered measuring cylinder into which 5 ml of toluene have first been placed.

(Caution—Nitrocellulose, if present, may be precipitated by the toluene with the resulting possibility of an explosion during the distillation. The benzyl alcohol, by keeping the nitrocellulose in solution, avoids this danger.)

Gas-chromatographic examination—Add 3·0 ml of propan-1-ol to the distillate and mix. If the solution is still cloudy following this addition because of the presence of a small amount of water, add sufficient propan-2-ol (usually 1 or 2 ml) to clear it. Inject a suitable volume into the gas chromatograph, which should conform to the description given under Apparatus. Operate the gas chromatograph, with the gas flow in the forward direction until the ethanol and propan-1-ol have both been eluted from the first column (this takes from 2 to 3 minutes) and then reverse the flow through this column. Calculate from the integrator counts the peak area ratio of ethanol to propan-1-ol for the sample. By using the value obtained, select two standard ethanol solutions whose concentrations are just above and just below that of the sample. Examine these on the gas chromatograph under the same operating conditions except that back-flushing need not be used.

Calculation—From the peak area ratios, calculate the ethanol content of the distillate from the equation given in the National Formulary XII,[10] which is as follows—

$$\text{Ethanol, per cent. v/v, in distillate} = \frac{A\,(Y - Z) + B\,(Z - X)}{Y - X}$$

where A and B are the volume percentages of ethanol in the lower and higher standards, respectively, and X, Y and Z are the peak area ratios of the lower standard, higher standard and sample, respectively. The concentration of ethanol in the original sample is probably most conveniently expressed in terms of the volume of ethanol per unit weight. A suitable form is millilitres of ethanol per 100 g, which, in commercial terms, is identical with litres of ethanol per 100 kg. The concentration in these terms is obtained as follows—

If the volume percentage of ethanol in the distillate is V, then

$$\text{ml of ethanol per 100 g sample} = \frac{V \times 25}{W}$$

where W is the weight of the sample.

Results and discussion

Before examining samples by this method, it was considered necessary to establish that the presence of toluene and other volatile solvents in the distillate would not adversely affect the precision of the determination of ethanol. A mixture containing methanol, ethanol, propan-2-ol, propan-1-ol and toluene was examined on the gas chromatograph five times. The peak area ratios of ethanol to propan-1-ol were calculated and the following results were obtained: 0·746 7, 0·746 6, 0·741 9, 0·741 7 and 0·735 3. The mean of these results is 0·742 4, with a standard deviation of 0·004 6, indicating that adequate precision was obtained.

The reliability of the method was further checked by distilling mixtures containing known amounts of ethanol in toluene, adding the internal standard to the distillate and determining the ethanol content with the gas chromatograph by using aqueous ethanol standards for the quantitation. The results in Table I show that satisfactory recovery of the ethanol was obtained.

Table I

Ethanol contents of toluene distillates

Ethanol, per cent. v/v		
As prepared	By distillation	Percentage recovery
4·90	4·91	100·2
9·86	9·86	100·0
20·19	20·10	99·5
40·17	39·52	98·3

The method was applied to the analysis of a series of typical samples. The actual ethanol contents of some were unknown, but a number of samples prepared in the laboratory, incorporating known amounts of ethanol, were also examined. Each sample was analysed in duplicate and from the results obtained estimates of the precision and accuracy of the method were made. The results of these experiments are shown in Table II; the ethanol contents of the samples prepared in the laboratory are given in the third column of the table.

TABLE II

ETHANOL CONTENTS OF PAINTS AND OTHER COMPOSITE PRODUCTS

Product		Ethanol, ml per 100 g			
			Found		
Number	Description	Actual			Mean
1	Floor coating	*	11·45	11·64	11·55
2	Nail lacquer	*	4·76	4·63	4·70
3	Printing ink	13·07	13·06	12·76	12·91
4	Paint	7·03	6·79	7·10	6·95
5	Acrylate copolymer solution ..	26·15	25·96	24·31	25·14
6	Paint	14·09	13·84	13·94	13·89
7	Varnish	*	13·63	13·52	13·58
8	Paint	9·42	9·16	9·31	9·24
9	Acrylate terpolymer solution ..	*	3·65	3·81	3·73
10	Lacquer	*	29·26	29·13	29·20
11	Printing ink	*	33·26	33·26	33·26
12	Lacquer	*	11·06	10·68	10·87
13	Varnish	13·39	13·05	13·17	13·11
14	Metallised lacquer	*	5·53	5·24	5·39
15	Edible ink	*	31·64	32·55	32·10
16	Varnish	7·92	7·81	7·80	7·81

* Not known.

From the duplicate results in Table II, the standard deviation was calculated to be 0·355 ml of ethanol per 100 g of sample. As a number of samples contained known amounts of ethanol the precision of the method was also estimated by using the relationship—

ml of ethanol per 100 g (found) = a + [b × ml of ethanol per 100 g (actual)]

The values of the terms a and b were found to be 0·34 and 0·952, respectively, and the residual standard deviation was estimated as 0·366 ml of ethanol per 100 g, which is of the same order as that for the variation between the results of replicate determinations.

CONCLUSION

The use of gas chromatography for the determination of ethanol has been successfully extended into the field of paints, inks and similar composite products. Compared with the existing methods, the procedure shows a great saving in time, and the examination of a sample can be completed within one hour.

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REFERENCES

1. Spirit Tables, H.M. Stationery Office, London, 1955.
2. British Pharmacopoeia, The Pharmaceutical Press, London, 1968, p. 1278.
3. British Pharmaceutical Codex, The Pharmaceutical Press, London, 1968, p. 1380.
4. Boorman, E. J., *Analyst*, 1939, **64**, 791.
5. Harris, J. R., *Ibid.*, 1970, **95**, 158.
6. Jones, J. H., Ritchie, C. D., and Newburger, S. H., *J. Ass. Off. Agric. Chem.*, 1958, **41**, 673.
7. Hoover, W. S., *Paint Varn. Prod.*, 1964, **54**, [7], 69; [8], 41; and [9], 61.
8. Haken, J. K., *J. Oil Colour Chem. Ass.*, 1966, **49**, 993.
9. Deans, D. R., *J. Chromat.*, 1965, **18**, 477.
10. National Formulary, 12th Edition, First Supplement, American Pharmaceutical Association, Washington, D.C., 1965, p. 20.

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The Determination of Residues of Dichlorvos and Malathion in Wheat Grain by Gas - Liquid Chromatography

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A procedure is described for the determination of malathion and dichlorvos in grain. After extraction with methanol, and clean-up on a charcoal column, if required, the pesticide residues are determined by gas - liquid chromatography on Apiezon L and butane-1,4-diol succinate columns with a phosphorus-sensitive detector.

Between the concentrations of 0·25 and 10 p.p.m. both pesticides were recovered from spiked samples with between 87 and 99 per cent. efficiency.

Malathion, added at a concentration of about 10 p.p.m., has been one of the chosen insecticides for the treatment of stored grain since about 1960. Its effectiveness at that level against granary pests, combined with its relatively low mammalian toxicity, is probably the main reason for its extensive use in countries, like Australia, where the climate favours the multiplication of insects in stored grain. The United Kingdom imports from Australia grain that has been treated in this fashion and that may have been given further treatment at the dockside. Hill and Thompson[1,2] have shown that, after the long voyage to the United Kingdom, including passage through the tropics, the malathion content has fallen by degradation to between 2 and 5 p.p.m. (mean value about 4 p.p.m.). Grain treated in a similar manner is also imported from the Argentine and the same authors have shown it to contain about 2 p.p.m. of malathion on arrival in the United Kingdom.

Dichlorvos, which also has a relatively low mammalian toxicity, has also been shown to be very effective against granary pests at the level of 10 p.p.m. in the treatment of cereal products. It is therefore necessary when examining grain for pesticide residues to allow for the presence of both malathion and dichlorvos, which may have been added at separate stages of storage or transportation. The Food and Agriculture Organisation and the World Health Organisation of the United Nations jointly have recommended tolerances of 8 p.p.m. of malathion and 2 p.p.m. of dichlorvos in cereals.[3]

Most procedures for the determination of malathion have been based on that of Norris, Vail and Averell,[4] in which malathion is hydrolysed by alkali to sodium *OO*-dimethylphosphorodithioate, which forms a yellow complex with copper(II) ions at about pH 5 and is soluble in carbon tetrachloride. These methods have been applied to fruit and vegetable crops[4,5] and cereals,[6,7] although interference from co-extracted materials has led to difficulties in some instances. Interference with the colorimetric finish from the presence of other organophosphorus insecticide residues has also been reported.[8] A form of the method has been recommended by the Joint Malathion Panel[9] for application to wheat grain, and was used for this purpose by Hill and Thompson.[1,2] Weisenberg, Gertner and Schoenberg[10] have recommended another variant for wheat. Instability of the complex may cause fairly rapid fading of the yellow colour; Hill[11] has studied the stability of this complex and devised a means of reducing dissociation to less than 1 per cent. Jeffs, Lord and Tuppen[12] used gas - liquid chromatography to determine carbophenothion and chlorfenvinphos on single wheat seeds by immersion in acetone - hexane (1 + 1) for several hours. Such a method has been shown in the present investigation to be unsuitable for dichlorvos. Elms,[13] by using gas - liquid chromatography for the determination, extracted malathion from wheat, after grinding it, by Soxhlet extraction with hexane for 4 hours. This extraction procedure also has been shown in the present investigation to be inadequate for dichlorvos. A spectrophotometric method is available for determining dichlorvos in air.[14] More recently, Ivey and Claborn[15] developed methods for extracting dichlorvos from milk, egg, and cattle and chicken

tissues. The extraction solvent used for muscle, blood and egg was acetonitrile; chicken was extracted with hexane and acetonitrile; and milk was extracted with a mixture of dichloromethane and hexane. Dichlorvos was determined by gas - liquid chromatography with a phosphorus-selective flame-spectrophotometric detector. St. Clair, Lamberton, Claeys and Goulding[16] developed methods for determining dichlorvos vapour emitted from PVC Vapona strips and the residue left in the strip. Various columns used for gas - liquid chromatography were compared.

When used in conjunction with the caesium bromide tipped thermionic detector, gas - liquid chromatography provides the means of determining very small residues of dichlorvos and malathion. Other advantages are that no chemical treatment is required before the chromatography, thus ensuring specificity, and the method is relatively unaffected by the presence of co-extractives. The method now proposed has therefore been developed for the simultaneous extraction of residues of both of these compounds from wheat, with their determination by gas - liquid chromatography by using this type of detector.

Malathion can be recovered from grain by most common solvents but dichlorvos is much more difficult to recover. Because of the volatility and high polarity of dichlorvos a more specific method is required.

Method

Apparatus—

High-speed coffee grinder.
High-speed food mixer—For macerating grain with methanol.
Centrifuge—This should be able to take 150 ml of solution and rotate at 2 000 r.p.m.
Evaporator—Kuderna-Danish, 500 ml.
Chromatographic column—This was 30 cm long and of 1·5 cm o.d., fitted with tap and 100-ml reservoir.
Conical flask—150-ml capacity, with B24 neck and side-arm.
Water-pump.
Two 100-ml calibrated flasks.
Pear shaped flask—25-ml capacity, with B14 neck.
Pipette, 0·1 ml.
An accurate balance.

Reagents—

Methanol, analytical-reagent grade.
Cotton-wool.
Celite 545 or 560—Available from Johns Manville Co.
Charcoal, Nuchar C-190.
Acetone, analytical-reagent grade.
Dichlorvos and malathion—Technical grades of known purity.

Preparation of standard solutions—

Weigh 0·1 g of pesticide into a 100-ml calibrated flask and dissolve it in 100 ml of acetone (solution A). With a pipette, introduce 0·1 ml of solution A into a 100-ml calibrated flask and dilute it to 100 ml with acetone (solution B). Use 5 μl of solution B to give a peak corresponding to 5 ng of pesticide on the gas - liquid chromatographic column.

Prepare separate solutions of dichlorvos and malathion. Use a freshly prepared solution B for each determination. Renew solution A for dichlorvos after 1 week and solution A for malathion after 1 month. Keep solutions A in a refrigerator.

The pesticides used in this work were specified by the manufacturers to be of 95·1 per cent. purity for dichlorvos and of 96·3 per cent. purity for malathion.

Gas - liquid chromatographic columns—

These consisted of (*a*) Apiezon L (1·3 per cent.) and Epikote (0·1 per cent.) on 100 to 120-mesh acid-washed silanised Chromosorb G, and (*b*) butane-1,4-diol succinate (1·3 per cent.) and Epikote (0·1 per cent.) on the same solid support as (*a*). The columns were circular, glass, 1·5 m long and 3 mm i.d., fitted to a caesium bromide tipped thermionic detector a

the same temperature as the columns. Gas flow-rates were: carrier gas (nitrogen) 15 to 20 ml minute^{-1}; hydrogen 8 to 10 ml minute^{-1}; and air 150 to 200 ml minute^{-1}. The columns were maintained at 220 °C for malathion and 190 °C for dichlorvos.

PROCEDURE—

Grind 20 g of grain in a high-speed coffee grinder and macerate with three 50-ml portions of methanol. Centrifuge the combined methanolic extracts for a few minutes at 2 000 r.p.m. Evaporate the clear solution to a small volume in a Kuderna-Danish evaporator.

For samples containing residues of less than 0·25 p.p.m., clean up the solution on a charcoal column prepared as follows: place a plug of cotton-wool in the chromatographic column, and cover with a 0·5 to 1·0-cm layer of Celite 545 or 560; place 3 g of charcoal in a 150-ml conical flask with a side-arm and stopper, cover with acetone and de-gas by suction from a water-pump for about 1 minute; pour this slurry into the column, allow it to settle, and run off the excess of acetone. Add the methanolic concentrate to the column and elute with 100 ml of acetone. Adjust the final volume as required. If a final volume of a few millilitres is required, concentrate the solution in a Kuderna-Danish evaporator.

Determine the pesticides in the final solution by gas - liquid chromatography with the columns as specified. Make injections of 5 μl of solution. Do not use a final volume of test solution of less than 5 ml, and for samples with residues in excess of 0·25 p.p.m. adjust the volume of the test solution to obtain a pesticide concentration of about 1 μg ml^{-1}.

DISCUSSION

PREPARATION OF STANDARD—

To attain a reasonable comparison with current commercial practice the untreated grain samples were spiked by covering the grain with a solution of pesticides in acetone and evaporating off the acetone with a gentle stream of air at room temperature. No significant difference was found between samples taken as a whole and as sub-samples.

METHOD OF EXTRACTION—

Three methods were compared: shaking by hand with a solvent for 1 minute; high-speed maceration with a solvent for 1 minute; and grinding followed by the same maceration procedure. In each instance three 50-ml portions of solvent were used and the grain was spiked with 0·25 p.p.m. of each pesticide. Typical recoveries are shown in Table I.

TABLE I

COMPARISON OF METHODS OF EXTRACTING THE PESTICIDES FROM GRAIN

Method	Solvent	Percentage recovery of dichlorvos	Percentage recovery of malathion
Shaking	Acetone	23	67
Maceration	Acetone	38	87
Maceration	Methanol	87	95
Grinding and maceration ..	Methanol	98	99

The figures for methanol suggest that some dichlorvos penetrates below the surface of the grain. The best of these methods is clearly grinding followed by maceration of the grain.

EXTRACTION SOLVENT—

Acetone and dichloromethane give good recoveries of malathion but hexane and acetonitrile were less effective; none of these solvents was efficient in extracting dichlorvos as Table II shows. Dichloromethane has to be removed completely after extraction, as small amounts of this solvent have a marked effect on the sensitivity of the thermionic detector. Evaporation to dryness is undesirable in view of the appreciable volatility of dichlorvos. Acetonitrile also must be removed so as not to interfere with the determination of dichlorvos, as the thermionic detector responds to nitrogen-containing compounds. Propan-2-ol was unsuitable for both dichlorvos and malathion. Methanol was found to be by far the best solvent for both pesticides.

TABLE II

COMPARISON OF SOLVENTS FOR EXTRACTING THE PESTICIDES FROM GRAIN

	Percentage recovery of dichlorvos	Percentage recovery of malathion
Acetone	38	87
Acetonitrile	43	58
Dichloromethane ..	33	100
Hexane	18	65
Methanol	87	96
Propan-2-ol	56	31

CLEAN-UP—

The clean-up stage may be unnecessary, especially for samples with high residue levels. Some grades of methanol contain impurities that give rise to early peaks that mask the dichlorvos peak; these impurities are removed by the charcoal.

On addition of the methanolic concentrate to acetone, a white material, which was shown by infrared spectroscopy to be mainly glucose-like in nature, was precipitated; it was found not to affect the method.

RECOVERIES—

Because of the high volatility of dichlorvos, a check was made by evaporating to dryness in a gentle stream of air at room temperature 5 ml of acetone solution containing 5 μg of dichlorvos and evaporating 5 μg of dichlorvos in 100 ml of acetone to 5 ml in a modified Kuderna-Danish evaporator. The recoveries were 98 and 91 per cent., respectively.

On eluting from a charcoal column with acetone 100 per cent. recovery for 5 μg of dichlorvos and 93 per cent. for 5 μg of malathion were achieved.

Recovery on the over-all method was found to be 98, 93 and 87 per cent. (3 runs) for dichlorvos and 99 per cent. for malathion with grain samples spiked at 0·25 p.p.m. and 88 per cent. for dichlorvos and 95 per cent. for malathion with grain samples spiked at 10 p.p.m.

SENSITIVITY—

At normal working amplification, full-scale deflection was obtained with 5 ng of malathion and 1·0 ng of dichlorvos. With a 20-g sample and a final test solution of 5 ml, residues of the order of 0·025 p.p.m. of malathion and 0·005 p.p.m. of dichlorvos can be conveniently determined by the method.

CONCLUSION

The proposed method is a rapid and sensitive procedure for determining dichlorvos and malathion in grain.

Permission to publish this paper has been given by the Government Chemist, Department of Trade and Industry.

REFERENCES

1. Hill, E. G., and Thompson, R. H., *J. Sci. Fd Agric.*, 1968, **19**, 119.
2. Thompson, R. H., and Hill, E. G., *Ibid.*, 1969, **20**, 293.
3. Report of the 1967 Joint Meeting of the FAO Working Party of Experts and the WHO Expert Committee on Pesticide Residues, Food and Agriculture Organisation, Rome, and World Health Organisation, Geneva, 1968.
4. Norris, M. V., Vail, W. A., and Averell, P. R., *J. Agric. Fd Chem.*, 1954, **2**, 570.
5. Rowlands, D. G., *Analyst*, 1964, **89**, 498.
6. Bates, A. N., Rowlands, D. G., and Harris, A. H., *Ibid.*, 1962, **87**, 643.
7. Bates, A. N., and Rowlands, D. G., *Ibid.*, 1964, **89**, 286 and 288.
8. Conroy, H. W., *J. Ass. Off. Agric. Chem.*, 1959, **42**, 551.
9. Joint Malathion Panel, *Analyst*, 1960, **85**, 915.
10. Weisenberg, E., Gertner, S., and Schoenberg, J., *Ibid.*, 1968, **93**, 443.
11. Hill, A. C., *J. Sci. Fd Agric.*, 1969, **20**, 4.
12. Jeffs, K. A., Lord, K. A., and Tuppen, R. J., *Ibid.*, 1968, **19**, 195.
13. Elms, K. D., *J. Stored Prod. Res.*, 1967, **3**, 393.
14. Hughes, J. T., *Analyst*, 1963, **88**, 318.
15. Ivey, M. C., and Claborn, H. V., *J. Ass. Off. Analyt. Chem.*, 1969, **52**, 1248.
16. St. Clair, A. D., Lamberton, J. G., Claeys, R. R., and Goulding, R. L., *Ibid.*, 1969, **52**, 1010.

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The Microbiological Assay of the Vitamin B_6 Complex (Pyridoxine, Pyridoxal and Pyridoxamine) with *Kloeckera Brevis*

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A complete re-investigation of the microbiological assay of the vitamin B_6 complex (pyridoxine, pyridoxal and pyridoxamine) with the yeast *Kloeckera brevis* has been made. The superiority of the proposed method over others *e.g.*, those involving *Saccharomyces carlsbergensis* 4228 (ATCC 9028), the protozoan *Tetrahymena pyriformis* and the X-ray mutant *Neurospora sitophila* 299, is discussed and suitable methods of extraction are described, particularly for blood, liver, human and rat brain, and urine.

Kloeckera brevis has also been found to be a more suitable organism for the cup-plate assay of the vitamin B_6 complex than *S. carlsbergensis*. The zones of exhibition are sharp and clear and background growth is reduced to a minimum, which is frequently not so with *S. carlsbergensis*.

Serious difficulties have been encountered in the author's laboratory with the microbiological assay of the vitamin B_6 complex with the "bottom" yeast *Saccharomyces carlsbergensis* 4228 (ATCC 9028) because of the frequent and inexplicable incidence of unrestricted growth in the blanks. This difficulty has apparently been encountered in other laboratories and Gare[1] has suggested that *S. carlsbergensis* should be completely depleted of pyridoxine before an assay by growing it in a pyridoxine-free liquid medium and then using this medium directly as the inoculum. This modification of technique was tested and proved to be successful for a few weeks, but later the blanks again showed unrestricted growth, which became progressively worse with time.

Baker and Frank[2] have used the protozoan *Tetrahymena pyriformis* for the microbiological assay of pyridoxine, pyridoxal and pyridoxamine in blood, brain, muscle and urine. This method, however, has many and serious disadvantages: the composition of the basal medium is complex; the assay as described occupies a great deal of incubator space; and the organism, while responding equally well to pyridoxal and pyridoxamine, has only 50 to 80 per cent. activity towards pyridoxine. This last criticism also applies to *S. carlsbergensis*, which responds equally well to pyridoxine and pyridoxal, but has only about 80 to 84 per cent. activity towards pyridoxamine.

The evidence for the behaviour of the X-ray mutant *Neurospora sitophila* towards the three forms of the vitamin B_6 complex is contradictory. In any event, the assay with this organism is time consuming, occupies much incubator space and is unsuitable for routine assay work.

The yeast *Kloeckera brevis* has been used in this laboratory for some years for the assay of the vitamin B_6 complex and, although unacceptably high blanks were occasionally encountered, nevertheless, most assays were successful. At no time was the massive growth encountered with *S. carlsbergensis* blanks found with *K. brevis*. The blanks, however, tended to be high, leading to an undesirable flattening of the standard curve.

It was, therefore, decided to test Gare's "depletion" technique with *K. brevis*, to ascertain if there was any improvement in the blanks. The attempt proved to be unsuccessful; the same massive growth occurred in the blanks as with *S. carlsbergensis* and unrestricted growth was even more marked when an inoculum was started up from a freeze-dried culture on a pyridoxine-free basal medium.

It therefore occurred to the author that the same situation obtains in *K. brevis* as in *Lactobacillus fermenti* 36, which, when used for the microbiological assay of thiamine, has to be maintained on an agar stock medium containing free thiamine, and has to have the

inoculum prepared by growing the organism in a liquid medium also containing free thiamine. This analogy was confirmed and no further difficulties were encountered with unacceptably high blanks.

The pyridoxine-free basal medium has also been modified in several respects; notably, by substituting a 10 per cent. charcoal-treated malt extract solution for vitamin-free casein hydrolysate, which often proved difficult to free completely from pyridoxine.

Experimental

Maintenance of organism—

Kloeckera brevis is maintained in liquid stock culture instead of on the conventional malt agar slope. The liquid stock culture medium has the following composition:

Malt extract (10 per cent.) solution	100 ml
Difco yeast extract	0·20 g
Glucose	2·00 g
Pyridoxine hydrochloride	1 000 μg

Dissolve 10 g of malt extract in approximately 150 ml of water, boil the solution gently until the volume has been reduced to less than 100 ml and allow it to cool. Make the volume up to 100 ml with water and filter under suction with filter aid. Re-adjust the volume of the solution, if necessary, add 0·2 g of Difco yeast extract, 2 g of glucose and 1 000 μg of pyridoxine hydrochloride, adjust the pH to between 4·8 and 5·0 by using bromocresol green as external indicator, and dispense the solution in 20-ml portions into 250-ml conical flasks plugged with cotton-wool; sterilise them by steaming for 30 minutes. Cool in the dark, since all three forms of the vitamin B_6 complex are photosensitive, especially in hot solution, and store at a temperature not exceeding 4 °C.

Slope cultures of the organism should also be maintained as a safeguard against accidental contamination of a liquid stock culture. The slopes are prepared by adding 2 g of agar to the liquid medium described above. The medium is steamed to melt the agar and dispensed in 5-ml aliquots in 1-oz screw-capped bottles, sterilised at 10 p.s.i. for 10 minutes and sloped in the dark.

Slope and liquid cultures are renewed at weekly intervals. A slope is prepared in the usual way and incubated overnight at any selected temperature between 25 and 30 °C, with the cap loose. After incubation, the cap is tightened and the culture kept in a refrigerator at a temperature not exceeding 4 °C.

The liquid stock culture is prepared by transferring 1·0 ml of a culture under strictly aseptic conditions to a fresh flask of medium and incubating for 24 hours on a shaker at any selected temperature between 25 and 30 °C.

A fresh liquid stock culture is prepared by suspending the cells from a slope in 10 ml of sterile water and then transferring 1·0 ml of the suspension to a fresh flask of medium and incubating for 24 hours on a shaker at any selected temperature between 25 and 30 °C.

Basal medium—

The pyridoxine-free basal medium (5× strength) has the following composition:

Charcoal-treated 10 per cent. malt extract solution (see below)	20·0 ml	DL-Isoleucine	20·0 mg
L-Asparagine	1·0 g	L-Lysine hydrochloride	20·0 mg
Ammonium sulphate	1·0 g	DL-Methionine	20·0 mg
Potassium dihydrogen orthophosphate	0·6 g	DL-Tryptophan	20·0 mg
Tripotassium citrate	5·0 g	DL-Valine	20·0 mg
Citric acid	1·0 g	Niacin (Nicotinic acid)	5 000 μg
Potassium chloride	0·5 g	Calcium *d*-pantothenate	5 000 μg
Calcium chloride (anhydrous)	0·2 g	Thiamine hydrochloride	5 000 μg
L-Arginine hydrochloride	20·0 mg	Biotin	40·0 μg
L-Histidine hydrochloride	10·0 mg	Inositol	30·0 mg
		Inorganic salt solution (see below)	6·3 ml
		Water to	100 ml

After mixing, adjust the pH of the medium to between 4·8 and 5·0 with hydrochloric acid, using bromocresol green as external indicator, store at a temperature not exceeding 4 °C and use within 7 days. Immediately before an assay, gently warm the solution, add 20 g of glucose and make up to a total volume of 200 ml.

Malt extract solution (10 *per cent.*)—Dissolve 10 g of malt extract in approximately 150 ml of water and then proceed exactly as described under "Maintenance of organism." After making the volume of the solution up to 100 ml, adjust the pH to between 2·5 and 3·0 with concentrated hydrochloric acid, using bromocresol green as external indicator, add 5 g of a good quality activated charcoal and stir the solution mechanically for 30 minutes. Filter under suction. Repeat the operation twice more with 5-g portions of charcoal. Steam the solution for 30 minutes after final filtration, cool, store under sulphur-free toluene at a temperature not exceeding 4 °C and use within 14 days of preparation.

Inorganic salt solution—This solution has the same composition as that devised by the author for microbiological assays with lactic organisms. Dissolve 10 g of magnesium sulphate ($MgSO_4.7H_2O$), 0·5 g of manganese(II) sulphate ($MnSO_4.4H_2O$) and 0·1 g of anhydrous iron(III) chloride in 250 ml of water and add 5 drops of concentrated hydrochloric acid. This solution will maintain its activity indefinitely at room temperature.

The recommended additions of trace amounts of boron, zinc, copper, molybdenum and iodate described in the literature have been found to be unnecessary (L. Gare, private communication).

ASSAY PROCEDURE—

A separate standard curve *must* be established for every assay. The amounts of pyridoxine hydrochloride required to establish such a curve are: 0·000, 0·001, 0·002, 0·004, 0·006 and 0·008 μg. Prepare a solution of pyridoxine hydrochloride containing 0·002 $\mu g\ ml^{-1}$ and add the following amounts to establish the range of standards: blank (4 ml of water); 0·5 ml of standard solution *plus* 3·5 ml of water; 1·0 ml of standard solution *plus* 3 ml of water; 2·0 ml of standard solution *plus* 2 ml of water; 3 ml of standard solution *plus* 1·0 ml of water; and 4·0 ml of standard solution. Adjust the level of liquid in all of the tubes to 5 ml by the addition of 1·0 ml of basal medium and place a glass bead in each tube.

Set up the test preparation at three concentrations by taking 1, 2 and 4 ml of the test extract, making the volume up to 4 ml with water and adding 1 ml of basal medium.

Add the water and basal medium to each tube with a 5-ml calibrated B-D Cornwall Luer-Lok syringe, and the standard and test solution with a 2-ml calibrated instrument. This will be found to be a more accurate and expeditious method than addition with a manual pipette.

Set up all concentration levels of standard and test solutions in triplicate, cap the tubes with aluminium thimbles, with coloured thimbles for the blanks, sterilise by steaming for 20 minutes, cool in the dark and inoculate.

PREPARATION OF INOCULUM—

Place 10-ml portions of the liquid stock medium described above into 1-oz screw-capped bottles and sterilise them at 10 p.s.i. for 10 minutes, cool in the dark and store at a temperature not exceeding 4 °C and use within 1 month of preparation.

The inoculum is prepared as follows: add 1 ml of a liquid stock culture, which should not be more than 48 hours old, under strictly aseptic conditions to a bottle of liquid medium, loosen the cap and incubate at between 25 and 30 °C for 24 hours.

Centrifuge aseptically, wash the deposit twice on the centrifuge with sterile distilled water and suspend the deposit in 10 ml of sterile distilled water, and dilute the suspension 1 to 100 with sterile distilled water. Add 1 drop of this final suspension to each tube, but omit to inoculate one of the blanks, so that it can be used to set the colorimeter.

Incubate the tubes at any selected temperature between 25 and 30 °C on a shaker in the dark for 24 hours. Whichever temperature is selected for incubation, it is essential that the temperature of the incubator remains constant to ± 1 °C, otherwise erratic results will be encountered among the replicates, especially among the lower levels of pyridoxine concentration in the standard tubes.

After incubation, add 5 ml of water to each tube, steam for 15 minutes and determine the response nephelometrically.

PREPARATION OF SAMPLES FOR ASSAY—

The vitamin B_6 complex occurs naturally not only as free pyridoxine, pyridoxal and pyridoxamine, but also as the phosphorylated derivatives of each form. To obtain an accurate estimate of the vitamin B_6 complex content of natural materials, it is necessary

that dephosphorylation should first be carried out. The conventional method recommended in the literature is to hydrolyse the material with 0·055 N hydrochloric acid for 4 hours at 15 p.s.i. This method proved satisfactory in our hands for meat and meat products and most plant materials, except cereals, in which the presence of any unhydrolysed starch led to filtration difficulties.

A modification of the technique recommended by Jones and Morris,[3] in which takadiastase was used, was found to be more satisfactory with all products, with the exception of those described below.

Weigh an appropriate amount of the sample (2 to 5 g) into a 250-ml conical flask and add 50 ml of 0·05 N hydrochloric acid. Autoclave for 1 hour at 15 p.s.i. and cool in the dark. Add 2 ml of a 1 per cent. sodium acetate solution and adjust the pH to 4·5, using bromocresol green as external indicator, add 0·3 g of takadiastase and incubate overnight under a thin layer of sulphur-free toluene at 37 °C. Steam the flask for 30 minutes to inactivate the diastase, make up to 100 ml or other suitable volume with water and filter. Further dilution may be necessary depending upon the pyridoxine content of the material. The final test solution should contain between 0·001 and 0·002 μg ml^{-1} of pyridoxine.

The following materials require special extraction treatment: blood, serum or plasma, liver, human and rat brain, and urine. The methods given in detail below are minor modifications of those described by Baker and Frank[2] and will be found to be satisfactory.

Blood, serum and plasma—Place 2-ml samples in three 30-ml Quickfit borosilicate glass centrifuge tubes and dilute each with 2 ml of a 0·02 M sodium monophosphate buffer solution (pH 4·5). The buffer is prepared by dissolving 2·76 g of sodium dihydrogen orthophosphate ($NaH_2PO_4.H_2O$) in 1000 ml of distilled water. Set up at the same time an enzyme blank consisting of 2 ml of distilled water and 2 ml of buffer solution. Stopper the tubes and autoclave for 30 minutes at 15 p.s.i., cool in the dark and add 6 ml of enzyme solution. The recommended enzyme is Clarase, and is prepared by dissolving it to give a concentration of 5 mg ml^{-1} in the buffer solution and filtering before use. Stir the mixture to distribute any coagulum and incubate overnight at 37 °C under a thin layer of sulphur-free toluene. After incubation, autoclave the tubes for 30 minutes at 15 p.s.i., cool in the dark and centrifuge to remove any debris. Bulk the contents of the three tubes and set up the assay in triplicate by using 1, 2 and 4-ml portions in the tubes.

Liver—Accurately weigh 10-mg portions of lyophilised liver powder into six 30-ml Quickfit centrifuge tubes and add 5 ml of water to each. Incubate the suspension under a thin layer of sulphur-free toluene for 3 days at 37 °C. Autoclave for 30 minutes at 15 p.s.i., cool in the dark and centrifuge. The contents of the tubes are bulked and 1, 2 and 4 ml of the supernatant liquid are transferred to the assay tubes. Liver contains all enzymes for liberating vitamin B_6 from proteins during the incubation period.

Human and rat brain—Accurately weigh 50-mg portions of lyophilised human or rat brain into two 30-ml Quickfit centrifuge tubes and add 1 ml of buffer solution to each. Set up at the same time an enzyme blank by placing 1-ml aliquots of buffer solution into two 30-ml centrifuge tubes. Stopper the tubes and autoclave for 30 minutes at 15 p.s.i., cool in the dark and add 4 ml of enzyme solution (Clarase dissolved in water to give a concentration of 4 mg ml^{-1} and filtered before use). Stir the mixture to disperse the coagulum and incubate for 3 days under a thin layer of sulphur-free toluene at 37 °C. After incubation, autoclave the tubes for 30 minutes at 15 p.s.i., cool in the dark and add 20 ml of distilled water to each tube. Centrifuge, bulk the contents of the tubes and add 1, 2 and 4-ml portions of the supernatant liquid in triplicate to the tubes.

Urine—With a pipette, introduce four 1-ml aliquots of a 24-hour sample of urine into four 30-ml Quickfit centrifuge tubes and dilute each with 1 ml of buffer solution, autoclave for 30 minutes at 15 p.s.i., cool in the dark and add 8 ml of distilled water to each tube. Centrifuge if necessary. Bulk the contents of the tubes and, by pipette, place in triplicate 1, 2 and 4-ml portions of the supernatant liquid into assay tubes. Enzyme hydrolysis to release the loosely bound vitamin B_6 is unnecessary.

CUP-PLATE ASSAY—

Add 20 g of glucose and 20 g of agar to the basal medium described above, make up to 1000 ml with distilled water, steam until the agar has dissolved, cool to 45 °C and add the inoculum.

The inoculum is prepared in exactly the same way as for the tube assay, but after centrifuging and re-suspending the deposit in 10 ml of sterile distilled water, the suspension is diluted so that the opacity corresponds to tube No. 9 or 10 of the Wellcome series of standard opacity tubes. One millilitre of this suspension is added for every 20 ml of medium.

After the addition of the inoculum, thoroughly mix and pour into Petri dishes. Cool the dishes on a flat surface and, after the agar has set, leave in a refrigerator overnight. The rest of the assay conforms with normal cup-plate procedure. The range of pyridoxine concentrations to establish a standard curve is: 0·25, 0·50, 1·0 and 2·0 $\mu g\ ml^{-1}$ and the dilutions of the test extract are 1 + 1, 1 + 3, 1 + 7 and 1 + 15. The plates are incubated at between 25 and 30 °C for 20 to 22 hours.

CALCULATION—

The main responses in the standard series are plotted against the doses and the results of the test samples evaluated by direct reading. The results should not differ among themselves by more than ±10 per cent. The method has the advantage of rapidity and shows immediately whether an assay is valid or not. A regular upward or downward drift in the figures shows the presence of interfering substances in the test solution and makes the assay invalid.

As the standard curves are invariably curvilinear by this method of assay, it is strongly advised that, after approximate values have been calculated by direct reading from the standard curve, exact values should be determined by the Wood[4] "log-log" procedure. If fiducial limits are required, they should be calculated by computer.

DISCUSSION

The microbiological assay of the vitamin B_6 complex with *Kloeckera brevis* has several advantages over previously recommended procedures with other microorganisms. Apart from the relatively simple composition of the basal medium and the straightforward assay technique, the great advantage of this method is that *K. brevis* shows virtually equal activity towards all three forms of the B_6 complex; there is, however, a slight but persistently higher activity (2 to 3 per cent.) towards pyridoxamine. This compares favourably with *Saccharomyces carlsbergensis*, which shows only 80 per cent. activity towards pyridoxamine, and *Tetrahymena pyriformis*, which shows equal activity towards pyridoxal and pyridoxamine but only about 50 to 80 per cent. activity towards pyridoxine.

REFERENCES

1. Gare, L., *Analyst*, 1968, **93**, 456.
2. Baker, H., and Frank, C., "Clinical Vitaminology," Interscience Publishers Inc., New York, 1968, p. 66.
3. Jones, A., and Morris, S., *Analyst*, 1950, **75**, 608.
4. Wood, E. C., *Ibid.*, 1947, **72**, 84.

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Communication

Material for publication as a Communication must be on an urgent matter and be of obvious scientific importance. Manuscripts must not exceed 300 words; rapidity of publication precludes the use of diagrams, but tables or formulae may be included if the length of text is reduced appropriately. Communications should not be simple claims for priority. This facility for rapid publication is intended for brief descriptions of work that has progressed to a stage at which it is likely to be valuable to workers faced with similar problems. A fuller paper may be offered subsequently, if justified by later work.

Manuscripts are *not* subjected to the usual examination by referees. Inclusion of a Communication is at the Editor's discretion; a manuscript not accepted as a Communication may, if the author wishes, be re-submitted as a possible paper and subjected to the usual scrutiny by referees.

POTENTIAL HAZARDS BY FORMATION OF SILVER ACETYLIDE UPON ASPIRATING SOLUTIONS CONTAINING HIGH CONCENTRATIONS OF SILVER TO AN ATOMIC-ABSORPTION SPECTROPHOTOMETER WHEN ACETYLENE IS USED AS FUEL

WE were recently required to determine traces of lead and copper in silver metal. The test solutions contained silver at levels ranging between 1 and 10 g per 100 ml, in the presence of an excess of nitric acid. The air - acetylene flame system was being used.

As soon as test solutions were aspirated to the atomic-absorption spectrophotometer, a white precipitate was observed in the effluent drain-line. Removal of the mixing chamber revealed it to be heavily coated with a white curd-like material that was eventually established as being silver acetylide, Ag_2C_2 (completely insoluble in ammonia solution, but slowly soluble in nitric acid).

This compound is well known to be highly unstable when dry, "exploding upon moderate rubbing."[1] The acetylides are generally stated to be formed in solution under alkaline conditions —in the case of silver, when acetylene gas is bubbled through an ammoniacal solution of silver nitrate. This experience indicates that silver acetylide is also formed in the aerosol phase when an acidic solution containing a sufficiently high concentration of silver is aspirated into an atmosphere of acetylene.

For the application described, the determination was satisfactorily accomplished with the air - hydrogen flame. When it is essential to use acetylene as fuel, it will be necessary to remove the silver before aspirating the solution to the atomic-absorption spectrophotometer.

REFERENCE

1. Ephraim, F., "Inorganic Chemistry," Gurney and Jackson, London. 1948, p. 863.

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Book Reviews

TOPICS IN ORGANIC MASS SPECTROMETRY. Edited by A. L. BURLINGAME. Pp. xii + 471. New York, London, Sydney and Toronto: Wiley-Interscience, a division of John Wiley & Sons. 1970. Price £10·55.

This book, one in the series "Advances in Analytical Chemistry and Instrumentation," shows how the scope of organic mass spectrometry has been enlarged in recent years. The book, which presents a wide range of topics in the field, is written by acknowledged experts in their particular areas.

There are nine chapters in the book, with an introductory chapter by Beckey and Comes on techniques of molecular ionisation. This is followed by chapters on gas - liquid chromatography - mass spectrometry combination by Ställberg-Stenhagen and Stenhagen, high resolution

mass spectrometry by Biemann, mass spectrometry of complex natural products by Das and Lederer, and applications of mass spectrometry in flavour and aroma chemistry by McFadden and Buttery. There is a chapter on correlation of fragment ion structures with energy of formation by Harrison and another on mechanics of ion decomposition reactions by McLafferty. Another chapter, which is possibly out of harmony with the size of the book, covers the subject of mass discriminations caused by electron-multiplier detectors. Finally, there is a chapter on the application of mass spectrometry to organic geochemistry.

The book will be in the main of interest to specialists in the field. The chapters are well written, the diagrams are clear, and the references will bring the reader very up-to-date in the topics covered. The book can be recommended because of its remarkable freedom from errors of any kind.

N. R. DALY

Notice to Authors

FURTHER NOTES ON THE WRITING OF PAPERS FOR *The Analyst*

THE Final Report of the Commission on Symbols, Terminology and Units of the International Union of Pure and Applied Chemistry's Division of Physical Chemistry, as adopted by I.U.P.A.C. on July 7th, 1969, was published in *Pure and Applied Chemistry*, 1970, **21**, 3–44. It is entitled "Manual of Symbols and Terminology for Physicochemical Quantities and Units," and reprints are available from Butterworths, London, price £1.

The Royal Society and the Chemical Society are supporting the use of the styles laid down in this Manual. These styles are to a large extent those already in use in papers in *The Analyst*. However, certain changes are now required to further the standardised presentation of terms and symbols.

Numbers—The Manual recommends that the decimal sign should in general be a comma (,), except that in English-language texts it should be a full-stop (.). This will NOT at present be adopted by *The Analyst*, which will continue to use the centred decimal point (·).

However, because of this recommendation, the use of a comma to divide the digits of long numbers into threes (*e.g.*, 34,500) is no longer acceptable. *The Analyst* will in future use a small space (technically a "hair space") instead (*e.g.*, 34 500).

Physical quantities, units and numerical values—The Manual lays down that a physical quantity equals the product of a *numerical value* and a *unit*. For example, a volume (*e.g.*, of titrant) would be—

$$V = 17 \text{ ml}.$$

This statement is to be treated as a mathematical relationship, and may equally well be written as—

$$V/\text{ml} = 17.$$

This style will be used in headings to tables and in labels on the axes of graphs, where the numbers represent *numerical values*—

Volume/ml
17
27
37

The diagonal line ("solidus") will not be used to present "per." In accordance with S.I., units such as grams per millilitre are already expressed in the form: g ml^{-1}. For a table (or graph), this would appear as—

Concentration of solution/g ml^{-1}.

It should be noted that the "combined" unit, g ml^{-1}, must not have any "intrusive" numbers. To express concentration in grams per 100 millilitres, the word "per" will still be required—

Concentration/g per 100 ml (*not* g 100 ml^{-1}).

It may be preferable for an author to express concentrations in grams per litre (g l^{-1}) rather than grams per 100 ml, *viz.*, instead of "3 per cent. *w/v*" being expressed as "3 g per 100 ml", it could well appear as "30 g l^{-1}."

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LECTURES AND COURSES

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OCTOBER 1971-1972

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There is specialisation in organic analysis at Chelsea College and in trace analysis at Imperial College.

Chelsea College,
Manresa Road,
London S.W.3.

Imperial College,
South Kensington,
London S.W.7.

Potentiometric Method for the Determination of Aromatic Monothiosemicarbazones

A method has been developed for the quantitative determination of aromatic monothiosemicarbazones, in which addition of silver nitrate to a solution of the organic compound leads to complex formation. The hydrogen ion liberated on complex formation is determined by potentiometric titration.

M. J. M. CAMPBELL, R. GRZESKOWIAK and I. D. M. TURNER
Department of Chemistry, Thames Polytechnic, London, S.E.18.

Analyst, 1971, **96**, 286–287.

The Determination of Glycerol by the I.U.P.A.C. Form of the Malaprade Method

The accepted periodate method for the determination of glycerol has come under review by the International Organisation for Standardisation (Sub-Committee ISO/TC47/GT2) and certain modifications have been suggested with regard to the different pH end-points for the sample and blank and the possible loss of formic acid from the system by volatilisation. The present paper summarises the view of the U.K. delegates to ISO/TC47/GT2, which are:

(*i*) The choice of pH about 8·0 for the sample is justified on the ground that it contains formic acid in addition to strong acids. The blank, which contains only strong acids, should, on general grounds and by calculation, be titrated to pH 7·0. The use of pH 6·5 instead of 7·0 for the blank is an empirical correction designed to compensate for some lack of stoicheiometry or other bias in the procedure and to bring the results into agreement with those obtained by independent moisture and specific gravity determinations. This "correction" amounts to 0·03 per cent.

(*ii*) There is a potential loss of formic acid by volatilisation from the system (estimated variously to be equivalent to 0·01 to 0·04 per cent. of glycerol), which partly explains the apparent lack of stoicheiometry.

(*iii*) Modifications have been proposed to minimise this loss of formic acid and (by adding formate ions to both solutions) to unify the end-points at or about pH 8·0. Unless these modifications lead to a pronounced improvement in reliability, it seems doubtful whether further extensive trials on an international basis would be justified.

R. F. BARBOUR
Newcastle Technical Centre, Procter & Gamble Ltd., Newcastle upon Tyne.

and J. DEVINE
Unilever Research Laboratory, Unilever Ltd., Port Sunlight, Cheshire.

Analyst, 1971, **96**, 288–295.

The Separation and Determination of Pentachlorophenol in Treated Softwoods and Preservative Solutions

A method is described for the extraction, separation and determination of pentachlorophenol or its sodium salt in softwoods and preservative solutions. Pentachlorophenol is separated from lower chlorophenols and wood extractives by adsorption on to Bio-Rad AG 2-x8 anion-exchange resin, eluted with glacial acetic acid, extracted into chloroform, and determined by spectrophotometric measurement of the blue 4-aminophenazone - pentachlorophenol complex.

The effect of time and temperature on the oxidation - condensation reaction of 4-aminophenazone with pentachlorophenol has been investigated.

The procedure is particularly useful for the study of the distribution of pentachlorophenol-containing preservatives in wood.

A. I. WILLIAMS
Department of the Environment, Forest Products Research Laboratory, Princes Risborough, Bucks.

Analyst, 1971, **96**, 296–305.

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Chromium	357.9	AIR/C_2H_2	0.03
Cobalt	240.7	AIR/C_2H_2	0.15
Copper	324.8	AIR/C_2H_2	0.075
	242.8	AIR/C_2H_2	0.10
	248.3	AIR/C_2H_2	
		AIR/C_2H_2	

The Determination of Ethanol in Paints, Inks and Adhesives by Gas Chromatography

A gas-chromatographic method for the determination of ethanol in paints, inks, adhesives and similar composite products has been developed by which the time required for the analysis of samples has been greatly reduced. The ethanol is distilled azeotropically from the sample in the presence of toluene, and the distillate is examined directly by gas chromatography without any further treatment other than mixing with an internal standard. The problem of the longer retention time of toluene, compared with those of the lower aliphatic alcohols, has been overcome by the use of back-flushing. From the results obtained by the analysis in duplicate of a series of samples, including a number prepared in the laboratory containing known amounts of ethanol, the accuracy and precision of the method have been established.

J. R. HARRIS

Department of Trade and Industry, Laboratory of the Government Chemist, Cornwall House, Stamford Street, London, S.E.1.

Analyst, 1971, **96**, 306–309.

The Determination of Residues of Dichlorvos and Malathion in Wheat Grain by Gas - Liquid Chromatography

A procedure is described for the determination of malathion and dichlorvos in grain. After extraction with methanol, and clean-up on a charcoal column, if required, the pesticide residues are determined by gas - liquid chromatography on Apiezon L and butane-1,4-diol succinate columns with a phosphorus-sensitive detector.

Between the concentrations of 0·25 and 10 p.p.m. both pesticides were recovered from spiked samples with between 87 and 99 per cent. efficiency.

S. CRISP and K. R. TARRANT

Department of Trade and Industry, Laboratory of the Government Chemist, Cornwall House, Stamford Street, London, S.E.1.

Analyst, 1971, **96**, 310–313.

The Microbiological Assay of the Vitamin B_6 Complex (Pyridoxine, Pyridoxal and Pyridoxamine) with Kloeckera Brevis

A complete re-investigation of the microbiological assay of the vitamin B_6 complex (pyridoxine, pyridoxal and pyridoxamine) with the yeast *Kloeckera brevis* has been made. The superiority of the proposed method over others *e.g.*, those involving *Saccharomyces carlsbergensis* 4228 (ATCC 9028), the protozoan, *Tetrahymena pyriformis* and the X-ray mutant *Neurospora sitophila* 299, is discussed and suitable methods of extraction are described, particularly for blood, liver, human and rat brain, and urine.

Kloeckera brevis has also been found to be a more suitable organism for the cup-plate assay of the vitamin B_6 complex than *S. carlsbergensis*. The zones of exhibition are sharp and clear and background growth is reduced to a minimum, which is frequently not so with *S. carlsbergensis*.

E. C. BARTON-WRIGHT

Galloway and Barton-Wright, Haldane Place, London, S.W.18.

Analyst, 1971, **96**, 314–318.

Potential Hazards by Formation of Silver Acetylide upon Aspirating Solutions Containing High Concentrations of Silver to an Atomic-absorption Spectrophotometer when Acetylene is Used as Fuel

Communication

R. J. REYNOLDS and D. S. LAGDEN

Evans Electroselenium Research Laboratories, Braintree, Essex.

Analyst, 1971, **96**, 319.

Microfiche Editions of *The Analyst* and *Analytical Abstracts*

As announced on page 88 of the current (April) issue of *Proceedings of the Society for Analytical Chemistry*, the Society proposes to produce a microfiche edition of *The Analyst*, and possibly of *Analytical Abstracts* also.

The Analyst for 1969 (volume 94) will be published on microfiche shortly, price £15; $36 (reference: *a*).

A reduced price (£7·50; $18) will be charged to customers who subscribed during 1969 to the "paper" edition of *The Analyst*.

The future programme of microfiche production depends on the response to this initial offer. Customers, or potential customers, are therefore urged to write to the publishers stating if they are interested in ordering—

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