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Summaries of Papers in this Issue

The Analytical Role of Ion-selective and Gas-sensing Electrodes in Enzymology—A Review

Summary of Contents

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Analyst, 1975, **100**, 609–619.

Nitrosophenol and Nitrosonaphthol Derivatives as Reagents for the Spectrophotometric Determination of Iron and Determination of Micro-amounts in Waters with 2-Nitroso-5-dimethylaminophenol

Twenty-five nitroso compounds have been examined for suitability as organic analytical reagents for the spectrophotometric determination of iron(II) ion. The complexes formed by 2-nitroso-5-dimethylaminophenol (nitroso-DMAP) and 2-nitroso-5-diethylaminophenol (nitroso-DEAP) with iron(II) are shown to have a 1:4 composition and the other iron(II) complexes a 1:3 composition. Nitroso-DMAP and nitroso-DEAP were found to be very sensitive reagents for iron(II) ion, the molar absorptivities of their complexes being about 4×10^4 at about 750 nm in aqueous solution. The colour of the nitroso-DMAP - iron(II) complex is very stable in aqueous solution at about pH 8, while that of the nitroso-DEAP complex is less stable. Nitroso-DMAP is more useful for the determination of micro-amounts of iron than nitroso-DEAP, because of the greater stability of the reagent and the complex formed and the solubility of the latter in aqueous solution.

Diverse ions, which are generally present in city and river waters, did not interfere in the direct spectrophotometric determination of micro-amounts of iron in these waters with nitroso-DMAP.

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Analyst, 1975, **100**, 629–636.

An Improved and Accurate Procedure for the Determination of Vitamin A

A rapid and accurate procedure is described for determining vitamin A with trichloroacetic acid. Water-free reagents are prepared less than 3 h before use and the colorimetric readings are made by using a fast-measurement accessory and a slave recorder. The use of this accessory enables the rapid mixing of the colour reagent with the solution to be tested and the reading of results to be carried out within 1 s.

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Analyst, 1975, **100**, 637–639.

Determination of Ammonia-, Nitrate- and Organic Nitrogen in Water and Waste Water with an Ammonia Gas-sensing Electrode

The use of the Orion ammonium ion selective electrode for the determination of ammonia-, nitrate- and organic nitrogen in a number of waters and effluents has been investigated. Adjustment of the pH to attain the optimum conditions for the determination was achieved by use of 2 M sodium hydroxide solution that was 1 M in ethylenediaminetetraacetic acid (EDTA) disodium salt. Evidence is produced which shows that the alkali present in excess of that required to give a pH value of 12 does not affect the calibration graphs or the recovery of ammonia from the water samples investigated.

Standard nitrate solutions and samples were reduced and measured as ammonia-nitrogen. Reagents used to bring about this reduction are discussed. Recoveries at a level of 1 mg l^{-1} of nitrate-nitrogen averaged 95 per cent., using the nascent hydrogen produced by Devarda's alloy in acidic conditions as the reductant. A contact time of 24 h was required for this percentage reduction. A coefficient of variation of $\pm 6{\cdot}8$ per cent. was achieved. Under these reducing conditions, the percentage recovery decreased as the concentration of nitrate-nitrogen increased but acceptable recoveries were obtained for concentrations of nitrate-nitrogen up to 5 mg l^{-1}. However, when ammonia-nitrogen was present at levels significantly higher than those of nitrate-nitrogen, the reliability of the results decreased. The method is convenient for the determination of ammonia-, nitrate- and organic nitrogen in the same sample.

Interference by metals that form insoluble hydroxides under the alkaline conditions of measurement of ammonia-nitrogen was studied. The high level of magnesium present in sea water is shown to interfere with electrode response, probably because of hydroxide formation and consequent precipitation. This potential interference is eliminated by the use of 1 M EDTA to complex magnesium, and other metals, present in sea water and estuarine waters.

It is further shown that organic nitrogen can be determined with the ammonium ion selective electrode. The method involves removal of free ammonia followed by acid digestion and final release and measurement of the ammonia produced in alkaline solution. Recoveries are shown to be acceptable at the relatively low levels measured and show good agreement with published figures for similar environments.

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Analyst, 1975, **100**, 620–628.

The Effect of Valency State on the Determination of Chromium in Perchloric Acid Media by Atomic-absorption Spectrophotometry

Measurements of the atomic absorption of chromium, present in perchloric acid solutions in both the chromium(III) and chromium(VI) states, show that the valency state of the chromium is important. Conversion of all of the chromium into the chromium(III) state by the addition of hydrogen peroxide overcomes this valency problem and also ensures that the maximum absorption is obtained for a given concentration of chromium.

Comparison of the atomic-absorption results obtained by using the air - acetylene and nitrous oxide - acetylene flames, with and without the addition of ammonium chloride, shows that the choice of these parameters has appreciable effects. Up to a 30 per cent. higher chromium response has been found to occur with the addition of ammonium chloride and a further 30 per cent. increase can be obtained by the use of the nitrous oxide - acetylene flame.

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Analyst, 1975, **100**, 640–642.

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The Analyst

The Analytical Role of Ion-selective and Gas-sensing Electrodes in Enzymology

A Review*

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Summary of Contents

Introduction

Among the many applications of ion-selective electrodes[1] is their use for monitoring various enzyme systems.[2–57] This use is made possible by the fact that many of the enzymolytic products from various substrates are directly detectable by use of ion-selective electrodes. Such products include carbon dioxide, ammonia, cyanide, thiocyanate and sulphide, while detectable ions, such as cyanide, sulphide and nitrate, can also function as substrates.

The mode of application of ion-selective electrodes in enzymology can be illustrated by the urea - urease system

$$CO(NH_2)_2 + 2H_2O \xrightarrow{\text{Urease}} 2NH_4^+ + CO_3^{2-} \quad \ldots\ldots\ldots\ldots \quad (1)$$

whereby the enzyme urease is added to a stirred, thermostatically controlled solution of urea substrate containing a pre-calibrated, glass cation-selective electrode - reference electrode pair in the conventional dipping mode, connected to a potentiometric recording system. In this way, Katz and Rechnitz[40] determined urea and urease after stoicheiometric deamination when the final steady potential recorded with a Beckman univalent cation-selective electrode was proportional to the concentration of ammonium ions, and hence to one half of the concentration of urea or urease activity. Alternatively, substrate can be added to the enzyme medium.[9]

Considerable progress has been made in the monitoring of various enzyme systems with ion-selective electrodes[2–57] and the main features, summarised in Table I, are discussed. In general, the sensors have been used with the objectives of determining Michaelis constants (K_m) and of carrying out assays of enzyme activities, enzyme inhibitors and substrate concentrations.

The various types of ion-selective electrodes used in enzymology include conventional glass, homogeneous solid-state, liquid ion-exchanger, neutral carrier complex and gas-sensing electrodes. These types have been modified in many instances[6,8,11,14,16,21,25,26] by sandwiching an enzyme- or substrate-soaked matrix between the sample solution and the sensing membrane of the electrode. Such modifications are termed enzyme electrodes, a term that might be deemed misleading for it is by means of the substrate or product that an enzyme is monitored (Table I). Indeed, Montalvo[10] proposed the term "substrate electrode" in the case of the Beckman glass cation-selective electrode that is used to sense ammonium ions. This alternative is restrictive in situations where the ion-selective electrodes sense a product, and it is therefore sensible to adopt the term enzyme electrode, especially as its definition[58]

* Reprints of this paper will be available shortly. For details see summaries in advertisement pages.

TABLE I: SOME ENZYME - SUBSTRATE SYSTEMS

Substrate	Enzyme*	Buffer(s)	Optimum pH†	Products‡
Amygdalin	β-Glucosidase (E.C. 3.2.1.21)	$NaOH - NaH_2PO_4$	6·4	Benzaldehyde, β-D-glucose and CN^-
$S_2O_3^{2-}$ and CN^- ..	Thiosulphate sulphur transferase or rhodanase (E.C. 2.8.1.1)	Tris - HCl, or $NaOH - NaH_2PO_4$	8·6–8·7 7·9	SO_3^{2-} and SCN^-
L-Cysteine and CN^-..	β-Cyanoalanine synthase (E.C. 4.4.1.9)	Tris - acetic acid	8·5	HS^- and β-cyanoalanine
Diphenylcarbamyl fluoride (DPCF) ..	α-Chymotrypsin (E.C. 3.4.4.5)	—	7·5	DPC-chymotrypsin and F^-
NO_3^-	Nitrate reductase (dissimilatory); isolated from *E. coli* (strain B) (E. 11303)	Phosphate	7·2	NO_2^-
NO_2^-	Nitrite reductase (E.C. 1.6.6.4)	Phosphate	7·2	NH_4^+
Creatinine	Creatine deaminase (E.C. 3.5.4.21)	$NaH_2PO_4 - Na_2HPO_4$, $NaOH - NaH_2PO_4$, or NaH_2PO_4 - Tris	8·5	*N*-Methylhydantoin and NH_3
Glutamine	Glutaminase (E.C. 3.5.1.2)	Tris	7·0	L-Glutamic acid and NH_4^+
Asparagine	Asparaginase (E.C. 3.5.1.1)	Tris	7·0	L-Aspartic acid and NH_4^+
L-Tyrosine L-Leucine	L-Amino-acid oxidase (E.C. 1.4.3.2)	Tris	7·0	NH_4^+, $RCOCOO^-$ and H_2O_2
D-Tyrosine D-Methionine	D-Amino-acid oxidase (E.C. 1.4.3.3)	Tris	7·0	NH_4^+, $RCOCOO^-$ and H_2O_2
Urea	Urease (E.C. 3.5.1.5)	Tris	7·0	NH_4^+ and CO_3^{2-}
Urea	Urease (E.C. 3.5.1.5)	Tris	7·0	NH_4^+ and CO_3^{2-}

* Sensor-reference electrodes dipped into substrate - enzyme mixture unless otherwise stated.

† These may vary from literature values owing to different rate measuring techniques as well as certain

‡ These must relate to the reaction stoicheiometry. Actual species often depends on pH, for example,

§ In conjunction with saturated calomel reference electrode unless otherwise quoted. Commercial gas

STUDIED WITH ION-SELECTIVE ELECTRODES

Electrode sensor type§	Comments/Results	Reference
Conditioned Orion 94–06A cyanide with Orion 90–01 reference electrode	Can detect 10 μg of enzyme per 100 μl in about 1 min with relative precision of 2 per cent. K_m = 0·25 mM at 37 °C and pH 6·4	28
Orion 94–06A cyanide with Orion 90–01 reference electrode. (Orion 94–58 thiocyanate electrode less satisfactory)	K_m = 1·2–8·7 mM depending on $[S_2O_3]_0$ and $[CN]_0$. Detects 0·1 enzyme unit in about 1 min, and 0·01 unit with longer incubation periods	29
Home-made silver sulphide model	—	18
Orion 94–09A fluoride. Reference type not mentioned	Detects as little as 0·03 μmol of enzyme with about 3 per cent. precision. Technique is three times more sensitive than previous methods	39
Orion 92–07 nitrate. Reference type not mentioned Home-made electrode comprising nonactin in a silicone rubber matrix sensor. Reference type not mentioned	The rates of formation of NH_4^+ ion and decrease in nitrate determined compare favourably with alternative tedious spectrophotometric methods	24
Orion 95–10 flow-through ammonia gas model	After incubation, the system is quenched with 1 M NaOH to establish the product as NH_3 (equation 2). Little interference from urea, arginine or creatine in a flow-measuring system	27
Beckman cation glass No. 39137, or No. 39047 micro-electrode model	All substrates and enzymes assayed with a precision of about 2·5 per cent. Any sodium or potassium interferents first "removed" by adding Dowex 50 ion exchanger. Subsequently, the Beckman electrode was coated[2,5] with D- or L-amino-acid oxidase in a gel matrix. The addition of catalase to the enzyme solution also improved the electrode performance	9
Beckman cation No. 39137 model with sensor glass covered by nylon netting soaked in polyacrylamide gel - urease solution. An uncoated Beckman glass electrode used for reference purposes	Used over 19 d at 25 °C with no loss of urease activity. Urea in blood and urine assayed with precision of about 2–3 per cent. provided Na^+ and K^+ interferents first removed with Dowex 50-X2	3

parameters of the electrode system (see text).

S^{2-}, HS^- or H_2S for the sulphur system.

electrodes incorporate suitable built-in reference electrodes.

[*continued*

TABLE I

Substrate	Enzyme	Buffer(s)	Optimum pH	Products
Urea	Urease (E.C. 3.5.1.5)	Tris	7·0	NH_4^+ and CO_3^{2-}
Urea	Urease (E.C. 3.5.1.5)	Phosphate	7·2–7·4 for 10^{-1} to 10^{-3} M substrate	NH_4^+ and CO_3^{2-}
Urea	Urease (E.C. 3.5.1.5)	Phosphate	6·2 (not necessarily optimum value)	NH_4^+ and CO_3^{2-}
Tyrosine	Tyrosine decarboxylase (E.C. 4.1.1.25)	Sodium citrate	5·5	Tyramine and CO_2
Catechol	Polyphenoloxidase (E.C. 1.10.3.1)	Acetic acid - sodium acetate	5·9	Quinone and Cu^{2+} (?)
SO_4^{2-} seeded with *Desulphovibrio desulphuricans* colony (NCIB8457)	Sulphate reductase	None	pH rose from 7·5–9 during 2-week incubation period	HS^-
Acetylcholine ..	Acetyl-cholinesterase (E.C. 3.1.1.7)	Saline	7·2	Choline and acetic acid
Sodium ampicillin Sodium dicloxacillin Sodium nafcillin Potassium penicillin G Potassium penicillin V Potassium cyclicillin	Penicillin β-lactamase I (E.C. 3.5.2.6)	Substrate media adjusted by adding NaOH or HCl	6·4 as a compromise	Corresponding penicilloic acid
β-D-Glucose	Glucose oxidase (E.C. 1.1.3.4)	—	5·1	D-Gluconic acid and H_2O_2

as a sensor "in which an ion-selective electrode is covered with a coating that contains an enzyme which causes the reaction of an organic or inorganic substance (substrate) to produce a species to which the electrode responds" is generally accepted and understood.

In all work involving ion-selective electrodes the choice of a suitable reference electrode is of paramount importance, but in the enzyme field the slow leakage of an anti-enzyme factor, possibly mercury(I) chloride, in the potassium chloride from certain saturated calomel reference electrodes is another vital parameter requiring consideration. Thus, an aryl sulphate sulphohydrolase (E.C. 3.1.6.1) is inactivated[59] in a pH-stat fitted with a glass electrode and any of three different models of saturated calomel electrode. During short runs the leakage rates may be too low for any significant effect in the type of work being reviewed here, but it is useful to know that the addition of cysteine (at a concentration of 1 mM) to the sulphatase gives complete protection against the calomel leak factor. By isolating the enzyme reaction from the sensor and reference electrodes with a flowing system[31,50] the possibility of such enzyme inactivation is, of course, essentially eliminated.

Direct Use of Cation-selective Glass Electrodes

The urea - urease system represented by equation (1) has been extensively studied by determining either the ammonia or carbon dioxide terminal products. As already mentioned,

continued

Electrode sensor type	Comments/Results	Reference
Nonactin - silicone rubber ammonium ion sensor matrix with urease - polyacrylamide gel	Nonactin sensor material almost entirely eliminates K^+ interference. No need to employ pre-treatment with Dowex ion exchangers. Also improves response times	14
Orion 95–10 ammonia flow-through gas model	Suitable for automated assay of serum urea after quenching with 0·1–0·5 M sodium hydroxide. Good precision in the physiological range of 10^{-1} to 10^{-3} M for urea	30
Instrument Laboratory Inc. carbon dioxide gas electrode coated with urease suspension	—	16
Instrument Laboratory Inc. carbon dioxide gas electrode coated with the enzyme suspension	Superb selectivity; only high levels of acetic acid interfere. Relatively long response times could be a disadvantage. Linear response range to tyrosine is about one decade compared with two for urea	16
Orion 94–29A copper coupled with Orion 90–02 reference	$K_m \approx 1$ mM at 25 °C and pH 5·9	47
Orion 94–16A sulphide coupled with 90–02 reference electrode	Samples pumped from culture media under nitrogen and quenched with 1 M sodium hydroxide solution + anti-oxidant to establish product as the S^{2-} species in order to elicit maximum response from sulphide electrode	46
Corning 476200 acetylcholine liquid membrane model	$K_m = 0{\cdot}255 \pm 0{\cdot}036$ mM compared with 0·28 mM by an alternative method. Performance greatly improved[35] by trapping the sensor acetylcholine tetra-*p*-chlorophenyl-borate (with phthalate ester as plasticiser) in a PVC matrix	32
Beckman No. 39303 or 39301 glass models coated with enzyme - polyacrylamide gel, coupled with a Beckman permaprobe solid-state reference electrode No. 39406	New electrodes respond within 15–30 s and function for about 2 weeks, in which time over 100 assays made show linear response down to about 10^{-4} M, but reproducibility is poor. Slopes depend on gel layer thickness	44
Orion 94–53A iodide with Orion 90–01 reference electrode to detect iodide (see equation 15)	Many samples can be processed on a continuous basis with a flowing electrode system	31

stoicheiometric deamination of urea by urease leads to a steady-state potential, which may be recorded with a glass cation-selective electrode - reference electrode pair. Studies of the urea - urease system with the Beckman 39137 glass cation-selective electrode have shown copper and zinc to be non-competitive inhibitors and nitrate to be an inhibitor, whereas phosphate exerts an activation effect.[42]

In applications of this kind it is necessary to recall that the performance of many ion-selective electrodes is impaired by the presence of high levels of hydrogen ions. In enzymology the problem is accommodated by exercising buffer control on the enzyme - substrate system (*viz.*, pH 6·5–8·5, as summarised in Table I), but problems caused by hydrolysis must also be borne in mind:

$$NH_4^+ + H_2O \rightleftharpoons NH_3 + H_3O^+ \quad .. \quad .. \quad .. \quad .. \quad (2)$$

However, the optimum pH of the urease - urea reaction is about 7, at which pH, fortunately, 99·7 per cent. of the ammonia product exists as the NH_4^+ cation, the only form that elicits a potential response from a Beckman glass cation-selective electrode.

Cation-selective glass electrodes are also notoriously subject to interferences from alkali-metal ions and the much used Beckman 39137 model is no exception ($K_{NH_4K} \approx 2{\cdot}5$;

$K_{NH_4Na} \approx 0{\cdot}25$). Any potassium or sodium ion interference introduced with the enzyme (or substrate) can easily be detected because of the large positive potential reading that it causes prior to the addition of urea. In such an instance, Dowex 50 cation exchanger (cationic form) is stirred into the solution for 5 min and the filtrate made 10^{-2} M in urea.[9] The change in potential with time due to ammonium-ion production is recorded and the urease originally present found from a calibration graph of ΔE min^{-1} *versus* concentration of urease. The same technique has been used to assay glutaminase, asparaginase and L- and D-amino-acid oxidases after addition of the appropriate substrate.[9] Alternatively, the substrates of the enzymes can be assayed by adding the appropriate enzyme and the resultant ΔE min^{-1} profile related to concentration from the ΔE min^{-1} *versus* concentration of substrate calibration.

The activities of purified L-asparaginase from both *Escherichia coli* and *Serratia marcescens*, when determined with a Beckman glass cation-selective electrode, compared favourably with those obtained by a coupled assay procedure as well as by nesslerisation.[53]

The potential, E, developed at the glass surface is proportional to the ammonium-ion activity, $[NH_4^+]$

$$E = \text{Constant} + \frac{RT}{nF} \ln [NH_4^+] \quad .. \quad .. \quad .. \quad .. \quad (3)$$

Differentiation[9] of equation (3) with respect to time gives

$$\frac{dE}{dt} = \frac{RT}{2{\cdot}303nF} \times \frac{1}{[NH_4^+]} \times \frac{d[NH_4^+]}{dt} .. \quad .. \quad .. \quad .. \quad (4)$$

and forms the basis for determining the initial rate of the enzyme reaction. Thus, assuming $1/[NH_4^+]$ to be changing relatively more slowly with time than the time differential of $[NH_4^+]$ in the initial stages of the reaction, dE/dt is considered[9] to be directly proportional to $d[NH_4^+]/dt$. This assumption[9] simplifies the calculation of the progress of the enzymolyses in that the initial rates of reaction can be obtained from tangents drawn at the steady zero potential on the E *versus* t graphs at $t = 0$. A similar method has been adopted for amygdalin[28] and rhodanase[29] substrate studies.

Although less sensitive than some other methods, for example by two orders of magnitude for amino-acid oxidases compared with fluorescence techniques, enzyme electrode methods can be up to 30 times faster. Fast dynamic response times of ion-selective electrodes are an associated and essential feature that can lead to a final result being obtained within about 100 s of a test run.

Immobilised Enzyme Systems

All of these direct mixing methods are expensive in terms of substrates and enzymes consumed in each assay. The immobilisation of an enzyme in a water-insoluble matrix close to the surface of, for instance, a Beckman glass cation-selective electrode, is an important innovation, and one in which enzyme integrity is preserved and repeated usage facilitated. In this manner several enzymes[2,3,5,8,11,13–15,21,25,26,28,44] have been successfully immobilised in a thin polyacrylamide gel adjacent to the ion-selective sensing surface. Some loss of enzyme activity can occur[5] during the polymerisation of the acrylamide monomer and the riboflavin initiator employed can inhibit amino-acid oxidases.[5]

The functional principles of these electrodes are illustrated by a Beckman univalent cation electrode (Model 39137) coated with urease. This electrode, when brought into contact with urea solution,[8] produces NH_4^+ cations (equation 1), which diffuse through the gel layer to the glass sensor surface and there produce a potential in the usual manner. Such an electrode has been operational for 19 days.[8] Factors such as gel thickness, enzyme concentration and the method of gel - enzyme matrix preparation all affect the quality of the ion-selective electrode.[21] The average differences between the values for urea in blood and urine as determined by the urea electrode and a spectrophotometric technique were 2·8 and 2·3 per cent. *m/m*, respectively.[3]

The Beckman glass cation-selective electrode has also been successfully used for assaying L- and D-amino-acid oxidase (AAO) in polyacrylamide gel[2,5] on the basis of the reaction

$$RC(H)N^+H_3COO^- + H_2O + O_2 \xrightarrow{\text{L- or D-AAO}} RCOCOO^- + NH_4^+ + H_2O_2 \quad .. \quad .. \quad (5)$$

and subsequently the non-enzymic reaction

$$RCOCOO^- + H_2O_2 \rightarrow RCOO^- + CO_2 + H_2O \quad .. \quad .. \quad .. \quad (6)$$

Electrodes that are suitable for the rapid assay of D-phenylalanine, D-alanine, D-valine, D-methionine, D-leucine, D-isoleucine, D-norleucine and asparagine, based on the Beckman 39137 cation-selective electrode coated with the corresponding enzyme, are stable for up to one month.[2] Some of the L-isomers can be similarly assayed and the sensitivity and stability improved[5] by incubation with catalase in order to destroy hydrogen peroxide (reaction 6), thereby releasing additional oxygen for reaction 5.

The immobilisation of the enzyme, or substrate,[10] in gel matrices is a major advance in sensor design and operation. However, the selectivity performance of, for example, the Beckman 39137 glass cation-selective electrode remains essentially unaltered after coating with urease - polyacrylamide gel.[8] Thus, sodium in serum ($\approx$153 mM) and potassium in urine ($\approx$38 mM) still present the same interference profiles in the urea assay whether an uncoated or coated Beckman 39137 cation-selective electrode is used, and pre-treatment of samples with Dowex 50W-X2 is still necessary.[13] This problem has been overcome by using alternative ion-selective electrodes with better univalent cation selectivity.[13–15,27,30] Thus, a silicone rubber matrix containing nonactin ($K_{NH_4K} \approx 1{\cdot}5 \times 10^{-3}$ and $K_{NH_4Na} \approx 1{\cdot}2 \times 10^{-3}$) and coated with urease - polyacrylamide gel provides[14] a long-life ammonium-ion sensor showing about a 2-mV drift over a period of 5 weeks and time responses of about 60 s. The assay of urea in serum by use of this electrode is claimed to be in good agreement with that by a standard spectrophotometric technique.[15]

Booker and Haslam[48] consider many methods for L-arginine ureohydrolase (E.C. 3.5.3.1) assay to be too cumbersome; for example, they involve the formation and isolation of a urea derivative or a considerable time for colour development, while others lack sensitivity, are too expensive for routine purposes or require elaborate, expensive equipment and specialist personnel. L-Arginine ureohydrolase, which converts L-arginine into L-ornithine and urea, can be quickly assayed in a second-stage reaction whereby the urea is converted into the ammonium ion by a urease-coated ammonium ion selective electrode. No blanks are necessary and the method conserves enzyme, although there is some loss of sensitivity. The K_m value of 1·26 mM compares favourably with 1·5 mM, the value obtained by use of a spectrophotometric method.[48] The same dual reaction technique has been used in order to measure arginine substrate rather than the enzyme.[54]

Use of Gas Sensors

The gas, or more correctly, diffusion electrode[60] represents the latest commercial effort in utilising ion-selective electrodes. This type of electrode, which senses a gas indirectly, involves the use of a gas-permeable, but ion-impermeable, membrane to separate the analytical solution medium from the internal solution. The gas molecules of, for example, sulphur dioxide, carbon dioxide or ammonia, diffuse through the membrane to effect a change in the activity level of an ion, H_3O^+ in this instance, contained in the internal solution, which is situated between the diffusion membrane and the inner glass pH-sensitive membrane

$$CO_2 + 2H_2O \rightleftharpoons H_3O^+ + HCO_3^- \quad .. \quad .. \quad .. \quad (7)$$

The activity change is then detected by the internal ion-selective electrode, in this instance the classical glass pH electrode. These electrodes have superb selectivity[60] because nearly all other important common ions, including NH_4^+, HCO_3^-, and CO_3^{2-}, cannot diffuse across the outer membrane barrier. Only very high concentrations of acetic acid interfere with the Instrument Laboratory Inc. carbon dioxide gas electrode.[13] Recently, the ammonia and carbon dioxide gas electrodes have been applied in enzymology, both in unmodified[27,30] and coated[13] gel formats.

The substrate molecules under assay have to diffuse to, and enter, the enzyme - gel surface, undergo enzymolysis and then the product, for example ammonium ions, diffuses yet further

to the ion-selective surface. This same product must also be capable of fast diffusion from the sensor surface to allow recovery before subsequent sampling. With gel - enzyme coated gas electrodes the product, in the form of ammonia in this example, has to traverse an additional gas-permeable barrier before eliciting any potential response.

The optimum pH level ($\approx$7) for many deaminases fixes the ammonia product as the ammonium cation (equation 2), a species totally foreign to the ammonia gas electrode. Raising the pH to about 12 shifts the equilibrium most favourably to the ammonia gas species but, unfortunately, inactivates most enzymes. Llenado and Rechnitz[30] solved this equilibrium problem by first completing the serum urea - urease incubations at the optimum pH of 7·4, in a static stage lasting for 20 min at 37 °C, and then bleeding off samples on a continuous basis, quenching the pH to about 12 by addition of 0·5 M sodium hydroxide solution and measuring the ammonia gas in a flow-through Orion 95–10 combination ammonia gas electrode. The potentials displayed on a recorder are again proportional to the concentration of ammonia and also to one half of the concentration of urea. The fact that some ion-selective electrodes and their associated enzymes function best at widely different pH levels is thus readily solved in a two-stage system.

This continuous, automatic, incubation - quenching technique for the rapid, low-cost assay of urea will be of considerable clinical importance for blood and urine analysis.[30] In principle, any metabolic process that terminates in the production of ammonia or carbon dioxide can be similarly quantified. Already some progress has been made with creatinine, another nitrogenous terminal metabolite, using an ammonia gas electrode,[27] while urea and tyrosine can be determined[16] by using the appropriate decarboxylase and a carbon dioxide gas electrode coated, in this instance, with an enzyme suspension. Decarboxylases are more specific for some amino-acids than their deaminases or oxidases for example, and Guilbault and Shu[16] consider that in such instances the amino-acids could best be assayed with a carbon dioxide gas electrode.

The air-gap electrode is based on the same principle as the other gas electrode except that the gas permeable membrane is replaced by an air gap, which separates the electrolyte layer (adsorbed as a very thin film on the surface of the glass pH electrode) from the sample solution, the entire system being contained in a gas-tight measuring chamber.[45] The chief advantage of this design is that the ion-selective electrode surface does not make contact with fouling proteins that are normally present in many clinical samples. Selectivity parameters compare favourably with the ammonia gas membrane electrode, while response times are even better. The rapid determination of urea in blood over the range 10^{-2}–10^{-4} M has been reported.[45] The values for blood urea using the air-gap electrode and the AutoAnalyzer differed by $\pm$2·2 per cent.[51] The excellent stability of the electrode facilitated the assay of over 460 samples during 4 weeks, the cost per assay being 2 per cent. of that of the AutoAnalyzer method, which really emphasises the economic benefits of ion-selective electrodes in this analytical context. However, the response time per sample did rise from about 3 min initially to a final time of about 5–6 min.[51]

Miscellaneous Monitoring

Cyanide as Substrate and Product

The rhodanase-initiated reaction is interesting in that both substrates and products are simple ions.

$$S_2O_3^{2-} + CN^- \xrightarrow{\text{Rhodanase}} SCN^- + SO_3^{2-} \quad .. \quad .. \quad .. \quad (8)$$

Rhodanase activity can be monitored as cyanide consumption with an Orion 94–06A cyanide-selective electrode, but the corresponding thiocyanate production cannot readily be followed with the Orion 94–58 thiocyanate electrode owing to uncertain interference from cyanide.[29]

Amygdalin, which is hydrolysed according to

$$C_6H_5CH(OC_{12}H_{21}O_{10})CN + 2H_2O \xrightarrow{\beta\text{-Glucosidase}} 2C_6H_{12}O_6 + HCN + C_6H_5CHO \quad .. \quad (9)$$

can be rapidly and conveniently assayed using an Orion 94–06A solid-state cyanide-selective

electrode that is coated with β-glucosidase - acrylamide gel.[25] As little as 0·01 mg of the enzyme per 100 μl can be monitored.[28] Because this electrode only senses free cyanide and not hydrocyanic acid, the pH of the reaction must be maintained at greater than 12.

$$CN^- + H_3O^+ \rightleftharpoons HCN + H_2O \quad .. \quad .. \quad .. \quad .. \quad (10)$$

Llenado and Rechnitz[26] have attached a Plexiglass extension to the cyanide-selective electrode surface to cover the gel - enzyme matrix, which, on inversion, becomes a sample container into which dips the upright Orion 90–01 reference electrode. The miniaturisation of sample that is possible with this method (0·2 ml) is reminiscent of the Durst - Taylor technique that was developed[61] for 25–50 μl of fluoride.

Sulphide as Substrate and Product

An Orion 94–16 sulphide-selective electrode, coupled with an Orion double-junction 90–02 reference electrode, has been successfully employed[55] to study the properties of partially purified *O*-acetylserine sulphydralase, from the germinating rape seed, which is involved in the following biosynthesis:

$$O\text{-Acetyl-L-serine} + S^{2-} \rightarrow \text{L-cysteine} + \text{acetate} \quad .. \quad .. \quad .. \quad (11)$$

Ngo and Shargool[55] proposed the use of the same set of electrodes to study similar syntheses, for example, homocysteine from *O*-acetyl-L-homoserine and sulphide with *O*-acetyl-L-homoserine sulphydralase.

In turn, a sulphide-selective model has been used to follow sulphide production from L-cysteine and cyanide substrates that are catalysed by β-cyanoalanine synthase.[18]

Monitoring of Bacterial Growth

The decrease in nitrate levels that occurs during the growth of *Escherichia coli* (strain B) has been followed with an Orion 92–07 nitrate-selective electrode, while an ammonium ion selective electrode (based on a silicone rubber - nonactin sensor membrane) proved suitable for assessing ammonium ion production in viable *E. coli* (strain Bn) cultures.[24] The production of sulphide from sulphate media inoculated with *Desulphovibrio desulphuricans* bacteria can be monitored with an Orion 94–32A sulphide-selective electrode.[46]

Assay of Acetylcholinesterase and Cholinesterase

The assay of acetylcholinesterase, which converts acetylcholine (ACh) into choline (Ch), has been followed with a Corning 476200 liquid membrane acetylcholine bromide selective electrode,[33,34] for which $K_{AChCh} \approx 0{\cdot}06$. An improved version was constructed from a poly(vinyl chloride) - acetylcholine tetra-*p*-chlorophenylborate membrane including phthalate plasticiser.[35]

The selectivity towards choline and acetyl-, propionyl-, acetyl-β-methyl-, butyryl-, valeryl- and benzoylcholine has been exploited for identifying the two clinically important compounds, acetylcholinesterase and cholinesterase.[36] This use is possible because both acetylcholinesterase and cholinesterase will hydrolyse acetylcholine, but only cholinesterase hydrolyses butyrylcholine while only acetylcholinesterase hydrolyses acetyl-β-methylcholine.[32,36] These different substrates thus permit identification, but it is essential to re-equilibrate the acetylcholine electrode when changing the substrate, or better, to use a separate equilibrated electrode for each substrate.[36]

The assay of serum cholinesterase has also been based on the measurement with a Corning glass pH electrode of the acetic acid produced from acetylcholine with a precision of 1·3–3·5 per cent.[56] The hydrolysis of acetylthiocholine

$$(CH_3)_3N^+CH_2CH_2SCOCH_3 \xrightarrow{\text{Cholinesterase}} (CH_3)_3N^+CH_2CH_2SH + CH_3COOH \quad .. \quad (12)$$

can also be used in a flowing system (Fig. 1) for the same purpose, as a sulphide-selective electrode responds to the thiocholine product.[52] The thermostatically controlled delay coil provides a 1-min reaction time lag before any change in thiocholine is monitored at the

sensor sulphide electrode (S_I), which is coupled to a second sulphide electrode as reference (S_r). Except for a relatively long reaction time (3–4 min) the method compares favourably with other spectrophotometric and fluorescence procedures.[52]

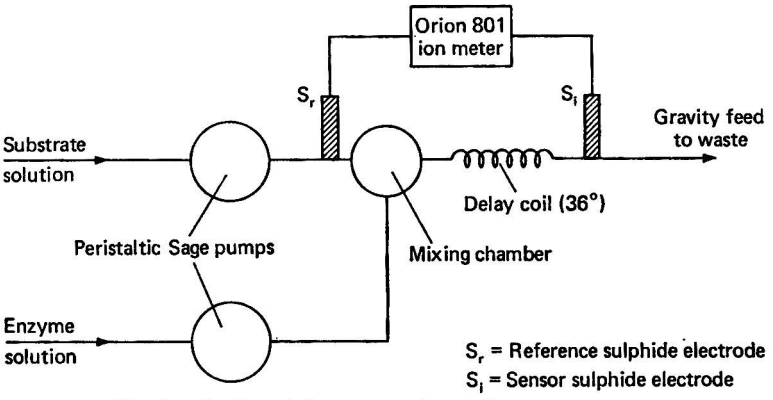

Fig. 1. Outline of flow system for cholinesterase assay.

Organophosphate pesticides are potent cholinesterase inhibitors. The extent of anti-cholinesterase activity exerted by organophosphate pesticides is a measure of their residual concentration. Use of the Corning acetylcholine electrode permits the assay of Paraoxan and Tetram in the concentration ranges 100–1000 ng ml^{-1} (standard deviation 12 ng ml^{-1}) and 50–300 ng ml^{-1} (standard deviation 19·2 ng ml^{-1}), respectively. For potent inhibitors the sensitivity compares favourably with that achieved by using gas - liquid chromatographic techniques.[37]

The same principle has been applied to assaying α-chymotrypsin, which is inactivated by diphenylcarbamyl fluoride (DPCF)

$$\text{DPCF} + \alpha\text{-chymotrypsin} \rightarrow \text{DPC-chymotrypsin} + F^- \quad .. \quad (13)$$

As little as 0·03 μmol of enzyme can be titrated[39] with an Orion 94–09A fluoride-selective electrode (precision about ±3 per cent).

Indirect Monitoring of Products

The analyses described so far depend on the availability of ion-selective electrodes that are compatible with the substrate and/or the product. Many enzyme systems are of the type

$$\beta\text{-D-Glucose} + H_2O + O_2 \xrightarrow{\text{Glucose oxidase}} \text{D-gluconic acid} + H_2O_2 \quad .. \quad (14)$$

where neither substrate nor product can be directly detected by the present-day ion-selective electrodes. Nevertheless the consumption of iodide by the hydrogen peroxide product at pH 5·1, according to the equation

$$H_2O_2 + 2H_3O^+ + 2I^- \xrightarrow{\text{Mo(VI) catalyst}} I_2 + 4H_2O \quad .. \quad .. \quad .. \quad (15)$$

which can be monitored with the Orion 94–53A iodide-selective electrode and Orion 90–01 reference electrode,[31] provides an excellent indirect assay of that product. Up to 40 glucose samples per hour can be processed by use of this method.

The same reaction has also been studied[50] in a flow system coupled with a sensor - reference pair of iodide-selective electrodes and substituting the more efficient peroxidase catalyst for molybdenum(VI). Maltose and cellibiose do interfere but samples containing the more serious interferents ascorbic acid, tyrosine or uric acid require pre-treatment.[50]

Conclusion

Developments of ion-selective electrodes and the application of automated flow systems will facilitate rapid, accurate, low-cost analyses of enzymes and substrates to an increasing extent and will make significant contributions to the efficiency of biochemical and clinical laboratories.

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Determination of Ammonia-, Nitrate- and Organic Nitrogen in Water and Waste Water with an Ammonia Gas-sensing Electrode

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The use of the Orion ammonium ion selective electrode for the determination of ammonia-, nitrate- and organic nitrogen in a number of waters and effluents has been investigated. Adjustment of the pH to attain the optimum conditions for the determination was achieved by use of 2 M sodium hydroxide solution that was 1 M in ethylenediaminetetraacetic acid (EDTA) disodium salt. Evidence is produced which shows that the alkali present in excess of that required to give a pH value of 12 does not affect the calibration graphs or the recovery of ammonia from the water samples investigated.

Standard nitrate solutions and samples were reduced and measured as ammonia-nitrogen. Reagents used to bring about this reduction are discussed. Recoveries at a level of 1 mg l^{-1} of nitrate-nitrogen averaged 95 per cent., using the nascent hydrogen produced by Devarda's alloy in acidic conditions as the reductant. A contact time of 24 h was required for this percentage reduction. A coefficient of variation of $\pm 6{\cdot}8$ per cent. was achieved. Under these reducing conditions, the percentage recovery decreased as the concentration of nitrate-nitrogen increased but acceptable recoveries were obtained for concentrations of nitrate-nitrogen up to 5 mg l^{-1}. However, when ammonia-nitrogen was present at levels significantly higher than those of nitrate-nitrogen, the reliability of the results decreased. The method is convenient for the determination of ammonia-, nitrate- and organic nitrogen in the same sample.

Interference by metals that form insoluble hydroxides under the alkaline conditions of measurement of ammonia-nitrogen was studied. The high level of magnesium present in sea water is shown to interfere with electrode response, probably because of hydroxide formation and consequent precipitation. This potential interference is eliminated by the use of 1 M EDTA to complex magnesium, and other metals, present in sea water and estuarine waters.

It is further shown that organic nitrogen can be determined with the ammonium ion selective electrode. The method involves removal of free ammonia followed by acid digestion and final release and measurement of the ammonia produced in alkaline solution. Recoveries are shown to be acceptable at the relatively low levels measured and show good agreement with published figures for similar environments.

The determination of the various nitrogen-containing constituents of water and waste water is considered to be an important test in the establishment of water quality. Three of the more important of these constituents are considered to be ammonia-, organic and nitrate-nitrogen. The presence of these nitrogen compounds in greater than normal amounts may be an indication of a polluted source.

Analytical methods recommended for the determination of these compounds at present involve time-consuming chemical separations, distillations, etc., with subsequent spectrophotometric or titrimetric measurements.[1]

It has been shown that a recently introduced ion-selective electrode for ammonia can be used for the direct determination of ammonia in water and in waste water.[2,3] It has also been reported that the electrode can be used for the determination of organic nitrogen (after Kjeldahl digestion of the sample[4,5]) and nitrate-nitrogen (after reduction to ammonia) in aqueous solution[6] although experimental details are brief and incomplete.

An investigation into the possible application of the electrode to the determination of ammonia-, nitrate- and organic nitrogen in water and waste water is described in this paper.

Experimental

Apparatus

A Radiometer, Model PHM 26C, meter equipped with a millivolt meter read-out together with an Orion ammonium ion selective electrode, Model 95–10, were used for all the determinations. The pH meter was in the millivolt expanded mode. When not in use the electrode was stored in 0·1 M ammonium chloride solution.

A magnetic stirrer with a PTFE-coated stirrer bar was used to mix the solutions. Insulation of the sample from heat produced by the stirrer motor is necessary and can be achieved by means of a piece of foam rubber placed between the stirrer and the beaker containing the sample and stirrer bar.

Method for the Determination of Ammonia- and Nitrate-nitrogen

Reagents

Use analytical-reagent grade chemicals unless otherwise stated.

Ammonia-free water. Freshly distilled, de-ionised water passed through a mixed ion-exchange column.

Stock standard ammonia solution, 1000 mg l^{-1} as nitrogen. Dissolve 3·821 g of ammonium chloride (dried at about 100 °C) in ammonia-free water and make up to the mark in a 1-l calibrated flask. Prepare working standard solutions of 100, 50, 25, 10 and 5 mg l^{-1} of ammonia-nitrogen by diluting suitable aliquots of the stock standard solution. For standards of concentrations below 5 mg l^{-1} of ammonia-nitrogen, make up daily as required.

pH adjuster. Dissolve 40 g of sodium hydroxide and 18·6 g of EDTA disodium salt in de-ionised water and dilute to 500 ml. For saline waters increase the concentration of EDTA to 186 g per 500 ml.

Standard nitrate solution, 100 mg l^{-1}. Dissolve 0·7218 g of potassium nitrate in ammonia-free de-ionised water and make up to 1 l in a calibrated flask. Store in the dark and make up fresh monthly.

Devarda's alloy. Laboratory-reagent grade powder.

Sodium fluoride. AnalaR.

Concentrated hydrochloric acid (sp. gr. 1·18). Laboratory-reagent grade.

Procedure

Calibration graph

Obtain a calibration graph by measuring the electrode potential of solutions in the concentration range 0·1–100 mg l^{-1} of ammonia-nitrogen using the following procedure.

Transfer 50 $\pm$ 1 ml of the lowest concentration standard into a 100-ml beaker, immerse the electrode in the standard solution and stir by means of the magnetic stirrer. Add 5·0 ml of pH adjuster before measurement and record the potential after a standard stabilisation time of 8 min. For this initial standard solution only, repeat the process until a reproducible reading is obtained.

After reading the potentials for all standards in the selected range construct a calibration graph of millivolt readings *versus* the logarithm of concentration. The graph is linear over the range 100–0·2 mg l^{-1} but curves increasingly below the latter value. Once prepared for a particular membrane the calibration graph will require only occasional checking for selected standard solutions. Care must be taken to ensure that no air bubbles are trapped on the under-surface of the membrane. It has been found that for concentrations above 1 mg l^{-1} a 4-min stabilisation period is adequate.

Determination of ammonia-nitrogen

The procedure for this determination is identical with that for the determination of ammonia-nitrogen in the standards. If consecutive electrode readings involve a difference in potential in excess of +60 mV, then repeated measurements on the second sample should be made. Read the concentration of ammonia-nitrogen in the sample directly from the graph. As a general rule the taking of measurements on samples in decreasing order of concentration should be avoided.

Reduction of nitrate

Transfer 100 ± 1 ml of standard or sample solution to a 100-ml stoppered flask. To this add 1·0 ml of concentrated hydrochloric acid and about 0·5 g of sodium fluoride. Mix the contents of the flask thoroughly. Add about 0·1 g of Devarda's alloy, stopper the flask and mix the contents again. Stand the flask in a water-bath at 100 °C and allow evolution of hydrogen to proceed. Remove the flask from the bath after 1 h and allow it to stand overnight. Then, either filter the sample using glass-fibre filter-paper or, if the carry-over of metal can be avoided, decant off the liquid.

Any undissolved metal in the alkaline solution in which the electrode is placed will lead to the production of hydrogen gas, which may interfere with the operation of the electrode. Measure the reduced nitrate-nitrogen as ammonia-nitrogen, using the method described above.

If necessary, nitrite can be selectively removed by addition of sulphamic acid or, alternatively, hydrazoic acid.

Results and Discussion

pH adjustment

Initially, a pH adjusting solution, 1 M in sodium hydroxide, was used in order to obtain pH values in excess of 12 for the determination of ammonia-nitrogen. Most samples of water and waste water have a pH of approximately 7, so few problems in adjusting the pH to 12 are experienced. One aim of this study was to determine nitrate-nitrogen, recovered as ammonia-nitrogen after reduction. It was found that reduction was most complete when using Devarda's alloy in acidic conditions. Addition of 5 ml of the 1 M sodium hydroxide solution to 50 ml of reduced nitrate-nitrogen standard was insufficient to give the conditions necessary for the measurement of the ammonia-nitrogen produced.

It is assumed that this was partly due to the formation of aluminium and zinc hydroxides, whose interference with the permeability of the electrode membrane is suspected.[3] This possible interference is eliminated by the addition of excess of sodium hydroxide in order to form soluble sodium zincate and sodium aluminate. The pH adjuster was 0·1 M with respect to EDTA so that any other metals present (*e.g.*, magnesium) that were likely to form insoluble hydroxides under the conditions of measurement of ammonia-nitrogen would be preferentially complexed.

It was found (Table I) that the calibration graphs prepared with either 1 or 2 M sodium hydroxide solution were superimposable in the region 0·1–50 mg l^{-1}. (In each instance 5 ml of pH adjuster were added to 50 ml of standard solution.) As no disadvantages were evident, 2 M sodium hydroxide solution was adopted as pH adjuster for the remaining work.

To obtain further evidence for the lack of any interference, samples were spiked with known concentrations of ammonia-nitrogen and measured using 2 M alkali as the pH adjuster (Table II).

The over-all coefficient of variation for the determinations in Table II was ±4·5 per cent. Determinations of ammonia-nitrogen using the ion-selective electrode have been carried out routinely for some time and as this work has already been well documented[2,3] no further investigation into the recovery of ammonia-nitrogen was considered necessary.

TABLE I

EFFECT OF 2 M SODIUM HYDROXIDE ON ELECTRODE RESPONSE

Concentration of NH_3-N/mg l^{-1}	Electrode response: In 1 M NaOH + 0·1 M EDTA	Electrode response: In 2 M NaOH + 0·1 M EDTA
50	76·0	75·0
10	35·0	34·0
5	16·0	17·0
2·5	−2·0	−3·5
1·0	−23·0	−27·0
0·1	−79·0	−79·0

Reduction of nitrate

In order to obtain the best possible reducing conditions, a number of combinations of metals with acid and alkali were studied. Initially 0·1 g of aluminium dust, 0·5 g of sodium fluoride

TABLE II

RECOVERY OF ADDED NH_3-N

Sample	Original concentration of NH_3-N in sample/mg l^{-1}	Spiked concentration of NH_3-N/mg l^{-1}	
		Added	Recovered*
Wool scour rinse water ..	5·9	10	10·4
		4	3·8
River water	0·01	10	10·5
		4	4·1
Primary sewage effluent ..	32	40	42·0

* Recovery figures are means of three determinations.

and 1 ml of concentrated hydrochloric acid were used for each 100 ml of sample or standard solution,[6] the contact time being 5–7 min.

Low recoveries were obtained by use of this method and other combinations were then tried. From the results given in Table III the following conclusions can be made:

1. that of the metals used, Devarda's alloy gives the best reduction if other conditions are constant;
2. the contact time[6] of 5–7 min is insufficient to effect good reduction, an overnight contact period producing the best recoveries;
3. the use of an alkali, such as 25 per cent. potassium hydroxide solution, in preference to concentrated hydrochloric acid is not recommended, owing to the loss of ammonia-nitrogen over the contact period.

TABLE III

RECOVERY OF NO_3-N BY MEANS OF VARIOUS REDUCING MEDIA

Volume of standard or sample solution, 100 ml.

Reductant	Contact time/h	Added/ mg l^{-1}	Recovery, per cent.
0·1 g of Zn + 0·5 g of NaF + 1 ml of conc. HCl	2	0·4	37·5
		2·0	50
0·1 g of Al + 0·5 g of NaF + 1 ml of conc. HCl	2	0·5	40·0
		2·0	45
0·1 g of Devarda's alloy + 0·5 g of NaF + 1 ml of conc. HCl	24	0·5	80·0
0·1 g of Zn + 0·5 g of NaF + 1 ml of conc. HCl	24	0·5	72·0
0·1 g of Al + 0·5 g of NaF + 1 ml of 25 per cent. KOH ..	24	0·5	46·0
0·1 g of Devarda's alloy + 0·5 g of NaF + 1 ml of 25 per cent. KOH	24	0·5	72·0

From these conclusions a standard procedure was derived. This procedure involved the use of 0·1 g of Devarda's alloy plus 0·5 g of sodium fluoride plus 1 ml of concentrated hydrochloric acid per 100 ml of standard or sample solution, with a 24-h contact period. Table IV summarises the recovery of nitrate-nitrogen at a level of 1 mg l^{-1} when the reduction conditions outlined were used. A comparison of the more finely divided BDH Devarda's alloy with a synthetic alloy made up from zinc and aluminium powder and copper filings (5 + 45 + 50) is made.

TABLE IV

RECOVERY OF 1 mg l^{-1} OF NO_3-N

Volume of standard or sample solution, 100 ml.

Reductant	Number of samples	Mean recovery, per cent.	Standard deviation, per cent.	Coefficient of variation, per cent.
0·5 g of NaF + 1 ml of conc. HCl + 0·1 g of BDH Devarda's alloy ..	13	95	±7·9	±8·3
+ 0·1 g of synthetic Devarda's alloy ..	17	86	±5·8	±6·8

From Table IV it can be seen that the BDH Devarda's alloy gave the best reduction of the nitrate-nitrogen standard solutions. It is considered that this was due to two factors: the smaller mesh size and consequent greater surface area; and the greater homogeneity of the commercial alloy.

The inclusion of 0·5 g of sodium fluoride[6] gave higher percentage recoveries of nitrate-nitrogen in six out of eight determinations.

Limitations of this method of reduction were noted, the percentage reduction decreasing as the concentration of the nitrate-nitrogen standard solution increased. From the results given in Table V it can be seen that the best recoveries of nitrate-nitrogen occur at about the 1 mg l^{-1} level and that recoveries of 75–80 per cent. can be achieved for levels up to 5 mg l^{-1}. It can be concluded that using this method for the determination of nitrate-nitrogen in waters and effluents, recoveries of 75–80 per cent. will be obtained for levels up to 5 mg l^{-1}. At these trace levels the recovery mentioned is considered to be acceptable.

TABLE V

RECOVERY OF NO_3-N USING BDH DEVARDA'S ALLOY AS REDUCTANT

Added/mg l^{-1}	Recovery, per cent.
1	95
2	77
4	79
5	76
10	68
50	64

In waters having low levels of ammonia-nitrogen (0·05 mg l^{-1}) and levels of nitrate-nitrogen up to 5 mg l^{-1}, a proportionately large positive millivolt response is produced in the measurement of nitrate-nitrogen and reliable results are obtained. However, for those effluents in which the levels of ammonia-nitrogen are higher, and of nitrate-nitrogen are relatively low, the millivolt response to the nitrate-nitrogen is small. When this response is within the percentage coefficient of variation of the ammonia-nitrogen value of the sample, little confidence can be held in the measured concentration of nitrate-nitrogen.

In spite of this shortcoming, the method has an important application in stream, river, estuarine and marine surveys where the values for nitrate-nitrogen are collated with those for ammonia- and organic nitrogen to be used as supporting evidence, together with bacteriological counts, for the presence of sewage contamination. Table VI shows the recovery of nitrogen compounds containing ammonia-, nitrite- and nitrate-nitrogen present in a sample spiked with 1 mg l^{-1} of nitrate-nitrogen. Column 1 gives the level of ammonia-

TABLE VI

RECOVERY OF NITROGEN COMPOUNDS PRESENT IN A SAMPLE WITH A 1 mg l^{-1} STANDARD ADDITION OF NO_3-N

	NH_3-N content			
Sample	I. Before reduction/mg l^{-1}	II. After reduction/mg l^{-1}	III. After spiking and reduction/mg l^{-1}	IV. Recovery of 1 mg l^{-1} of NO_3-N, per cent.
Waste-receiving ditch ..	0·8	1·0	1·51	71
Stream	0·13	1·13	1·55	65
River I	0·05	0·78	1·33	71
River II	0·31	0·71	1·26	70
River III	0·03	0·79	1·39	76

nitrogen in the sample prior to reduction. Column II gives the level of ammonia-nitrogen after reduction and this value includes the nitrite- and nitrate-nitrogen as well as the ammonia-nitrogen content of the sample. Values in column III include the nitrate-, nitrite- and ammonia-nitrogen content of the sample as well as the 1 mg l^{-1} standard addition of nitrate-nitrogen, the standard addition having been made prior to reduction. Column IV shows the apparent percentage recovery of the 1 mg l^{-1} standard addition. For column III values, 20 ml of a 5 mg l^{-1} standard solution of nitrate-nitrogen were added to 80 ml of sample

solution. Corrections were made for this change in sample volume when deriving the percentage recovery of added nitrate-nitrogen standard. Because of the volume change, only 80 per cent. of the values in column II contributes to the column III values, *i.e.*, together with the standard nitrate-nitrogen addition. By subtracting the corrected column II values from those in column III, the true percentage recoveries of added nitrate-nitrogen are obtained. From the 24 samples analysed, an over-all recovery of 70·6 per cent. was achieved, with a standard deviation of ±5·99 per cent. and a coefficient of variation of ±8·48 per cent. Nitrite, which may have been present, was not removed as levels of nitrite in this catchment area are typically very low. If, however, nitrite is of concern it can be removed by the use of sulphamic acid[7] or hydrazoic acid.[8] To obtain an actual value for the concentration of nitrate-nitrogen in an individual sample, a correction factor, derived from nitrate recovery trials, can be applied. This factor decreases as the concentration of nitrate-nitrogen increases. Although probably not the most accurate method for the determination of nitrate-nitrogen, it is convenient when ammonia-, organic and nitrate-nitrogen are to be determined in the same sample.

To obtain better reduction of nitrate to ammonia when present at levels in excess of about 5 mg l^{-1} the use of more highly reactive alloys is suggested. For example, it is reported that nitrates can be reduced quantitatively to ammonia in neutral solution by means of Arndt's alloy (magnesium, 60 per cent.; copper, 40 per cent.).[9]

It is intended to investigate the use of alloys such as Arndt's alloy with the aim of effecting more complete reduction.

Method for the Determination of Organic Nitrogen

Ammonia-nitrogen is first removed by boiling the sample solution with phosphate buffer solution. The sample is then digested in a mixture containing sulphuric acid and potassium sulphate with mercury(II) sulphate as catalyst in order to convert the amino-nitrogen of the organic materials into ammonium hydrogen sulphate. The sample is then made alkaline and the ammonia released measured with the ion-selective electrode.

Reagents

Use analytical-reagent grade chemicals unless otherwise stated.

Phosphate buffer solution, pH 7·4. Dissolve 14·3 g of potassium dihydrogen orthophosphate in 68·8 g of dipotassium hydrogen orthophosphate in ammonia-free distilled water and dilute to 1 l.

Digestion reagent. Dissolve 134 g of potassium sulphate in about 500 ml of ammonia-free distilled water plus 200 ml of concentrated sulphuric acid. Add, with stirring, 2 g of mercury(II) sulphate and dilute to 1 l. Keep the solution at temperatures above 14 °C in order to prevent crystallisation.

Alkaline reagent. Dissolve 400 g of sodium hydroxide and 300 g of sodium iodide in ammonia-free distilled water and dilute to 1 l.

Stock standard ammonia solution, 1000 mg l^{-1} as nitrogen. Dissolve 3·821 g of ammonium chloride (dried at 100 °C) in ammonia-free distilled water and dilute to 1 l.

Procedure

Transfer a 500-ml aliquot of the sample to a clean, dry 800-ml Kjeldahl distillation flask, together with 25 ml of phosphate buffer solution. Add a few boiling chips and boil off about 300 ml of water. Cool, and carefully add 100 ml of digestion reagent, mix and boil the solution in a fume cupboard until it clears (*i.e.*, becomes colourless or a pale straw colour), then digest for a further 30 min. Allow the flask and contents to cool, transfer to a 500-ml calibrated flask and make up to the mark with ammonia-free distilled water. Transfer a 20-ml aliquot to a 150-ml beaker, add 78 ml of distilled water and 5 ml of alkaline reagent. Use a magnetic stirrer and stirring bar to effect mixing, place the ion-selective electrode in the solution and record the reading when stable.

Prepare a blank solution containing exactly the same amounts of reagent as the sample and take it through the same procedure as the sample.

Calculate the concentration of organic nitrogen in the sample from the calibration graph.

Calibration graph

Transfer 20-ml aliquots of the reagent blank solution to a number of 150-ml beakers together with 78 ml of water and aliquots of the ammonia stock solution to give final concentrations of 0·2, 0·5, 1·0 and 2·0 mg l^{-1} of ammonia-nitrogen. Add sufficient alkaline reagent to make the solution alkaline and read the electrode potential of the solution when the reading is stable. Construct a calibration graph of the logarithm of the concentration *versus* the millivolt reading. This graph in part closely follows the calibration graph used for the determination of ammonia. Interferences would therefore appear to be minimal, provided that care is taken to exclude all possible sources of contamination by ammonia and ammonium ions.

Results and Discussion

The main types of organic nitrogen in waste water, and in waters receiving such waste, are proteins and their metabolic decomposition products, including urea. It is not considered likely that products other than ammonia-nitrogen and oxidised nitrogen will be lost by the initial distillation with phosphate buffer solution. Urea and other forms of organically bound nitrogen are decomposed by boiling with acids and alkalis. Any rise in the content of organic nitrogen in waters is often related to sewage or industrial waste pollution.

Table VII shows results obtained for effluents from sewage works and for adjacent areas of estuarine and river waters. The trend is towards higher levels of organic nitrogen in what are considered to be the more polluted areas.

TABLE VII

ORGANIC NITROGEN AT VARIOUS SAMPLING LOCATIONS

Sampling location	Organic nitrogen content/mg l^{-1}	Recovery after addition of 2 mg l^{-1} of ammonia-nitrogen/mg l^{-1}
Sewage works effluent	3·4	5·4
Sewage works outfall to estuary	0·8	2·6
Point in river mouth adjacent to outfall	0·7	2·8
Point ½ mile downstream of outfall and in estuary	0·3	2·4
Point in river about 3 miles from outfall and across estuary	0·1	1·9

A 20-ml aliquot from all digested samples was spiked with 2 mg l^{-1} of ammonia-nitrogen and the determination carried out. Recoveries are shown in Table VII. In all instances satisfactory recoveries were obtained showing the accuracy of the method to be acceptable at the levels measured. These figures for the organic nitrogen content of sewage effluent and of adjacent estuarine and river waters compare well with published data for a similar set of environments. Quoted figures for organic nitrogen levels are 4 mg l^{-1} for effluent from sewage works, 2 mg l^{-1} for an adjacent polluted river, 0·25 mg l^{-1} for an adjacent unpolluted river and 0·4 mg l^{-1} for sea water in a nearby estuary.[10]

Interferences

Estuarine and ocean outfalls are frequently used by municipalities for the discharge of domestic wastes and effluents. A frequently employed means of gauging the extent of pollution from such outfalls in the receiving water is to enumerate bacteria of sanitary significance, *i.e.*, faecal coliforms. However, rapid die-off of such organisms in saline water is probable and their use as indicators of pollution is limited.

Levels of ammonia-, nitrate- and organic nitrogen that are above those considered to be normal can be used, in a similar manner to the use of bacteria, to indicate pollution by sewage or animal wastes. It has been reported[2] that interference with the permeability of the electrode membrane arises from hydroxide flocs precipitating on to the membrane surface. Initially, 0·1 M EDTA solution was added in order preferentially to complex those metals which form insoluble hydroxides under the alkaline conditions of measurement of ammonia-nitrogen. It was found that this concentration of EDTA was insufficient to complex the metals present when determining ammonia-nitrogen in sea water and estuarine waters. The major contributor to the formation of the insoluble hydroxides was considered to be

magnesium. It is reported[8] that 1·294 g kg^{-1} of magnesium are present in sea water of 35 per cent. salinity.

A series of determinations of ammonia in sea water, designed to demonstrate the effect of insoluble hydroxides, was carried out. Four sets of experiments, with eight replicate readings in each, were performed. A standard solution of 10 mg l^{-1} of ammonia-nitrogen was made up in: 1, ammonia-free de-ionised water; and 2, sea water containing 0·01 mg l^{-1} of ammonia-nitrogen. The pH values of these two standard solutions were adjusted by using two solutions that were 2 M in sodium hydroxide and (*i*), 0·1 M in EDTA and (*ii*), 1·0 M in EDTA.

The 1·0 M EDTA solution contained EDTA in excess of that required to complex the metals present. The most important of these metals when determining ammonia-nitrogen in sea water is magnesium, which forms a heavy precipitate under the conditions of measurement. A response time of 4 min was allowed for each reading of sea water and de-ionised water standards.

From these determinations it was shown that stabilisation of the sea water standard reading took six replicate readings to be achieved. This occurred in the presence of magnesium hydroxide precipitate (*i.e.*, when the 0·1 M EDTA solution was used), but when 1·0 M EDTA was incorporated in the alkali no precipitate was apparent, the electrode giving a stable response after the first reading. This would appear to indicate that metals forming insoluble hydroxides under the conditions of measurement of ammonia-nitrogen present a potential interference. When de-ionised water standards were read immediately following those containing the hydroxide precipitate the interference appeared to be carried over. Stabilisation of the de-ionised water standard occurred after the fifth replicate when this was done. The presence of 1·0 M EDTA when reading sea water standards eliminated the carry-over.

It may therefore be concluded that 1·0 M EDTA can be successfully used to remove interferences with the electrode response that arise from metals forming insoluble hydroxides, *e.g.*, on addition of alkaline pH adjuster to a sea water sample. This removal of interference makes possible the determination of ammonia-nitrogen in estuarine waters and in sea water by use of the electrode technique.

Conclusion

In order to measure nitrate-nitrogen as ammonia-nitrogen after reduction in acid conditions, an alkali of higher concentration than that initially used was necessary to achieve a pH of 12. The use of a 2 M sodium hydroxide solution permits the measurement of ammonia-nitrogen produced under the conditions of reduction already described. The necessity of making corrections for larger volumes of alkali of lower concentration is avoided, and a dilution of 5·0 ml of alkali with 50 $\pm$ 1 ml of sample is maintained. No interference from the use of 2 M sodium hydroxide solution was evident and therefore it is recommended for use when measuring nitrate-nitrogen by reduction.

To utilise fully the ammonium ion selective electrode, its use for the measurement of nitrate- and organic nitrogen was investigated. A simple, yet viable method for the reduction of nitrate-nitrogen to ammonia-nitrogen with nascent hydrogen, produced *in situ* by the action of acid on Devarda's alloy, has been described. Recoveries of 95 per cent. at the 1 mg l^{-1} of nitrate-nitrogen level and 75–80 per cent. up to 5 mg l^{-1} were obtained. Recoveries in this region were also obtained when samples were spiked with known amounts of nitrate-nitrogen standards.

It was shown that the presence of metals producing insoluble hydroxides when the alkali pH adjuster was added to the sample interfered with the permeability of the membrane, affecting the response time of the electrode. This interference was avoided by the use of EDTA in excess of the amount required preferentially to complex such metals, mainly magnesium.

The measurement of ammonia-nitrogen in estuarine and sea water is much simplified by this method of pH adjustment and it is likely that other forms of nitrogen that can be converted to ammonia-nitrogen in sea water can also be determined. The use of the electrode for the determination of nitrate-nitrogen in sea water has added significance, owing to the interference of chloride ions in some of the other methods of nitrate determination, *e.g.*, the phenoldisulphonic acid method.[1]

A commercially produced electrode[6] is available for the measurement of nitrate in groundwater, plant tissue and soils. However, this electrode will not operate in the presence of high levels of other ions, and therefore it cannot be used for the measurement of nitrate in sea water and in brines.

It is considered that, by eliminating the possible interference from insoluble hydroxides by use of 1·0 M EDTA as a complexing agent, and by presenting a viable method for the reduction of nitrate-nitrogen to ammonia-nitrogen, the measurement of these compounds in sea water is much simplified.

The ammonia electrode is further utilised for the determination of organic nitrogen in water and waste-water samples. The method does not in itself show any startling improvements in time and measurement over the conventional method but when organic nitrogen is determined in conjunction with ammonia- and nitrate-nitrogen in the same sample the method has the inherent advantage that a single final measuring technique involving the determination of ammonia can be used for all three nitrogen compounds.

The ammonia gas-sensing electrode can be used to measure ammonia, nitrate- and organic nitrogen in a wide range of water samples.

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Nitrosophenol and Nitrosonaphthol Derivatives as Reagents for the Spectrophotometric Determination of Iron and Determination of Micro-amounts in Waters with 2-Nitroso-5-dimethylaminophenol

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Twenty-five nitroso compounds have been examined for suitability as organic analytical reagents for the spectrophotometric determination of iron(II) ion. The complexes formed by 2-nitroso-5-dimethylaminophenol (nitroso-DMAP) and 2-nitroso-5-diethylaminophenol (nitroso-DEAP) with iron(II) are shown to have a 1:4 composition and the other iron(II) complexes a 1:3 composition. Nitroso-DMAP and nitroso-DEAP were found to be very sensitive reagents for iron(II) ion, the molar absorptivities of their complexes being about 4×10^4 at about 750 nm in aqueous solution. The colour of the nitroso-DMAP - iron(II) complex is very stable in aqueous solution at about pH 8, while that of the nitroso-DEAP complex is less stable. Nitroso-DMAP is more useful for the determination of micro-amounts of iron than nitroso-DEAP, because of the greater stability of the reagent and the complex formed and the solubility of the latter in aqueous solution.

Diverse ions, which are generally present in city and river waters, did not interfere in the direct spectrophotometric determination of micro-amounts of iron in these waters with nitroso-DMAP.

Since 1-nitroso-2-naphthol was first applied by Ilinski and von Knorre[1] in 1885 as an organic chelating reagent for cobalt, many analytical studies of nitroso compounds have been carried out. Nitrosophenol and nitrosonaphthol derivatives are known to be selective and sensitive colorimetric reagents for cobalt and iron, and the application of nitroso compounds such as the nitrosonaphthols and nitroso-R salt to the determination of cobalt has frequently been studied. On the other hand, the application of nitroso compounds to the determination of iron has received less attention.

In earlier papers,[2,3] the authors reported the synthesis of many nitroso compounds and their reaction with some metal ions. Of twenty-five nitroso compounds examined, 2-nitroso-5-dimethylaminophenol (nitroso-DMAP) and 2-nitroso-5-diethylaminophenol(nitroso-DEAP) were found to be excellent reagents for the spectrophotometric determination of cobalt.[4–11]

Up to the present time, the iron(II) complexes formed with several nitroso compounds such as nitrosophenol derivatives,[12–17] nitroso-R salt,[18–28] 2-nitroso-1-naphthol-4-sulphonic acid[29–31] and nitrosonaphthol[22,32–35] have been studied, but only a few of these reagents have been applied to the spectrophotometric determination of iron.

Recently, the authors reported the extraction of the ternary complexes of iron(II) - nitrosophenol - rhodamine B and the application of this technique to the spectrophotometric determination of trace amounts of iron in waters[36] and sea water.[37] This method was very sensitive (molar absorptivity 9×10^4 l mol^{-1} cm^{-1}) and selective for determining iron(II) ion. However, the procedure involved is more troublesome because of the need to extract the iron from aqueous solution.

In this work, the iron(II) complexes of twenty-five nitroso compounds were examined spectrophotometrically in aqueous solution and after extraction into an organic solvent. The reagents 4-chloro-2-nitroso-1-naphthol, 7-hydroxy-1-nitroso-2-naphthol-3,6-disulphonic acid and 4,6-dichloro-2-nitrosophenol are new, and about twenty of these were first studied for suitability as analytical reagents for iron(II) ion. Nitroso-DMAP was found to be an excellent reagent for iron, and capable of application to the determination of micro-amounts of iron in natural waters.

Experimental

Reagents

Nitroso-DMAP and other nitroso compounds. These reagents were prepared by nitrosation of the parent compounds in aqueous solution with sodium nitrite.[2,3] The reagent 2-nitroso-phenol-6-carboxylic acid was obtained by the method described previously.[3] Neutral, alkaline or acidic aqueous solutions of all of these reagents were prepared, and were stable for at least 1 week. A solution of nitroso-DMAP in 0·1 N hydrochloric acid was stable for at least 1 month.

Standard iron(II) solution. Dissolve Mohr's salt [$FeSO_4(NH_4)_2SO_4.6H_2O$] in distilled water containing a small amount of concentrated sulphuric acid (1 ml per 100 ml of solution) and 1 per cent. *m/m* of hydroxylammonium chloride to give a 1×10^{-2} M solution. Standardise it by titration against standard EDTA solution. Use this stock solution after accurate dilution.

Tetradecyldimethylbenzylammonium chloride, aqueous solution. Dotite zephiramine (Dojindo Co. Ltd., Research Laboratories) was used. Prepare this solution as described previously.[38]

Buffer solutions. Acetic acid - sodium acetate, potassium dihydrogen orthophosphate - disodium hydrogen orthophosphate, ammonia - ammonium chloride, sodium hydrogen carbonate - sodium carbonate and potassium hydroxide solutions.

All the reagents used were of analytical-reagent grade.

Apparatus

A Hitachi - Perkin-Elmer, Model 139, spectrophotometer and a Hitachi, Model EPS-3T, recording spectrophotometer were used for measuring absorbances in glass cells of 10-mm and 100-mm path length. A Hitachi-Horiba, Model F-5ss, pH meter equipped with a combined electrode, 6026–05T, was used for measuring the pH and an Iwaki, Model KM, shaker for shaking the solutions.

General Procedure

Aqueous solution

The solution containing iron(II) was pipetted into a 25-ml measuring flask. The solution of the nitroso compound, buffer solution and distilled water were then added in that order by pipette. After mixing, the solution was left for about 30 min. The absorbance was measured in a glass cell of 10-mm path length against a reagent blank as reference.

Extraction

The solution containing iron(II), the nitroso compound and buffer solution were pipetted in that order into a stoppered 25-ml test-tube. The solutions were mixed and the zephiramine solution was added by pipette. After 30 min the mixture was shaken with 5 ml of chloroform for a further 30 min. After the phases had separated, the absorbance of the organic phase was measured in a glass cell of 10-mm path length against a reagent blank as reference.

Results

Effect of pH of the Aqueous Solution and the Chloroform Extract

The above general procedure was used in examining the effect of pH. The concentration of iron(II) ion used was 2×10^{-5} M and those of nitroso-DMAP were 1×10^{-3} and 2×10^{-4} M; the optimum pH range was found to be 6·5–11 for the aqueous solution and 6·5–10 for the chloroform extract when the nitroso-DMAP concentration was 1×10^{-3} M. For comparison, in Table I the optimum pH ranges in which the absorbances of the iron(II) complexes with the other nitroso compounds were at a maximum and constant are shown. The optimum pH ranges obtained in the extraction with zephiramine (1×10^{-3} M), using chloroform as the extraction solvent, are also listed in this table. In all extraction experiments, the volumes of aqueous and organic phases used were identical (5 ml). Almost all of the nitroso compounds tested were found to give a strong colour with iron(II) ion at about pH 7.

Absorption Spectra

The absorption spectra of the iron(II) complexes were examined at the optimum pH (8) in both the aqueous solution and after extraction into chloroform with zephiramine, the concentrations of iron(II), reagent and zephiramine being 2×10^{-5}, 2×10^{-4} and 1×10^{-3} M, respectively. The absorption spectra of nitroso-DMAP and its iron complex showed that maximum absorption of the complex occurred at 750 nm in aqueous solution and at 730 nm in chloroform. The absorption of the reagent itself at these wavelengths was very small. For comparison, in Table I the wavelengths of maximum absorption of the iron complexes with other reagents are listed.

TABLE I

MOLAR ABSORPTIVITIES (ϵ) OF IRON(II) COMPLEXES IN AQUEOUS SOLUTION AND CHLOROFORM

	Aqueous solution				Chloroform			
Reagent	Optimum pH*	pH†	$\lambda_{max.}$/ nm	$\epsilon \times 10^4$/ l mol^{-1} cm^{-1}	Optimum pH*	pH†	$\lambda_{max.}$/ nm	$\epsilon \times 10^4$/ l mol^{-1} cm^{-1}
2-Nitroso-1-naphthol								
Unsubstituted ..	5·0–8·5	7	724	1·49	5·0–8·5	6	724	1·65
4-chloro	4·0–8·0	6	740	1·43	4·0–7·5	6	732	1·72
5-hydroxy	5·0–8·0	6	766	1·20	5·0–8·0	6	750	1·15
4-sulphonic acid ..	4·0–8·5	6	720	2·12	5·5–8·5	7	716	2·12
5-sulphonic acid ..	4·5–9·0	6	728	2·02	5·5–8·5	7	724	1·97
1-Nitroso-2-naphthol								
Unsubstituted ..	4·5–8·5	7	732	1·60	4·5–8·5	6	730	1·80
4-hydroxy	4·0–6·5	5	670	1·22	4·0–6·5	5	660	1·16
7-hydroxy	5·0–7·5	6	712	1·69	4·5–7·0	6	708	1·65
3-carboxylic acid ..	5·0–9·5	7	740	1·77	3·5–9·0	7	728	1·75
6-sulphonic acid ..	4·5–8·5	6	720	2·12	5·5–8·5	7	720	1·86
7-sulphonic acid ..	5·0–7·5	6	724	2·00	5·5–8·0	7	724	1·85
3,6-disulphonic acid ..	4·0–8·0	6	724	2·02	5·0–8·0	6	720	2·02
7-hydroxy-3,6-disulphonic acid ..	4·5–8·0	6	728	2·12	5·0–7·5	6	716	1·93
2-Nitrosophenol								
4-chloro	3·5–9·0	6	712	1·14	3·5–9·0	6	724	1·15
4-bromo	3·5–8·5	6	712	1·20	3·5–8·5	6	720	1·15
4-methyl	3·5–9·0	6	712	1·14	3·5–9·0	6	724	1·14
5-chloro	3·5–6·5	5	710	$0{\cdot}68_4$	3·5–5·5	5	708	$0{\cdot}68_4$
5-methoxy	3·5–8·5	6	708	1·82	3·5–8·0	7	704	1·81
5-hydroxy	3·5–8·0	6	672	1·38	4·0–7·5	6	686	1·26
5-dimethylamino ..	6·5–9·5	8	750	3·95	6·5–9·5	8	730	2·87
5-diethylamino ..	7·5–10·0	8	755	4·21	6·5–10·0	8	735	3·23
6-carboxylic acid ..	3·5–9·0	6	698	1·07	3·5–6·5	4	712	1·00
3,5-dimethyl ..	3·5–7·5	5	692	$0{\cdot}56_4$	3·5–7·5	5	700	$0{\cdot}56_0$
4-chloro-5-methyl ..	3·5–8·0	6	708	$0{\cdot}69_5$	3·5–8·0	6	720	$0{\cdot}70_4$
4,6-dichloro	3·5–9·5	6	690	1·32	4·5–9·0	6	684	1·24

* The pH range in which the absorbances of the complex were constant and at a maximum when the concentrations of reagent and iron were 2×10^{-4} and 2×10^{-5} M, respectively.

† The pH at which the molar absorptivities were measured.

Molar Absorptivity of the Iron(II) Complex

The molar absorptivity of the iron(II) complex at the wavelength of maximum absorption was measured at the optimum pH from the slope of a calibration graph. The general procedure given above was also used. The concentrations of iron(II) ion and nitroso-DMAP were 0–2×10^{-5} M and 1×10^{-3} M, respectively. The absorbances were measured in a cell of 10-mm path length at 750 nm in aqueous solution and 730 nm in chloroform against the reagent blank. The graphs obtained were straight lines that passed through the origin. For comparison, the calibration graphs for the complexes with the other reagents were prepared in the same way as that for the complex with nitroso-DMAP and the absorbances of these complexes in aqueous and chloroform solutions also obeyed Beer's law, at least over the iron(II) concentration range 0–$2{\cdot}5 \times 10^{-5}$ M; their molar absorptivities were obtained from the slopes of their calibration graphs. The values obtained, which were all reproducible, are listed in Table I.

Composition of the Iron(II) Complex in Aqueous Solution and in Chloroform

The composition ratios [iron(II) to nitroso-DMAP] of the complex in aqueous solution and of the extract in chloroform (zephiramine concentration 1×10^{-3} M) were determined by the molar ratio [variable iron(II) concentration and reagent 8×10^{-5} M (pH 8); and variable reagent concentration and iron(II) 2×10^{-5} M (pH 8)] and continuous variation [iron(II) plus reagent concentrations 8×10^{-5} M (pH 8)] methods. The results obtained showed that the ratio of iron(II) to reagent in the complex was 1:4. The ratio for the nitroso-DEAP complex was also determined by the molar ratio method [variable iron(II) concentration and reagent 8×10^{-5} M (pH 8)] and was also found to be 1:4. For comparison, the ratios of the other iron(II) complexes were determined by the molar ratio and continuous variation methods and the results obtained are summarised in Table II. All of the complexes examined, except those of nitroso-DMAP and nitroso-DEAP, had a 1:3 composition in aqueous solution and in chloroform. The proportion of zephiramine in the extracted nitroso-DMAP and nitroso-DEAP complexes was also determined by the molar ratio method [iron(II) concentration 2×10^{-5} M and reagent 2×10^{-4} M (pH 8)], and the ratio of iron(II) ion to zephiramine was found to be 1:1. From the above results, the extracted species was shown to consist of iron(II), nitroso-DMAP (or nitroso-DEAP) and zephiramine in the proportions of 1:4:1. For comparison, the proportion of zephiramine in extracted complexes with some other reagents were determined by the molar ratio method and the results obtained are summarised in Table III.

TABLE II

RATIO OF IRON(II) TO NITROSO COMPOUND IN COMPLEXES IN AQUEOUS SOLUTION AND CHLOROFORM

	Aqueous solution		Chloroform	
Reagent	Molar ratio method	Continuous variation method	Molar ratio method	Continuous variation method
2-Nitroso-1-naphthol				
Unsubstituted ..	1:3·1	1:2·9	1:2·9	1:3·1
4-chloro	1:3·2	1:3·2	1:3·0	1:3·2
5-hydroxy	1:3·4	1:3·2	1:3·4	1:3·4
4-sulphonic acid ..	1:3·1	1:3·1	1:2·9	1:3·0
5-sulphonic acid ..	1:3·1	1:3·1	1:3·2	1:3·1
1-Nitroso-2-naphthol				
Unsubstituted ..	1:2·9	1:2·7	1:3·1	1:3·0
4-hydroxy	1:3·4	1:3·2	1:3·3	1:3·4
7-hydroxy	1:2·9	1:3·2	1:3·0	1:3·2
3-carboxylic acid ..	1:3·1	1:3·0	1:3·1	1:3·0
6-sulphonic acid ..	1:3·2	1:3·2	1:3·2	1:3·1
7-sulphonic acid ..	1:2·8	1:3·0	1:3·2	1:3·2
3,6-disulphonic acid ..	1:2·9	1:2·9	1:3·0	1:3·1
7-hydroxy-3,6-disulphonic acid ..	1:3·3	1:3·2	1:3·4	1:3·3
2-Nitrosophenol				
4-chloro	1:2·9	1:3·2	1:3·1	1:3·1
5-chloro	1:3·3	1:3·5	1:3·5	1:3·4
5-methoxy	1:3·0	1:3·2	1:3·1	1:2·9
5-hydroxy	1:3·2	1:3·1	1:3·0	1:3·1
5-dimethylamino ..	1:4·0	1:4·0	1:4·0	1:4·0
5-diethylamino ..	1:4·1	1:4·1	1:4·0	1:4·1
6-carboxylic acid ..	1:3·3	1:3·3	1:3·4	1:3·3
4,6-dichloro	1:3·3	1:3·4	1:3·2	1:3·4

Effect of Time

The time necessary for complete formation of the iron(II) complexes with nitroso-DMAP and nitroso-DEAP in aqueous solution was examined. Even when a ten-fold molar excess of nitroso-DMAP was added to iron(II) solution, about 20 min were sufficient for complete reaction at 20 °C, and the absorbance of the complex did not change for at least 1 week. When the concentrations of the reagent and iron(II) were 1×10^{-3} and 2×10^{-5} M, respectively, 10 min were sufficient for complete reaction at 20 °C. Measurement of the absorbance was therefore carried out more than 20 min after addition of the nitroso-DMAP. The colour

of the iron complex formed with nitroso-DMAP was found to be very stable and the absorbance of the complex in chloroform was also constant during at least 1 week. The colour of the iron complex of nitroso-DEAP was, however, less stable, and the absorbance was constant only for 50 min after addition of nitroso-DEAP. Although the molar absorptivity of the nitroso-DEAP complex was larger than that of the nitroso-DMAP complex, the latter was found to be more useful because of the greater stability of the colour.

TABLE III

RATIO OF IRON(II) TO ZEPHIRAMINE IN CHLOROFORM

Reagent	Fe to zephiramine by molar ratio method
2-Nitroso-1-naphthol-4-sulphonic acid	1:4·2
1-Nitroso-2-naphthol	1:0·9
1-Nitroso-2-naphthol-3-carboxylic acid	1:1·2
1-Nitroso-2-naphthol-3,6-disulphonic acid	1:7·2
4-Chloro-2-nitrosophenol	1:0·9
5-Methoxy-2-nitrosophenol	1:1·0
5-Hydroxy-2-nitrosophenol	1:1·2
2-Nitroso-5-dimethylaminophenol	1:1·0
2-Nitroso-5-diethylaminophenol	1:1·1

Determination of Iron with Nitroso-DMAP in Aqueous Solution

From the above results, nitroso-DMAP is considered to be an excellent reagent for determining iron because of the large molar absorptivity and the stability of the colour of its iron complex and the very wide pH range for complete reaction. Accordingly, the following procedure for the determination of iron with nitroso-DMAP is recommended. The sample solution containing less than 5 μg of iron is pipetted into a 50-ml measuring flask. To this solution 0·5 ml of 1 per cent. hydroxylammonium chloride solution and 5 ml of a 0·2 per cent. solution of nitroso-DMAP in 0·1 N hydrochloric acid are added. After mixing thoroughly, buffer solution is added. When the pH is below 7, dilute ammonia solution is added until it is between 7 and 9·5. Distilled water is then added to the mark and the absorbance measured against the reagent blank at 750 nm in a cell of 10-mm path length. When the amount of iron(II) is very small (below about 0·1 μg), a cell of 100-mm path length can be used. The calibration graph prepared in the same way was linear, passing through the origin, when cells of both 10-mm and 100-mm path length were used.

Effect of Co-existing Ions

The effect of co-existing ions was examined by the recommended method and the results obtained are shown in Table IV, from which it can be seen that nickel and copper at concentrations above 1×10^{-5} M and cobalt above 5×10^{-5} M give rise to positive errors.

TABLE IV

EFFECT OF DIVERSE IONS

[Fe] = 1×10^{-5} M and [Nitroso-DMAP] = 1×10^{-3} M; cell of 10-mm path length was used.

Ion	Maximum permissible concentration/M
Na^+, K^+, Cl^-, Br^-, I^-, NO_3^-, SO_4^{2-}, ClO_4^-, SCN^-	10^{-1} (at least)
Mg^{2+}, Ca^{2+}, Sr^{2+}, F^-	10^{-2}
Ba^{2+}	10^{-3}
Zn^{2+}, Cd^{2+}, Pb^{2+}, Hg^{2+}, Cr^{3+}, Al^{3+}, Ag^+	10^{-4}
Mn^{2+}, Co^{2+}	5×10^{-5}
Ni^{2+}, Cu^{2+}	1×10^{-5}

Effect of EDTA

The effect of EDTA was examined, the EDTA being added before the addition of nitroso-DMAP and buffer solution. When the concentration of iron is 1×10^{-6} M and that of EDTA is above 3×10^{-4} M, a negative error results. The reaction time was determined without the addition of EDTA and in its presence at a concentration of $1{\cdot}5 \times 10^{-4}$ M. A reaction time of about 80 min is necessary in order to obtain constant absorbance of the iron complex in the presence of EDTA, 5 min being sufficient when it is absent. The effect of EDTA

($1{\cdot}5 \times 10^{-4}$ M) in masking cobalt, nickel and copper ions was also examined. The absorbances of the solutions tested were measured 2 h after the buffer solution had been added. It was found that cobalt at concentrations below 75-fold, nickel below 100-fold and copper below 5-fold do not interfere when the concentrations of iron, nitroso-DMAP and EDTA are 1×10^{-6}, 1×10^{-3} and $1{\cdot}5 \times 10^{-4}$ M, respectively, and a cell of 100-mm path length is used.

Application to the Determination of Micro-amounts of Iron in City and River Waters

As iron generally exists in waters in both soluble and insoluble forms, the latter must be dissolved by the addition of an acid such as hydrochloric acid. The recommended procedure for determining iron in waters was therefore accordingly modified.

The sample solution (40 ml or less) is transferred to a 50-ml beaker and diluted to 40 ml with water; 2 ml of 20 per cent. hydrochloric acid, which is free from iron (below 0·1 p.p.m. of Fe), and 0·5 ml of 10 per cent. hydroxylammonium chloride solution are then added. A few pieces of sintered glass are added in order to prevent bumping and the beaker is covered with a watch-glass. The beaker is heated on an electric hot-plate (about 300 W) for 30 min; 5 ml of nitroso-DMAP solution (0·2 per cent.) are added and the pH is adjusted with 3 N ammonia solution. (When 6 ml of ammonia solution were added, the pH of the solution was about 9·2.) The solution is then transferred into a 50-ml measuring flask, diluted to the mark with water and the absorbance at 750 nm measured in a cell of 10-mm or 100-mm path length. If necessary, the solution is filtered through a quantitative filter-paper (Toyo filter-paper No. 5B) before measuring the absorbance. A calibration graph is prepared by using solutions containing known amounts of iron(II). It is not, however, necessary to heat the solution.

The amount of 20 per cent. hydrochloric acid added to the sample was checked by using several kinds of city and river waters, and was varied from 0·5 to 3 ml, the amounts of iron determined being almost identical. In the procedure given above, 2 ml of hydrochloric acid were added as a safety measure. The heating time was varied from 0 to 35 min; constant values were obtained when the sample was heated for more than 10 min. The solution was therefore heated for 30 min in order to ensure complete reaction and, for convenience, to reduce the 40-ml volume of the solution to about 10 ml. Even when the amount of 3 N ammonia solution added was varied from 5 to 9 ml, the pH of the solution ranged from about 8·5 to 9·6, the absorbances of the reagent blank were $0{\cdot}017 \pm 0{\cdot}004$ and the absorbances of the iron complex were identical, using a cell of 100-mm path length. By using a cell of 10-mm path length, the absorbance of the reagent blank was negligible (0·002). As the amounts of diverse ions generally present in river and city waters are much smaller than those given in Table IV, they do not interfere and it is not necessary to add EDTA.

The results obtained by the above method are shown in Table V.

TABLE V

DETERMINATION OF IRON IN WATERS

City water: waters of Okayama city; river water: Asahi river. Cell of 100-mm path length was used.

Sample	Date of sampling	Sample taken/ml	Absorbance*	Iron, p.p.m.*
City water ..	October 5th, 1974	20	$0{\cdot}171 \pm 0{\cdot}001$	$0{\cdot}59 \pm 0{\cdot}01$†
	October 9th, 1974	20	$0{\cdot}211 \pm 0{\cdot}006$	$0{\cdot}072 \pm 0{\cdot}002$
	October 16th, 1974	20	$0{\cdot}188 \pm 0{\cdot}002$	$0{\cdot}064 \pm 0{\cdot}001$
	October 17th, 1974	20	$0{\cdot}526 \pm 0{\cdot}005$	$0{\cdot}18 \pm 0{\cdot}00$
River water ..	October 18th, 1974	20	$0{\cdot}526 \pm 0{\cdot}013$	$0{\cdot}18 \pm 0{\cdot}00$
		20	$0{\cdot}057 \pm 0{\cdot}003$	$0{\cdot}20 \pm 0{\cdot}01$†
		40	$0{\cdot}117 \pm 0{\cdot}003$	$0{\cdot}20 \pm 0{\cdot}00$†
	October 23rd, 1974	10	$0{\cdot}627 \pm 0{\cdot}020$	$0{\cdot}43 \pm 0{\cdot}02$
		10	$0{\cdot}064 \pm 0{\cdot}004$	$0{\cdot}44 \pm 0{\cdot}03$†
		40	$0{\cdot}246 \pm 0{\cdot}005$	$0{\cdot}43 \pm 0{\cdot}01$†

* Average values of three determinations.
† Cell of 10-mm path length was used.

Reagent Blank

In order to determine whether the absorbance of the reagent blank in city and river waters differed from that obtained with distilled water, and to check the results of the quantitative

determination of iron in these waters, the volume of sample taken was varied from 5 to 40 ml and distilled water was added to each so as to give a constant volume of 40 ml. For all samples, linear graphs were obtained and the lines could be extrapolated to the same point, which coincided with the point obtained for 40 ml of distilled water. Accordingly, distilled water can be used for the reagent blank.

Conclusions

The possibility of using nitroso compounds for the determination of iron was examined. Most of the iron(II) complexes with nitroso compounds have a 1:3 composition in aqueous solution and when extracted into chloroform with zephiramine. However, it is of interest to note that only the complexes of nitroso-DMAP and nitroso-DEAP have a 1:4 composition. The iron(II) complexes of nitroso-DMAP and nitroso-DEAP are considered to be $[FeR_3(HR)]^-$ or $[FeR_4]^{2-}$ in aqueous solution and $[FeR_3(HR)]^-[zeph]^+$ in chloroform, where HR is nitroso-DMAP or nitroso-DEAP. With those reagents which have no sulphonic acid group, the iron(II) complexes are assumed to be $[FeR_3]^-$ and $[FeR_3]^-[zeph]^+$ in aqueous and organic solutions, respectively, and with those which have sulphonic acid groups, they are found to be extracted as the ternary complexes $[FeR_3]^{(3n+1)-}[zeph_{3n+1}]^{(3n+1)+}$, where n is the number of sulphonic acid groups in the reagent.

The molar absorptivities of the iron complexes of nitroso-DMAP and nitroso-DEAP are very large, about 4×10^4 and 3×10^4 l mol^{-1} cm^{-1} in aqueous solution and chloroform, respectively. This is because the ligands possess strongly electron-donating groups and the complexes have a 1:4 composition. Although the molar absorptivity of the nitroso-DEAP complex is slightly larger than that of nitroso-DMAP complex, nitroso-DMAP is more useful for the determination of micro-amounts of iron than nitroso-DEAP because it is more stable than nitroso-DEAP, and the colour of its complex with iron(II) in aqueous solution is stable for at least 1 week, while that of the nitroso-DEAP complex is stable during only 1 h. Also, part of the nitroso-DEAP complex precipitates at about pH 5–7, even at the concentration of 2×10^{-5} M.

In this work, a spectrophotometric method using nitroso-DMAP was applied to the determination of iron in city and river waters. The method described possesses the following advantages: (*a*) the complex formed has a large molar absorptivity (4×10^4), which is about four times as large as that with 1,10-phenanthroline, (*b*) high selectivity, that is, diverse ions, which are generally present in natural waters, do not interfere in the determination, (*c*) the wide pH range in which the full colour develops and (*d*) the simplicity of the procedure, that is, measurements can be carried out in aqueous solution.

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An Improved and Accurate Procedure for the Determination of Vitamin A

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A rapid and accurate procedure is described for determining vitamin A with trichloroacetic acid. Water-free reagents are prepared less than 3 h before use and the colorimetric readings are made by using a fast-measurement accessory and a slave recorder. The use of this accessory enables the rapid mixing of the colour reagent with the solution to be tested and the reading of results to be carried out within 1 s.

The methods most widely used for the micro-determination of vitamin A involve colorimetric procedures that employ the Lewis acids antimony(III) chloride, trichloroacetic acid and trifluoroacetic acid, which are the most sensitive reagents for determining vitamin A known today. These methods, however, suffer from strong interference from water, the presence of which causes a loss of colour intensity and also the solution to become turbid when the Carr - Price technique with antimony(III) chloride is used. Consequently, the reagents trichloroacetic acid[1] and trifluoroacetic acid[2] have recently been preferred. In order to obtain the maximum blue colour the reagents applied should, of course, be water free. It follows, therefore, that they must always be stored in a desiccator over phosphorus(V) oxide.

For the routine determination of vitamin A, trichloroacetic acid seems to be the most suitable reagent because of its ready availability, inexpensiveness and low toxicity, together with the ease of preparation of the colour reagent and the cleaning of cells.

When Lewis acid chromogens are used the absorbance reaches a maximum in approximately 5 s[1,2] and then decreases rapidly. In practice, it is often measured after 10–15 s.

In this paper we report improvements that permit the ready preparation of a water-free reagent, and the precise recording of the maximum absorption using special equipment for making fast measurements.

Method

Apparatus

Colorimetric measurements are made with a Unicam SP1800 ultraviolet spectrophotometer, using 1-cm cells, Type 21. Spectra are displayed on the Unicam AR25 slave recorder by using the fast-measurement accessory shown in Fig. 1. Use of this accessory ensures that measurements of absorption spectra are made immediately after the addition of the colour reagent. It comprises a lid for the cell compartment with a start - stop screw and a funnel with a long stem (110 × 8 mm) that is constricted at its lower end in order to facilitate introduction of the funnel into the cell through an appropriate hole in the lid.

The reagent solution is added from a fast-delivery pipette coupled to a syringe, which arrangement enables the trichloroacetic acid reagent to be mixed with the test solution within 1 s.

Reagents

Magnesium chloride dehydrating reagent. Dry a thin layer of $MgCl_2.6H_2O$ overnight at 105 °C and store it in a tightly closed vessel.

Chloroform, anhydrous. Wash twice about 250 ml of analytical-reagent grade chloroform with half its volume of redistilled water. To 200 ml of the washed chloroform add about 10 g of dehydrating reagent, shake the container occasionally and filter the chloroform before use.

Trichloroacetic acid, saturated solution in chloroform. Dissolve 50 g of analytical-reagent grade trichloroacetic acid in 25 ml of the washed chloroform, add 30 g of dehydrating reagent, mix for about 3 h and filter the solution before use. This procedure yields approximately 35 ml of chromogen reagent, which is stable for 5 h when allowed to stand with daylight excluded.

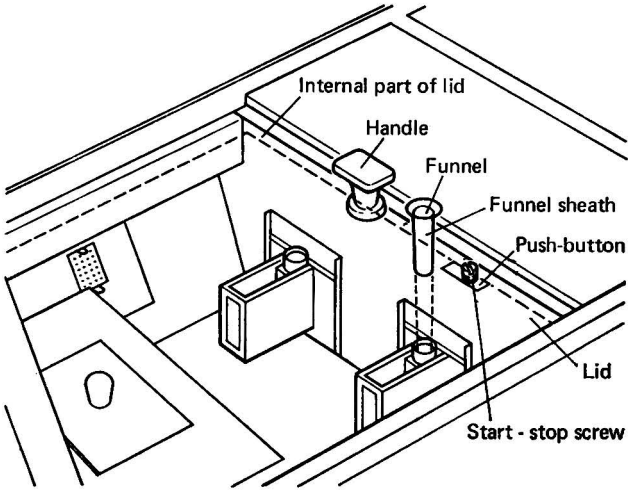

Fig. 1. Fast-measurement accessory in sample compartment. Dimensions: lid, external part 24 × 25 × 0·3 cm and internal part 20·4 × 22·6 × 1·2 cm; funnel sheath, 3 × 0·8 cm i.d.; and funnel, 110 × 0·8 cm o.d. (i.d. of narrowed end 0·3 cm).

Procedure

Place 1 ml of chloroform solution[3] containing from 0·2 to 10 μg of vitamin A in a cell with which a chloroform blank has previously been run at 620 nm. Cover the cell compartment with the fast-measurement accessory and insert the stem of the funnel into the neck of the cell. Select the meter range, set the chart speed to 0·1 cm s^{-1}, start the recorder, introduce into the cell 2 ml of reagent solution and scan the spectrum for 30 s.

Results

Colour Stability of the Vitamin A - Trichloroacetic Acid Complex

Fig. 2 shows the absorption spectrum produced with 9 μg of retinol. The maximum absorbance of the blue coloured complex (1·155) is reached in 1·6 s. In addition, two transient pauses that occur in the reaction about 2 s (duration 0·4 s) and 4 s (duration 0·2 s) after the addition of the trichloroacetic acid can also be noted. After 5 s the absorbance decreases to 74 per cent. of its maximum value, which finding differs significantly from the results reported by previous authors.[1,2]

Precision and Accuracy

In order to verify the precision of the recommended procedure a series of ten determinations on a 9-μg retinol standard were performed. The calculated standard error of the estimate of 1·8 per cent. agrees well with the findings of Bradley and Hornbeck,[2] who used an improved method with trifluoroacetic acid. The blank value is very low (0·005) and so does not influence the results significantly, even at low concentrations of vitamin A. The absorbance of 0·635 obtained with 5 μg of retinol per 3 ml of final solution, when multiplied by the factor 0·74, is identical with that noted by Bayfield[1] 5 s after the addition of trichloroacetic acid reagent. However, taking an absorbance reading at maximum deflection of the galvanometer is more precise than noting the results at a fixed time, because a time lag of 1 s corresponds to a difference of 5 per cent. in the absorbance reading.

In order to check the accuracy of the method solutions containing 10 μg ml^{-1} of vitamin D_3 and β-carotene were analysed; 1 ml of the vitamin D_3 solution gave the same result as the blank, and therefore this vitamin does not interfere. The wavelength of maximum absorption for the product of the reaction of vitamin D_3 with trichloroacetic acid was found to lie in the region 366–368 nm. The maximum absorbance value of 0·085 was reached in 8 min and was stable for 1 min.

With β-carotene solution, 1 ml gave an absorbance value of 0·105 at 1·6 s, and therefore the absorbance obtained with 12 μg of β-carotene corresponds to that given by 1 μg of retinol.

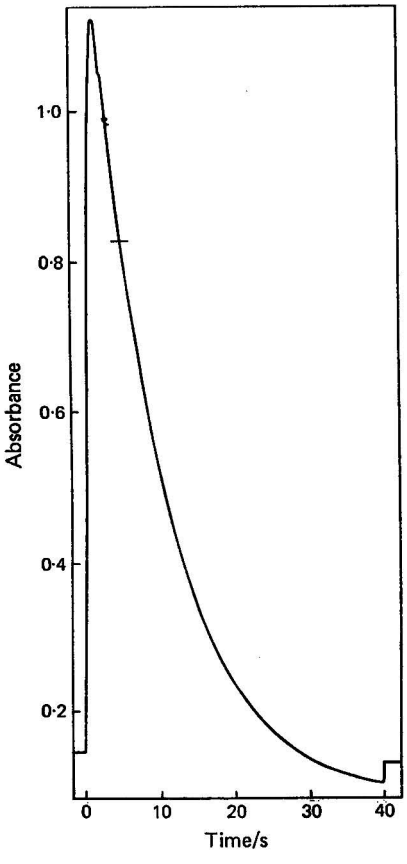

Fig. 2. Spectrum of 9 μg of retinol. Reference, chloroform; chart speed, 0·1 cm s^{-1}; absorbance range, 0–2; and optical path length, 10 mm. Dividing mark on the slope indicates the absorbance after 5 s.

Conclusions

The technique presented for the determination of vitamin A is rapid, simple and precise and enables vitamin A to be determined at low concentrations, together with the possibility of recording kinetic spectra for various derivatives. The method is also accurate because vitamin D_3 does not interfere, and the effect of carotenes (mainly β-carotene) can be eliminated by measurement of the carotene concentration and calculation of the contribution made by the carotenes in the determination by the Carr - Price reaction.

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The Effect of Valency State on the Determination of Chromium in Perchloric Acid Media by Atomic-absorption Spectrophotometry

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Measurements of the atomic absorption of chromium, present in perchloric acid solutions in both the chromium(III) and chromium(VI) states, show that the valency state of the chromium is important. Conversion of all of the chromium into the chromium(III) state by the addition of hydrogen peroxide overcomes this valency problem and also ensures that the maximum absorption is obtained for a given concentration of chromium.

Comparison of the atomic-absorption results obtained by using the air - acetylene and nitrous oxide - acetylene flames, with and without the addition of ammonium chloride, shows that the choice of these parameters has appreciable effects. Up to a 30 per cent. higher chromium response has been found to occur with the addition of ammonium chloride and a further 30 per cent. increase can be obtained by the use of the nitrous oxide - acetylene flame.

For the determination of chromium in ferrous materials by atomic-absorption spectrophotometry the use of the nitrous oxide - acetylene flame has been recommended in the literature. It was further recommended that the chromium in both samples and standards should be in the same oxidation state and that ammonium chloride should be added so as to reduce major interference by iron.[1]

In order to ascertain the effect and relative importance of carrying out these recommendations when determining chromium in perchloric acid media, a series of solutions of known concentrations was prepared and their atomic absorptions were measured. All instrument conditions were kept constant except for the changes necessary in using both the air - acetylene and nitrous oxide - acetylene flames.

Experimental and Results

Preparation of Standard Solutions

Chromium(VI) solution (A). Pure chromium metal (0·1000 g) (Koch-Light) was dissolved in 50 per cent. hydrochloric acid; 20 ml of perchloric acid (sp. gr. 1·54) were added, and the solution was evaporated to fumes of perchloric acid. A cover was placed on the beaker and the heating continued for 10 min with the perchloric acid refluxing down the sides of the beaker.

After allowing the solution to cool until no further fumes were evolved, but not below 100 °C, the solution was diluted with water. If the dilution with water is delayed until the solution reaches room temperature there is a risk of formation of trace amounts of hydrochloric acid, which would then be present in the solution [but not in the chromium(III) solution (see below) owing to the peroxide treatment] and thus give rise to an uncontrollable variable throughout the experiment. After dilution with water, the solution was cooled to room temperature and diluted in a calibrated flask to exactly 500 ml. This solution contained 200 p.p.m. of chromium in the chromium(VI) state.

Chromium(III) solution (B). A 100-ml volume of chromium(VI) solution (A) was transferred into a 250-ml beaker and 5 ml of 20-volume hydrogen peroxide were added. The solution was then boiled for 20 min in order to destroy the excess of peroxide, cooled to room temperature and diluted in a calibrated flask to exactly 100 ml. This solution contained 200 p.p.m. of chromium in the chromium(III) state.

Apparatus

A Techtron, Model AA5, atomic-absorption spectrophotometer, fitted with a chromium hollow-cathode lamp containing neon gas and having a Pyrex glass window, was used.

Procedure

Six test solutions were prepared with chromium solutions A and B such that each contained the same amount of chromium, but with varying proportions of chromium(III) and chromium(VI) ions. Table I shows the concentrations of each solution employed. Each of these test solutions was adjusted to a total volume of 100 ml after the addition of 5 ml of perchloric acid (sp. gr. 1·54). The solutions were aspirated into either the air - acetylene or nitrous oxide - acetylene flame under the optimum instrument conditions found, which are given in Table II.

TABLE I

TEST SOLUTIONS WITH CONCENTRATIONS OF CHROMIUM(III) AND CHROMIUM(VI) FOR ATOMIC-ABSORPTION DETERMINATION

Test solution (100 ml)	Chromium solution (A)/ml	Chromium as Cr(VI), p.p.m.	Chromium solution (B)/ml	Chromium as Cr(III), p.p.m.	Total chromium, p.p.m.
1	10·0	20	0	0	20
2	8·0	16	2·0	4	20
3	6·0	12	4·0	8	20
4	4·0	8	6·0	12	20
5	2·0	4	8·0	16	20
6	0	0	10·0	20	20

TABLE II

INSTRUMENT CONDITIONS WITH TECHTRON AA5 INSTRUMENT

Wavelength/nm	357·9
Lamp current/mA	6
Spectral band width/nm	8
Burner height/mm	8
Air or N_2O flow-rate/l min^{-1}	5·75 at 210 kPa
C_2H_2 flow-rate/l min^{-1}	3·25 (air) at 80 kPa 7·0 (N_2O) at 80 kPa
Burner slot (AB 50 for both gas mixtures)/mm	50
Damping position	C

After the absorption of each solution had been measured, exactly 1 g of solid ammonium chloride was added to exactly 50 ml of each solution. When the reagent had dissolved, the solutions were aspirated under the same instrument conditions as before, using both gas mixtures. The four sets of readings obtained are shown in Table III in terms of absorption scale units. Finally, a series of solutions, each solution containing a total of 20 p.p.m. of

TABLE III

ABSORPTION SIGNALS FOR MIXED CHROMIUM(III) - CHROMIUM(VI) SOLUTIONS

Absorption units equivalent to absorbance × 100.

	C_2H_2 - air flame		C_2H_2 - N_2O flame	
Solution	No NH_4Cl	2 g of NH_4Cl per 100 ml	No NH_4Cl	2g of NH_4Cl per 100 ml
1	29·0	38·0	47·5	50·5
2	30·0	41·0	50·0	54·0
3	32·5	44·0	53·0	58·0
4	34·5	45·5	56·0	62·5
5	36·5	46·5	59·5	64·0
6	37·5	47·0	61·0	64·5

chromium with equal concentrations of chromium ions in the two valency states, was prepared and different amounts of perchloric acid (sp. gr. 1·54) were added to the various solutions. The absorption values in the presence of 2 g of ammonium chloride per 100 ml were measured for the nitrous oxide - acetylene flame and the results obtained are shown in Table IV.

TABLE IV

RESULTS ON SOLUTIONS (100 ml) CONTAINING 10 p.p.m. EACH OF CHROMIUM(III) AND CHROMIUM(VI) IONS WITH VARIOUS CONCENTRATIONS OF $HClO_4$ USING C_2H_2 - N_2O FLAME (2 g OF NH_4Cl PER 100 ml)

Volume of $HClO_4$ (sp. gr. 1·54) in solution/ml	Absorbance × 100
0·4	61·5
2·4	65·0
5·4	66·0
10·4	67·0
20·4	69·0

Discussion of Results

Valency effects observed when determining chromium in perchloric acid media had been reported to the author and these effects were confirmed by the results indicated above. The improvement in sensitivity achieved by the addition of ammonium chloride was also confirmed for both types of flame. However, it was not expected before these measurements were taken that, irrespective of the type of flame used as atomiser, the addition of ammonium chloride would cause the calibration graph to be displaced to a parallel line. This finding indicated that the effect of ammonium chloride was proportional, and identical, for both valency states of chromium. In order to obtain reliable results for chromium the major step needed is to ensure that all of the chromium is in one valency state and that the chromium in the standards used is in the same state.

Use of ammonium chloride increases the absorbance for any given chromium content in the air - acetylene flame by up to 30 per cent. and up to 12 per cent. for the nitrous oxide - acetylene flame. The use of this latter flame, instead of the air - acetylene flame, can increase the absorbance by up to 30 per cent. By employing hydrogen peroxide to reduce the chromium(VI) to chromium(III) no further ions are introduced into the solution, but again a 30 per cent. improvement in absorbance can be obtained. These improvements are accumulative. It can be seen that 20 p.p.m. of chromium as chromium(VI) give an absorbance of 0·29 when measured without ammonium chloride and using an air - acetylene flame. Simply by reduction of the chromium, addition of ammonium chloride and the use of a high-temperature flame this value can be increased to 0·645, an improvement of 120 per cent.

Changes in perchloric acid concentration, in slit width and in lamp current give rise to only minor variations in the absorbance of any particular solution and such improvements as might be obtained by very careful control of these parameters would be marginal.

Conclusions

The valency state of the chromium is of paramount importance when determining this element in perchloric acid media. Reduction of chromium(VI) to chromium(III) can be achieved satisfactorily by the addition of hydrogen peroxide, the use of which avoids the introduction of some other ion to the solution, as would occur with most reducing agents. Maximum absorption for chromium is given by chromium(III) ions, measured using the nitrous oxide - acetylene flame. The addition of ammonium chloride provides a worthwhile increase in sensitivity. A sensitivity (1 per cent. absorption) of 0·15 p.p.m. was obtained by using the optimum instrument and solution conditions. The concentration of perchloric acid in the solutions to be aspirated is not critical.

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Determination of Aluminium, Calcium, Manganese and Titanium in Ferrosilicon Alloys by Atomic-absorption Spectrophotometry

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A method for the determination of aluminium, calcium, titanium and manganese in ferrosilicon alloys is described. After elimination of silicon by the addition of hydrofluoric acid and subsequent dissolution of the residue, the solution is ready to be analysed by means of atomic-absorption spectrophotometry. The proposed method is free of interferences from the most common elements used in the production of ferrosilicon alloys, and is suitable for the determination of concentrations of aluminium and titanium down to 0·1 per cent., manganese down to 0·01 per cent. and calcium down to 0·02 per cent. These limits can be reduced by varying either the dilution of the sample solution or the amount of sample taken.

Ferrosilicon alloys find widespread use in ferrous metallurgy; however, their main and typical use lies in the manufacture of silicon-containing steels in the form of sheets for the laminated cores of transformers. The more recently developed silicon steels are characterised by very low magnetic losses. As this property is directly influenced by impurities, interest in the control of the latter has consequently increased. In order to achieve the highest degree of control over the amounts of minor elements present in the final product, it is therefore necessary first to have accurate knowledge of the amounts of the minor constituents of the ferrosilicon alloys used. The usual methods for the determination of these elements involve the use of gravimetric, titrimetric and photometric techniques.[1]

In this work an analytical method for the determination of calcium, titanium, aluminium and manganese in ferrosilicon alloys has been developed.

With the aim of achieving greater speed of analysis and of simplifying the treatment required, bearing in mind the needs of the production workshops, the atomic-absorption technique has been adopted. As is well known, atomic-absorption spectrophotometry not only enables rapid and selective analysis to be carried out but also the determination of the required elements on the same sample solution.

Experimental

Sample Dissolution

Dissolution of the ferrosilicon alloy was carried out with a mixture of hydrofluoric and nitric acids, thus allowing the silicon, one of the main sources of interference in the atomic-absorption measurement, to be volatilised in the first step of the analysis.

Occasionally, especially with ferrosilicon alloys that contain large amounts of calcium and aluminium, the solution obtained may be cloudy; this difficulty can easily be overcome by filtering the solution and fusing the residue. A preliminary trial showed that fusion with anhydrous sodium carbonate at 1000 °C, followed by leaching with acid, enables complete dissolution of the sample to be achieved.

Atomic-absorption Behaviour of Aluminium, Titanium, Calcium and Manganese

Aluminium

Aluminium is determined in the nitrous oxide - acetylene flame with low noise and few interferences. It is partially ionised in this flame and good sensitivity is therefore achieved, reducing the ionisation by the addition of alkali-metal salts (1000–2000 μg ml^{-1}) to the sample and standard solutions.[2]

Titanium

A nitrous oxide - acetylene flame is recommended for the determination of titanium. Endo[3] has reported that the addition of aluminium increases the sensitivity and reduces interference.

Calcium

The determination of calcium in an air - acetylene flame is subject to interference by several anions (sulphate, phosphate, aluminate and silicate). Willis[4] and David[5] have reported that lanthanum or strontium at concentrations of 0·1–1 per cent. can be added to the sample and standards in order to control these interferences.

Manganese

Manganese is easily determined in an air - acetylene flame. However, Platte and Marcy[6] found that silicon interfered. This interference can be suppressed by the addition of calcium chloride (200 μg ml^{-1}).

Method

Apparatus

Experimental results were obtained with a Perkin-Elmer, Model 403, atomic-absorption spectrophotometer equipped, according to the element to be determined, with a hollow-cathode lamp of aluminium, manganese, titanium, calcium or magnesium. A standard Perkin-Elmer pre-mixing burner was used. The optimum conditions adopted for the various elements are given in Table I.

TABLE I

WORKING CONDITIONS FOR ATOMIC-ABSORPTION SPECTROPHOTOMETRY

Element	Wavelength/nm	Slit setting	Heating current/mA	Flame type
Aluminium	309·3	4	30	Nitrous oxide - acetylene Flow setting 40, 30 p.s.i.g. Flow setting 60, 10 p.s.i.g.
Manganese	279·5	3	20	Air - acetylene Flow setting 60, 30 p.s.i.g. Flow setting 40, 10 p.s.i.g.
Titanium	365·3	3	40	Nitrous oxide - acetylene Flow setting 40, 30 p.s.i.g. Flow setting 60, 10 p.s.i.g
Calcium	422·7	4	15	Air - acetylene Flow setting 60, 30 p.s.i.g. Flow setting 40, 10 p.s.i.g.

Reagents and Solutions

Hydrofluoric acid, sp. gr. 1·15.
Nitric acid, sp. gr. 1·40.
Hydrochloric acid, sp. gr. 1·19.
Perchloric acid, sp. gr. 1·68.
Sulphuric acid, sp. gr. 1·83.
Sodium carbonate.

Hydrochloric acid, 2 *per cent.* V/V. To 98 ml of water add 2 ml of hydrochloric acid (sp. gr. 1·19) and mix.

Hydrochloric acid (1 + 3). Dilute 250 ml of hydrochloric acid (sp. gr. 1·19) to 1 l with water.

Standard solution of manganese, 2000 $\mu g\ ml^{-1}$. Dissolve 1 g of manganese (more than 99 per cent. pure) in 30 ml of hydrochloric acid (sp. gr. 1·19) and dilute the solution to 500 ml.

Dilute standard solution of manganese, 100 $\mu g\ ml^{-1}$. Dilute 50 ml of the above standard solution to 1 l with hydrochloric acid (2 per cent. *V/V*).

Standard solution of aluminium, 5000 $\mu g\ ml^{-1}$. Dissolve 0·5 g of aluminium (99·5 per cent. pure) in 20 ml of hydrochloric acid (1 + 3). Dilute the solution to 100 ml.

Dilute standard solution of aluminium, 1000 $\mu g\ ml^{-1}$. Dilute 20 ml of the above standard solution to 100 ml with hydrochloric acid (2 per cent. *V/V*).

Standard solution of titanium, 5000 $\mu g\ ml^{-1}$. Dissolve 1 g of titanium (98 per cent. pure) in 30 ml of sulphuric acid (sp. gr. 1·83). Dilute the solution to 200 ml.

Dilute standard solution of titanium, 50 $\mu g\ ml^{-1}$. Dilute 5 ml of the above standard solution to 500 ml with hydrochloric acid (2 per cent. *V/V*).

Standard solution of calcium, 100 $\mu g\ ml^{-1}$. Dissolve 0·25 g of calcium carbonate, dried at 105 °C, in 10 ml of water plus a few drops of hydrochloric acid (sp. gr. 1·19). Bring the solution to the boil, cool and dilute it to 1 l.

Iron solution, 1000 $\mu g\ ml^{-1}$. Dissolve 1 g of pure iron (Merck) in 20 ml of hydrochloric acid (1 + 3). Dilute the solution to 1 l.

Strontium chloride solution, 50 $mg\ ml^{-1}$ *of Sr*. Dissolve 18·10 g of strontium chloride ($SrCl_2$), dried at 105 °C, in water and dilute the solution to 200 ml.

Aluminium chloride solution, 5 $mg\ ml^{-1}$ *of Al*. Dissolve 0·5 g of aluminium (pure for analysis) in 20 ml of hydrochloric acid (sp. gr. 1·19). Dilute the solution to 100 ml.

Procedure

A blank determination should be run when carrying out the following procedure.

Place 1·0 g of the very finely pulverised ferrosilicon alloy in a platinum crucible and moisten it with a few drops of water. Add, continuously, 6 ml of hydrofluoric acid and wait until fumes are given off. Add about 5 drops of nitric acid and, after the reaction has subsided, add about 10 drops more of nitric acid. Place the crucible on a hot-plate. When dissolution is complete, add 5 ml of perchloric acid and evaporate the solution until dense white fumes appear. Allow the residue to cool, transfer it into a 400-ml beaker and add about 50 ml of 2 per cent. *V/V* hydrochloric acid. Boil the mixture so as to obtain a clear solution. If the solution is cloudy or contains minute particles of graphite, filter it on a medium filter and fuse the residue in a platinum crucible with 1 g of anhydrous sodium carbonate at 1000 °C for about 15 min.

Place the cooled melt in the same 400-ml beaker into which the filtered solution has been transferred and dissolve it. Add 10 ml of hydrochloric acid (sp. gr. 1·19) and transfer the solution into a 250-ml calibrated flask, washing the beaker with 2 per cent. *V/V* hydrochloric acid and making the volume up to the mark with the same acid (solution A). For the determination of the elements transfer into four 100-ml calibrated flasks suitable aliquots of solution A (the final concentration for each element must be within the range of the calibration graph for that element).

For the titanium determination add to a calibrated flask 3·2 ml of aluminium chloride solution (5 mg ml^{-1}), and for the calcium determination, 10 ml of strontium chloride solution (50 mg ml^{-1}). Make the solutions up to volume with 2 per cent. *V/V* hydrochloric acid. Four "sample solutions" are thus obtained. The absorbances were then measured.

Calibration Graphs

Manganese

The calibration graph was prepared by adding to 100-ml calibrated flasks various volumes (0–2 ml) of the dilute standard manganese solution, an appropriate volume of the 1000 $\mu g\ ml^{-1}$ iron solution and the necessary amount of anhydrous sodium carbonate to match the iron and sodium concentrations of the sample solution. The volume was then made up to the mark with 2 per cent. *V/V* hydrochloric acid and the absorbance measured.

Aluminium

Various volumes (0–4 ml) of the dilute standard aluminium solution were added to 100-ml calibrated flasks and were treated as described for manganese.

Titanium

Various volumes (0–4 ml) of the dilute standard titanium solution were added to 25-ml calibrated flasks and the procedure described for manganese was followed, except that 0·8 ml of aluminium chloride solution (5 mg ml^{-1} of Al) was added before making the volume up to the mark.

Calcium

Volumes varying from 0 to 4 ml of the standard calcium solution were added to 100-ml calibrated flasks. The procedure described for manganese was then followed, except that 10 ml of strontium chloride solution (50 mg ml^{-1} of Sr) were added before making the volume up to the mark.

Interferences

The influence of the individual minor elements in the ferrosilicon alloy on the absorbance of aluminium, titanium, calcium and manganese was studied. An amount of each interfering element equal to its maximum concentration in the ferrosilicon alloys examined was added to synthetic solutions containing the element to be determined; no interference was found. The results obtained are summarised in Table II.

TABLE II

EFFECT OF VARIOUS ELEMENTS ON THE DETERMINATION OF MANGANESE, ALUMINIUM, CALCIUM AND TITANIUM

Element	Added, per cent.	Found, per cent.	Interfering elements, per cent. Ca	Al	Ti	P	Mn
Manganese	0·5	0·496	6·0				
	0·5	0·503		2·0			
	0·5	0·496			0·2		
	0·5	0·500				0·2	
Aluminium	1·0	0·98	6·0				
	1·0	1·00					1·0
	1·0	1·00			0·2		
	1·0	1·00				0·2	
Titanium	0·1	0·098	6·0				
	0·1	0·105					1·0
	0·1	0·098				0·2	
Calcium	3·0	3·14		2·0			
	3·0	2·97					1·0
	3·0	3·06			0·2		
	3·0	3·14				0·2	

Results

The proposed method was checked on a series of BCS, NBS, ISS and IRSID standards. The composition of the samples examined is shown in Table III, and the values obtained with the adopted procedure are given in Table IV.

TABLE III

TYPICAL COMPOSITION OF TESTED SAMPLES

Sample	Element content, per cent. Si	Al	Ca	P	C	Fe	Mn	S	Cr	Ni	B	Ti
BCS 305/1	75·0	1·38	0·33	0·030	0·040	—	—	—	—	—	—	—
NBS 59e	48·10	0·35	0·42	0·016	0·046	50·05	0·75	0·002	0·052	0·033	0·058	—
JSS 720/2	76·9	1·564	—	0·0337	0·0382	—	—	—	—	—	—	—
IRSID 501/1	79·3	—	—	0·016	0·30	16·2	0·11	—	—	—	—	0·15

It can be seen that the experimental results are in excellent agreement with the certified values. For the estimate of the reproducibility of the method we repeated the analysis of the BCS 305/1 standard sample ten times. The estimate of the standard deviation and the mean of the value obtained for this sample are reported in Table V.

TABLE IV

RESULTS FOR DETERMINATION OF CALCIUM, ALUMINIUM, MANGANESE AND TITANIUM

	Element content, per cent.							
	Calcium		Aluminium		Manganese		Titanium	
Sample	Certified	Found	Certified	Found	Certified	Found	Certified	Found
BCS 305/1	0·33	0·317	1·38	1·39	—	—	—	—
NBS 59e	—	—	0·35	0·37	0·75	0·76	—	—
JSS 720/2	—	—	1·564	1·51	—	—	—	—
IRSID 501/1	—	—	—	—	0·11	0·10	0·15	0·15

TABLE V

ESTIMATE OF THE REPRODUCIBILITY FOR BCS 305/1 STANDARD SAMPLE

Element	Mean value, per cent.	Standard deviation, per cent.
Aluminium	1·43	0·0141
Calcium	0·317	0·0031
Manganese	0·129	0·0023
Titanium	0·104	0·004

Conclusions

The method described is applicable to ferrosilicon alloys that contain concentrations of aluminium of not less than 0·1 per cent., titanium and manganese of not less than 0·01 per cent. and calcium of not less than 0·02 per cent. The method is not subject to interference from elements that are usually present in ferrosilicon alloys. By varying the amount of sample taken and/or the dilutions used it is possible to lower the range of concentrations examined.

The proposed method can be considered to be rapid compared with the methods already given in the literature and is of similar accuracy and precision. Further, it is based on the use of a single sample solution and, in consequence, is particularly suitable for routine analysis.

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The Gas-chromatographic Determination of Selenium in Steel with 4-Chloro-1,2-diaminobenzene

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A simple and practical method is described for the determination of small amounts of selenium in steel. A diluted mixture of equal volumes of hydrochloric and nitric acids is used to dissolve the steel sample, the selenium is completely converted into the quadrivalent state in the presence of perchloric acid and the large amount of iron(III) is masked with phosphoric acid. No loss of selenium has been encountered when using this treatment. Selenium(IV) reacts with 4-chloro-1,2-diaminobenzene to form 5-chloropiaselenol, which is extracted into toluene and determined by means of a gas chromatograph equipped with a thermal conductivity detector. When samples of 0·1 g are used, the standard deviation at the 0·1–0·3 per cent. of selenium level is 0·002–0·003 per cent.

Recently, various kinds of steel containing selenium have appeared on the market in which a relatively large amount of selenium (about 0·1 per cent.) is present. A rapid and accurate method for its determination is therefore required that avoids interferences from iron and the other elements that are present in steels.

Spectrophotometric or fluorimetric methods with 3,3′-diaminobenzidine[1–3] or 2,3-diaminonaphthalene[4] have been reported, but these methods are subject to serious interference from iron(III), vanadium(V) and manganese(VII) ions. The Japan Industrial Standards Committee recommends the use of an extraction - spectrophotometric method with 2,3-diaminonaphthalene.[5] In this method, the remainder of the acid that was used for the dissolution of the steel affects the coloration, while vanadium(V) or manganese(VII) ions interfere in the determination owing to oxidation of the reagent.

However, 1,2-diaminobenzene derivatives react with selenium(IV) to form piaselenol derivatives. It is well known that the reaction is scarcely affected by any other elements.[6] One group of workers[7,8] has proposed a gas-chromatographic method to determine an ultramicro-scale amount of selenium using this reaction.

The present paper describes an investigation of the determination of more than 10^{-2} per cent. of selenium in a steel by means of a gas chromatograph equipped with a thermal conductivity detector, using 4-chloro-1,2-diaminobenzene as reagent. Although the sensitivity of this method is not as high as that of the spectrophotometric method, selenium is determined successfully even in the presence of a 100-fold excess of vanadium(V) or manganese(VII). The proposed method provides simplicity and precision.

Experimental

Reagents

4-Chloro-1,2-diaminobenzene dihydrochloride solution. This was used as a 0·3 per cent. aqueous solution. 4-Chloro-1,2-diaminobenzene dihydrochloride was obtained from commercial 4-chloro-1,2-diaminobenzene by repeated recrystallisation from solution in hydrochloric acid.[9] As this solution becomes brownish after a few days, it should be freshly prepared.

Selenium(IV) stock solution. An amount (1·4053 g) of selenium(IV) oxide was accurately weighed and dissolved in 1 l of distilled water (1·00 mg ml^{-1} of selenium). The concentration was determined by gravimetry.[10] Working solutions were prepared by dilution of the stock solution, which was stable for at least six months.

Standard selenium(0) solution. An amount (6·0 mg) of elemental selenium was dissolved in 100 ml of carbon disulphide (60 μg ml^{-1} of selenium).

Mixed acid A. Concentrated hydrochloric acid (sp. gr. 1·18) and nitric acid (sp. gr. 1·38) were mixed in equal volumes. This mixture was then diluted (1 + 1) with distilled water.

Mixed acid B. Equal volumes of 60 per cent. perchloric acid and concentrated orthophosphoric acid (sp. gr. 1·69) were mixed.

The other reagents were of analytical-reagent grade.

Apparatus

A Shimazu, Model GC-5A, gas chromatograph, equipped with a thermal conductivity detector, was used. The glass column (1 m long, 3 mm bore) was packed with 15 per cent. SE-30 on 60–80-mesh Chromosorb W. The column and the detector temperatures were maintained at 200 °C, the helium flow-rate was 20 ml min^{-1} and the bridge current was 150 mA.

Procedure

A 100-mg sample of the steel is weighed accurately, placed into a 100-ml conical flask and dissolved completely in about 5 ml of mixed acid A. Next, 5 ml of mixed acid B are added and the solution is heated on a sand-bath (to about 230 °C). A brownish vapour is increasingly evolved and the solution comes to the boil. After several minutes, both the evolution of the brownish vapour and the boiling of the solution cease; the solution is heated further until white fumes of perchloric acid are evolved. The conical flask is then removed from the sand-bath. If the heating is continued after the evolution of white fumes erroneous results may be obtained.

After a few minutes the flask is cooled in water. The contents are transferred into a 100-ml separating funnel and the conical flask is rinsed with distilled water, the rinsings being transferred into the funnel. Then the total volume of liquid in the funnel is adjusted to about 50 ml. The final pH of the solution is found to be about 1 as a result of the remaining mixed acid B.

A 3-ml volume of the 0·3 per cent. 4-chloro-1,2-diaminobenzene dihydrochloride solution is added to the funnel and the solution allowed to stand for 1 h. Next, the 5-chloropiaselenol formed is extracted into 1 ml of toluene by shaking for 5 min on a mechanical shaker, then 5 μl of the toluene extract are injected into the gas chromatograph and the peak height is measured.

Results and Discussion

Dissolution of Steels and Oxidation of Selenium

When the dissolution of the steel is attempted by using nitric acid, a long period of heating is needed. Also, when a mixture of equal amounts of hydrochloric and nitric acids is used, vigorous bumping is observed. However, the diluted mixed acid A can dissolve the steel and oxidise the selenium to the quadrivalent state completely without the bumping. A sample of electrolytic iron (0·1 g) and 5 ml of standard selenium(0) solution [containing 300 μg of selenium(0)] are heated to dryness. The residue is dissolved and oxidised with various amounts of mixed acid A. Fig. 1 shows that more than 2 ml of mixed acid A can oxidise the selenium(0) completely, and that a good recovery is obtained.

It is found that unless the evaporation is continued very nearly to dryness, the selenium does not oxidise completely to the quadrivalent state. The remaining nitric acid does not interfere with the formation of 5-chloropiaselenol but oxidises the reagent, therefore the heating should be continued in the presence of perchloric acid until the expulsion of mixed acid A is complete. However, prolonged heating after the evolution of white fumes of perchloric acid caused a loss of selenium. For instance, if the heating is continued for 5 min and 10 min following the evolution of white fumes, the loss of selenium is 7 and 34 per cent., respectively. Therefore, the heating must be stopped as soon as white fumes are evolved.

The interference of the iron(III) ion with the formation of 5-chloropiaselenol is shown in Fig. 2. Even if only a small amount of iron(III) is present, the reaction of the selenium(IV) with 4-chloro-1,2-diaminobenzene is seriously affected. Ascorbic acid, oxalic acid, potassium dihydrogen orthophosphate and orthophosphoric acid are usually used for masking the effect of iron(III). Orthophosphoric acid is the most convenient masking agent, as the interference is completely eliminated by the addition of more than 0·6 ml (Fig. 3). As the orthophosphoric acid does not affect the oxidation of the selenium, it is added together with per-

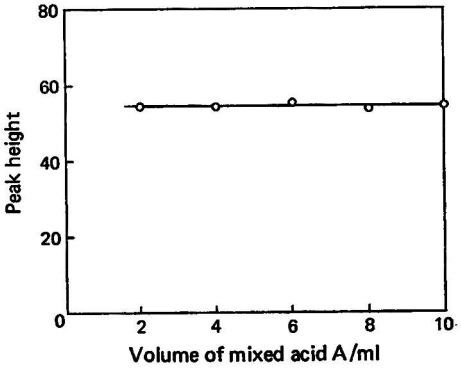

Fig. 1. Effect of mixed acid A on the dissolution of 100 mg of electrolytic iron and 300 μg of Se(0) (amount of mixed acid B = 5 ml).

chloric acid in a mixture in the volume ratio 1:1 (mixed acid B). As is shown in Figs. 1 and 4, 5 ml of mixed acid A are used for the dissolution of steel and for the oxidation of selenium to the quadrivalent state, and 5 ml of mixed acid B for the expulsion of mixed acid A and the masking of iron(III) ions. The decomposition time is about 20 min.

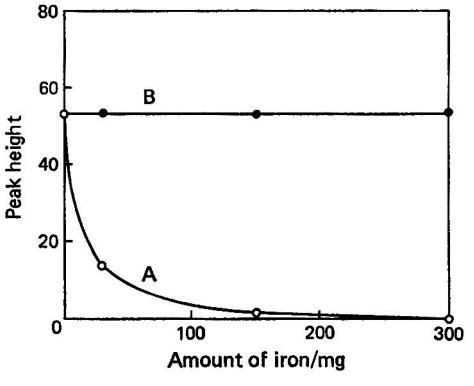

Fig. 2. Effect of iron(III) ions [300 μg of Se(IV)]. A, with no masking agent added; and B, with 2·5 ml of orthophosphoric acid added.

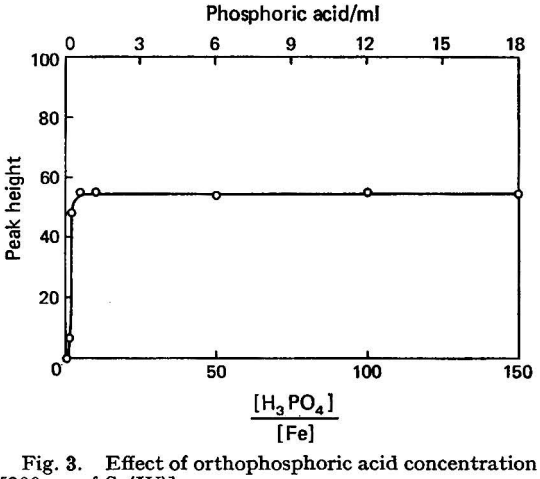

Fig. 3. Effect of orthophosphoric acid concentration [300 μg of Se(IV)].

1,2-Diaminobenzene Derivatives

1,2-Diaminobenzene and its 4-substituted derivatives have been studied as reagents for the determination of selenium. 1,2-Diaminobenzene reacts quantitatively with selenium(IV) in the pH range 1–2·5 and forms the piaselenol, while its 4-chloro- and 4-nitro- derivatives form the 5-substituted piaselenol in the pH range 0–2·5. Tanaka and Kawashima[6] have reported that increase of the distribution ratio of the 5-substituted piaselenols with toluene is in the order $H<NO_2<CH_3<Cl$, and that the distribution ratio of 5-chloropiaselenol is 2580 at pH 1 and 2. 5-Chloropiaselenol is extracted quantitatively from less than 60 ml of aqueous solution into 1 ml of toluene, but 5-nitropiaselenol is not extracted completely. Enhancement of the gas-chromatographic sensitivity of the 5-substituted piaselenols is in the order $CH_3O<CH_3<NO_2<Cl<H$. On the basis of these results, 4-chloro-1,2-diaminobenzene was chosen as the most favourable reagent for the determination.

Fig. 5 shows the gas chromatogram of 5-chloropiaselenol, which was obtained by use of the procedure described above. It is very clearly defined and only the peaks of toluene and 5-chloropiaselenol are found. Although the oxidation of 4-chloro-1,2-diaminobenzene may occur during the procedure, no oxidation product is extracted into the toluene and unknown peaks are not observed in the gas chromatogram.

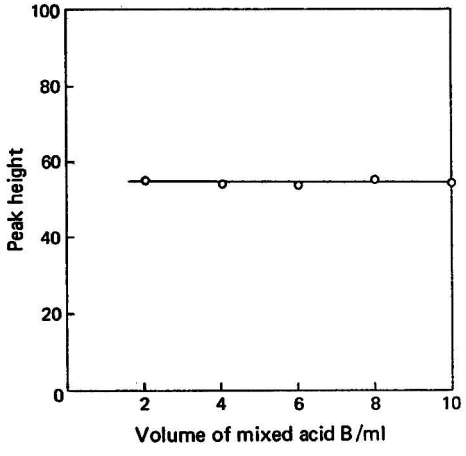

Fig. 4. Effect of mixed acid B on 300 μg of Se(0) plus 5 ml of mixed acid A.

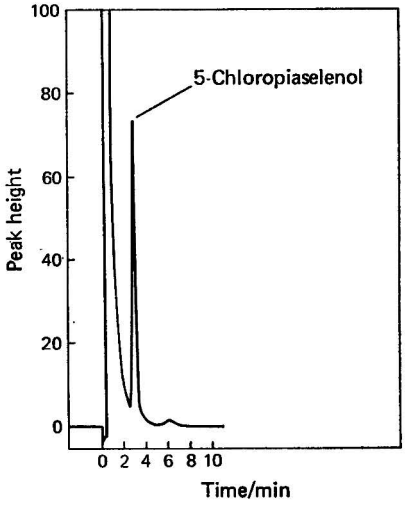

Fig. 5. Gas chromatogram of 5-chloropiaselenol (383 μg of Se).

Reaction

The effect of the standing time, following the addition of 4-chloro-1,2-diaminobenzene, is shown in Fig. 6; the standing time required is more than 20 min at room temperature. The reaction of the reagent with selenium is the longest stage in the procedure. As expected, heating hastens the reaction and if 4-chloro-1,2-diaminobenzene is added just before cooling is begun the reaction progresses to completion during the cooling time. Therefore, shortening of the reaction time is possible. At this stage, some of the reagent decomposes and the solution turns dark red. However, the determination of selenium is not affected because the decomposition products are not extracted into the toluene. The reagent concentration that is required to convert completely the selenium(IV) into 5-chloropiaselenol was investigated on 300 μg of selenium in solution in hydrochloric acid after leaving it to stand for 1 h at room temperature. As is shown in Fig. 7, it was found that the selenium reacts quantitatively with the reagent if the concentration of the reagent is more than four times in excess

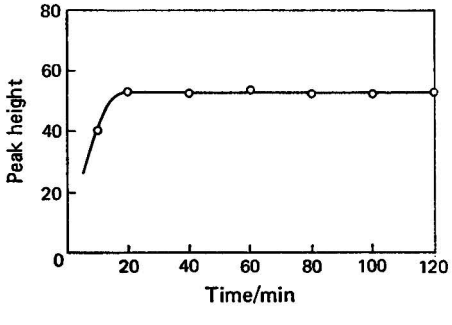

Fig. 6. Effect of standing time (300 μg of Se).

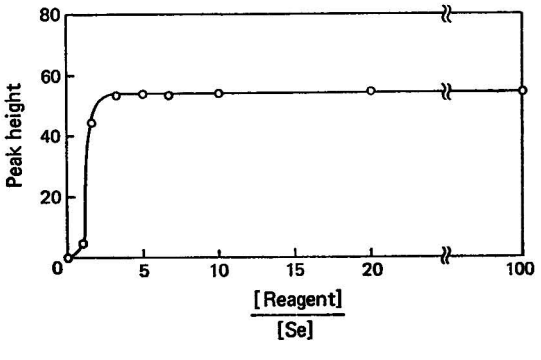

Fig. 7. Effect of 4-chloro-1,2-diaminobenzene reagent concentration (300 μg of Se).

of the concentration of selenium(IV). Therefore, 3 ml of the reagent solution are used in the method.

The effect of reaction pH is shown in Fig. 8. In the pH range 0–2·5, a constant peak height is obtained. In the recommended procedure, if 5 ml of mixed acid B are added, the pH of the solution, when diluted to 50 ml, is about 1. Therefore, no addition of a buffer solution is necessary.

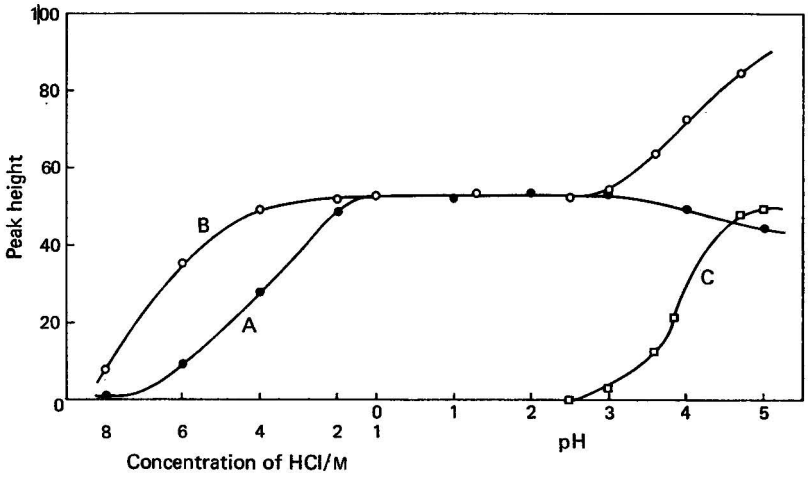

Fig. 8. Effect of the pH (300 μg of Se). A, reaction of 4-chloro-1,2-diaminobenzene with Se(IV); B, extraction of 5-chloropiaselenol; and C, extraction of 4-chloro-1,2-diaminobenzene.

Extraction

Fig. 8 shows the effects on the peak height of the reaction pH and the extraction pH. 4-Chloro-1,2-diaminobenzene reacts quantitatively with selenium(IV) over the pH range 0–2·5. As the excess of reagent is extracted together with the piaselenol and its peak overlaps with that of the piaselenol on the gas chromatogram, the peak height of 5-chloropiaselenol appears to become higher above pH 2·5. As the optimum pH range of the reaction is the same as that of the extraction, adjustment of pH for the extraction is not necessary.

In view of the large distribution ratio of 5-chloropiaselenol with toluene, the constant peak height is obtained by extraction with 1 ml of toluene from 10–60 ml of aqueous solution. (The final volume of the aqueous solution is usually about 50 ml in this procedure.)

As a shaking time of 1 min is the minimum that is sufficient for complete extraction, mechanical shaking for 5 min is used in this method.

Effect of Foreign Ions

In the spectrophotometric method the oxidising agent, particularly vanadium(V) or manganese(VII), interfered seriously in the determination of selenium. Cheng[2] reported that vanadium(V) reacted with 3,3′-diaminobenzidine. Therefore, even if a small amount of vanadium or manganese co-exists with the product, the amount of selenium determined must be corrected by reference to the calibration graph obtained from a solution containing the same amount of vanadium or manganese. Moreover, when more than 3 mg of manganese or 0·3 mg of vanadium is present in 100 mg of the steel, the spectrophotometric method cannot be used to determine the selenium.[5] With the gas-chromatographic method, 3 mg each of vanadium and manganese do not interfere in the determination of the selenium. If 30 mg each of vanadium and manganese are contained in 100 mg of the steel, selenium is recovered only slightly by use of the described procedure. However, as such interference consists in the oxidation of 4-chloro-1,2-diaminobenzene, the addition of a 100-fold excess of 4-chloro-1,2-diaminobenzene over selenium facilitates the quantitative determination of the selenium. With this modification, large amounts of oxidation products are produced, but as they are not extracted into toluene, the chromatogram is unaffected and the determination

is successful. Table I shows the effect of foreign ions. No interference is found, even if a 10-fold excess of the foreign ion is present.

TABLE I

EFFECT OF FOREIGN IONS

Foreign ion	Peak height: 10-fold excess over Se	Peak height: 100-fold excess over Se
None	55·0	
Mn^{7+}	55·3	0
		54·7*
V^{5+}	55·2	2·7
		54·9*
Ag^{+}	54·9	
Ca^{2+}	55·2	
Al^{3+}	54·8	
Cr^{3+}	55·1	
Cd^{2+}	54·8	
Mg^{2+}	55·3	
Mo^{6+}	55·2	
Ni^{2+}	55·0	
Sb^{3+}	54·9	
Zn^{2+}	55·0	
Cu^{2+}	54·8	

* 3 ml of 3 per cent. 4-chloro-1,2-diaminobenzene dihydrochloride solution added.

Calibration

The calibration graphs are constructed by use of two standard samples. One of the samples consists of an acidic solution containing only a known amount of selenium(IV) in the absence of iron, which does not undergo the oxidation procedure. The other is prepared by treating a mixture of elemental selenium and electrolytic iron powder according to the procedure described. A known amount of elemental selenium is obtained from the evaporation to dryness of a carbon disulphide solution of elemental selenium.

The graphs obtained are exactly coincident, both being straight-line graphs passing through the origin. This fact shows that no loss of selenium takes place in the procedure adopted and that the calibration graph can be obtained by use of the standard solutions of selenium(IV).

Determination

The determination of selenium in steel was carried out for three samples of a steel prepared from pure iron and elemental selenium by the Japan Industrial Standards Committee (JIS). The Committee reported on the selenium contents of the samples, determined by means of the extraction - spectrophotometric method using 2,3-diaminonaphthalene.[5] Table II shows the mean values determined at 14 institutes in Japan by the JIS method and also the mean values of six measurements by the proposed method. The results show good agreement.

TABLE II

SELENIUM CONTENT OF STEEL

These samples contained: C, 0·01 per cent.; Si, 0·01 per cent.; and Mn, 0·2 per cent.

Steel	Mean value for selenium by gas-chromatographic method, per cent.* (± standard deviation)	Value for selenium by spectrophotometric method: Mean, per cent.[5]	Value for selenium by spectrophotometric method: Error, per cent.
1	0·107 ± 0·003	0·105	0·019
2	0·215 ± 0·002	0·206	0·030
3	0·329 ± 0·002	0·312	0·038

* Mean value of six measurements.

In the proposed method a gas chromatograph equipped with a thermal conductivity detector is used. The interference caused by vanadium and manganese, which cannot be tolerated by the spectrophotometric method, is of no consequence with this method. The procedure is very simple and rapid, and no special skill is necessary in its use.

The authors thank Mr. Kazuro Kawamura of Nippon Steel Corporation for supplying the steel samples.

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Simple Titrimetric Methods for the Assay of Sulphurated Sodium Borohydride

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Back-titration procedures involving the use of oxidants such as chloramine T, potassium iodate and iodine are described for the accurate assay of sulphurated sodium borohydride. Quantitative reactions are completed within 1 h at room temperature for the concentrations and proportions described. The methods are simple, reproducible and accurate within the limits described.

Sulphurated borohydrides show selective properties as reducing and sulphurating agents that are of great interest in synthetic chemistry. The reduction of several organic functional groups with sulphurated sodium borohydride ($NaBH_2S_3$) has been studied extensively of late[1,2] and the preparation, structure and hydrolysis of this compound have been studied in detail by Lalancette *et al.*[1] The present paper describes some simple, accurate and reproducible oxidative methods for the assay of sulphurated sodium borohydride. In the event that kinetic or stoicheiometric studies are to be carried out on reactions with this compound, the methods of determination described here should be useful as there are no reports on the analytical determination of sulphurated sodium borohydride in the literature.

For the investigation reported here a very pure sample of sulphurated sodium borohydride was prepared according to the method reported in the literature[1] and its purity was checked by infrared and nuclear magnetic resonance spectrometry and elemental analysis. It is established that the $NaBH_2S_3$ is quantitatively oxidised when a large excess of an oxidising agent, *e.g.*, chloramine T, potassium iodate or iodine, is used in a highly alkaline medium (3 M sodium hydroxide). Iodine and iodate are, of course, classical oxidising agents and chloramine T has recently been used as an oxidising agent for the accurate assay of several organic and inorganic compounds.[3–10]

Experimental

Sulphurated Sodium Borohydride ($NaBH_2S_3$)

A mixture of 3·78 g (0·10 mol) of sodium borohydride and 9·60 g (0·30 mol) of sulphur was placed in a three-necked, 500-ml flask that was fitted with a condenser carrying a calcium chloride guard tube at neck 1, the flask being kept in an ice - water bath. Nitrogen was passed into the system through neck 2 in order to prevent the decomposition of the product. Through neck 3, 20 ml of anhydrous tetrahydrofuran were added and the mixture was stirred magnetically. The evolution of gas was brisk and the reaction exothermic. After the initial reaction had subsided, stirring at 25 °C was continued for a further 45 min. The solvent was then evaporated at room temperature under vacuum. In order to remove trace amounts of unreacted sodium borohydride, the product was washed at least ten times with light petroleum each time shaking the flask vigorously. Next, the washed sulphurated sodium borohydride sample was kept overnight in an excess of light petroleum under an atmosphere of nitrogen so as to ensure the complete removal of unreacted materials. The solvent was then decanted and the solid product dried under vacuum at room temperature. The compound obtained was a pale yellow solid and its purity was checked by spectral measurements.

Infrared spectrometry. Measurements were made with a Perkin-Elmer, Model 337, grating spectrometer, with the sample mounted in Nujol. Bands were observed at 1060 (medium), 930 (strong) and 900 (weak) cm^{-1}. No bands were noted at 2300 or 1120 cm^{-1}, clearly showing the absence of even trace amounts of sodium borohydride.

Elemental analyses. Analyses to determine sodium and boron were made with a Beckman DU flame photometer and microanalysis for sulphur was performed by Werby Laboratories, Boston. Found: sodium, 17·2, boron, 8·15 and sulphur, 73·1 per cent. Calculated: sodium, 17·4, boron, 8·2 and sulphur, 72·9 per cent.

Nuclear magnetic resonance spectrometry. Measurements were made with a Varian T-60 instrument. For the prepared $NaBH_2S_3$ in hexamethylphosphoramide (internal standard tetramethylsilane) peaks were observed at 11·20, 11·32 and 11·49 τ. The quadruplet of $NaBH_4$ was absent and a triplet appeared at 11·32 τ. For $NaBH_4$ in hexamethylphosphoramide (internal standard tetramethylsilane) peaks were observed at 8·30, 9·66, 11·00 and 12·37 τ.

Reagents

Sulphurated sodium borohydride solution. A 200–300-mg amount of the sample was weighed accurately and transferred to a dry calibrated flask (500-ml capacity), diluting to the mark with 5 M sodium hydroxide solution. All transfer operations were carried out in an atmosphere of nitrogen; the concentration of the solution was calculated from the mass and relative molecular mass (132·01) of the sample.

Standard chloramine T solution. A 30-g amount of chloramine T (Eastman Kodak Co.) was dissolved in distilled water, diluted to 500 ml, transferred to an amber-coloured bottle and standardised by use of an iodimetric procedure according to the method reported in the literature.[9]

Standard sodium thiosulphate solution, 0·1 N. This solution of sodium thiosulphate in carbon dioxide free distilled water was used and standardised iodimetrically against standard potassium dichromate solution.

Standard potassium iodate solution, 0·04 M. This solution was used and standardised iodimetrically.

Standard iodine solution, 0·1 M. The iodine solution was prepared in water containing the required amount of potassium iodide, stored in a dark brown bottle and standardised against standard thiosulphate solution prior to use.

Potasium iodide solution, 10 per cent.

Sulphuric acid, 5 M.

Amylose (soluble starch) indicator. A 1 per cent. solution was used for all titrations.

The reagents used were all of analytical-reagent grade.

Procedure

An aliquot of the sulphurated sodium borohydride solution (10 ml containing 4 mg of the sample) was added to a 500-ml stoppered conical flask. To this solution about 30 ml of 5 M sodium hydroxide solution were added in order to provide optimum stability (see Results and Discussion), followed by a known excess of the oxidant (for the actual amounts, see Table I) and the mixture was kept for a minimum period of 1 h. The unreacted oxidant was then allowed to react with 20 ml of 10 per cent. potassium iodide solution and 25 ml

TABLE I

OPTIMUM AMOUNTS OF REAGENTS FOR THE ASSAY OF 1 mg OF SULPHURATED SODIUM BOROHYDRIDE

Method	Amount of oxidant for the oxidation*/mg	Reagents for back-titration	
		KI (10 %)/ml	H_2SO_4 (5 M)/ml
Chloramine T	160–220	20–30	25–40
Potassium iodate	90–130	20–30	25–40
Iodine	250–350	—	25–40

* Over-all concentration of 3 M sodium hydroxide should be maintained.

of 5 M sulphuric acid when the oxidant was chloramine T or potassium iodate. The iodine liberated was determined by titrating it against the standard sodium thiosulphate solution, using starch as indicator. For the determination using iodine as the oxidant there was no addition of potassium iodide, but instead the excess of iodine was titrated with sodium thiosulphate solution following the addition of 25 ml of 5 M sulphuric acid. The amount of sulphurated sodium borohydride was calculated from the values of the amount of the oxidant reacted by using the reported stoicheiometry (refer to Table III).

Product analysis was carried out in the instance of the reaction of chloramine T with sulphurated sodium borohydride. It is known that chloramine T undergoes reduction to

toluene-4-sulphonamide ($CH_3C_6H_4SO_2NH_2$) as the product.[5–8,10] By carrying out the reaction on a preparative scale, the above product was isolated in quantitative yield by ether extraction of the reaction mixture and its identity was checked from its melting-point (137 °C). Mixed melting-point determination with an authentic sample of toluene-4-sulphonamide showed no depression.

Results and Discussion

Decomposition Studies

Decomposition studies were performed in order to ascertain the optimum concentration of sodium hydroxide to be used so as to avoid decomposition of the sulphurated sodium borohydride sample. It was noticed that the decomposition of the $NaBH_2S_3$ at lower concentrations of sodium hydroxide (1–2 M) was quite rapid, although at higher concentrations of sodium hydroxide (3–6 M) the compound was highly stable, with negligible decomposition over a period of 10 h. Hence, experiments on the assay were conducted by keeping an over-all sodium hydroxide concentration of 3 M in the reaction medium. Typical results for decomposition at various concentrations of sodium hydroxide are given in Table II (determination of the amount of $NaBH_2S_3$ in each instance was carried out according to the method described under Procedure, using potassium iodate as the oxidant).

TABLE II

DECOMPOSITION STUDIES ON SULPHURATED SODIUM BOROHYDRIDE IN VARIOUS CONCENTRATIONS OF SODIUM HYDROXIDE

Concentration of NaOH/M	Amount of sample* taken/mmol	Amount† of $NaBH_2S_3$ found/mmol	Error, per cent.
1·0	6·02	4·512	25·06
	12·04	9·058	24·76
2·0	6·02	5·286	12·19
	12·04	10·52	12·62
3·0	6·02	5·908	1·86
	12·04	11·82	1·83
4·0	6·02	5·912	1·79
	12·04	11·80	1·99
5·0	6·02	5·909	1·84
	12·04	11·81	1·91

* Actual amount of the sample ($NaBH_2S_3$) calculated from its mass.
† Amount of $NaBH_2S_3$ was determined after 10 h.

Stoicheiometry

In order to establish the stoicheiometry of the reaction, two different experiments, each with different amounts of the reactants, were conducted. In each instance an accurately known amount of the $NaBH_2S_3$ was allowed to react with a large excess of the oxidant solution at a 3 M sodium hydroxide concentration (see Decomposition Studies). The reaction mixture was kept for 10 h in order to ensure completion of the reaction. The amount of the unreacted oxidant was then determined according to the method described under Procedure and the stoicheiometry calculated from the reacted amounts of the reactants. Typical results are given in Table III.

In Table IV the results of the assay of sulphurated sodium borohydride by use of the three oxidants suggested, chloramine T, potassium iodate and iodine, are summarised. Reaction times varying from 15 min to 8 h were examined in each instance. As a result of this study, using different proportions of sulphurated borohydride and the oxidant, the following conclusions were reached.

1. A large excess of the oxidant is required for a quantitative determination. For 1 mg of the $NaBH_2S_3$ sample, 160–220 mg of chloramine T, 90–130 mg of potassium iodate or 250–350 mg of iodine must be used. Further increasing the amount of oxidant in order to decrease the reaction time, *i.e.*, to make the reaction instantaneous, leads to erroneous results.

2. Reduction of these oxidants by sulphurated sodium borohydride is slow, unlike their reduction with sodium borohydride.[9,11,12] For the proportions of the reactants suggested above, it was observed that the reported stoicheiometry in each instance was reached in 1 h.

TABLE III

STOICHEIOMETRY FOR THE REACTION BETWEEN SULPHURATED SODIUM BOROHYDRIDE AND THE OXIDANT

Oxidant	Amount of sample of $NaBH_2S_3$ taken/mmol	Amount of oxidant consumed/mmol	Stoicheiometry,* $NaBH_2S_3$: oxidant
Chloramine T ..	6·02	11·96	1 : 1·986
	12·04	23·98	1 : 1·992
Iodate	6·02	6·012	1 : 0·9987
	12·04	12·03	1 : 0·9992
Iodine	6·02	12·00	1 : 1·994
	12·04	23·96	1 : 1·990

* Stoicheiometry was found to be steady for a period of more than 10 h.

3. A change in the stoicheiometry was noticed after a period of contact between sulphurated sodium borohydride and the oxidant lasting for 24 h, provided that the oxidant was chloramiue T or iodine. This change can be accounted for by the fact that the SH^- ion formed by the hydrolysis of the $NaBH_2S_3$ (equation 4a) can undergo further oxidation. Such difficulties, however, are not encountered in the experimental conditions suggested for the proposed methods because the reported stoicheiometry in each instance would be reached in 1 h; the stoicheiometry is also constant for a period of contact of more than 10 h. When potassium iodate was used as the oxidant, however, no change in the stoicheiometry was noticed, even after a period of 48 h, clearly showing that all the possible reducing agents produced in the system must have been completely oxidised. As iodate is the most powerful oxidising agent of the three under the experimental conditions used, it is very likely that the SH^- ion produced could easily be oxidised by IO_3^- to sulphur.

TABLE IV

ASSAY OF SULPHURATED SODIUM BOROHYDRIDE

Amount of sample taken*/mmol	Time/h	Amount of sample found†/mmol		
		A	B	C
6·02	0·25	4·822	5·024	4·382
	0·50	5·254	5·548	5·028
	0·75	5·908	5·964	5·828
	1	5·922	5·982	5·932
	2	5·918	5·976	5·928
	4	5·920	5·968	5·926
	6	5·916	5·972	5·934
	8	5·920	5·980	5·930
9·03	1	8·882	8·972	8·892
	2	8·890	8·967	8·884
	4	8·886	8·970	8·889
12·04	1	11·84	11·96	11·88
	2	11·78	11·92	11·80
	4	11·80	11·94	11·82

* Actual amount of the sample ($NaBH_2S_3$) calculated from its mass.
† A, when chloramine T was used as the oxidant.
B, when potassium iodate was used as the oxidant.
C, when iodine was used as the oxidant.

Chloramine T as Oxidant

It is known[13,14] that in alkaline solution, chloramine T is hydrolysed as shown in reactions (1) and (2).

$$CH_3C_6H_4SO_2NNaCl + H_2O \rightleftharpoons CH_3C_6H_4SO_2NHCl + NaOH \quad .. \quad .. \quad (1)$$

$$CH_3C_6H_4SO_2NHCl + NaOH \rightleftharpoons CH_3C_6H_4SO_2NH_2 + NaOCl \quad .. \quad .. \quad (2)$$

Under the highly alkaline conditions used in the present investigations (3 M sodium hydroxide),

the hydrolysis reaction could be represented as a one-step reaction:

$$CH_3C_6H_4SO_2NNaCl + H_2O \rightarrow CH_3C_6H_4SO_2NH_2 + NaOCl \quad .. \quad .. \quad (3)$$

Lalancette *et al.*[1] have definitely established that the sulphurated sodium borohydride is hydrolysed according to equation (4):

$$BH_2S_3^- + 3H_2O \rightarrow SH^- + 2S + H_3BO_3 + 4H^+ + 4e^- \quad .. \quad .. \quad (4)$$

From equations (3) and (4), the following mechanism involving a redox couple could be proposed to account for the observed stoicheiometry of chloramine T:$NaBH_2S_3 = 2:1$ and also to explain the over-all reaction. Multiplying equation (3) by 2 we obtain:

$$2CH_3C_6H_4SO_2NNaCl + 2H_2O \rightarrow 2CH_3C_6H_4SO_2NH_2 + 2NaOCl \quad .. \quad (5)$$

Then, recognising that OCl^- is the true oxidant, rather than chloramine T, we have as the reduction half of the couple:

$$4e^- + 4H^+ + 2OCl^- \rightarrow 2Cl^- + 2H_2O \quad .. \quad .. \quad .. \quad (6)$$

which, in alkaline solution, could be written as:

$$4e^- + 2H_2O + 2OCl^- \rightarrow 2Cl^- + 4OH^- \quad .. \quad .. \quad .. \quad (6a)$$

Equation (4) represents the oxidation half of the couple, which, in alkaline solution, can be written as:

$$BH_2S_3^- + 7OH^- \rightarrow SH^- + 2S + 4H_2O + BO_3^{3-} + 4e^- \quad .. \quad .. \quad (4a)$$

Addition of equations (6a) and (4a) results in equation (7):

$$BH_2S_3^- + 2OCl^- + 3OH^- \rightarrow 2H_2O + 2Cl^- + 2S + BO_3^{3-} + SH^- \quad .. \quad (7)$$

Hence, the over-all reaction, obtained by summing equations (5) and (7), is:

$$2CH_3C_6H_4SO_2NNaCl + NaBH_2S_3 + 3NaOH \rightarrow 2CH_3C_6H_4SO_2NH_2 + Na_3BO_3 + NaSH + 2S + 2NaCl \quad .. \quad (8)$$

Iodate as Oxidant

Taking into account all of the observed facts and the usual behaviour of iodate as an oxidising agent, the reaction between $BH_2S_3^-$ and IO_3^- in alkaline solution can be represented by the redox couple

$$BH_2S_3^- + 8OH^- \rightarrow BO_3^{3-} + 3S + 5H_2O + 6e^- \quad .. \quad .. \quad (9)$$

and

$$6e^- + 3H_2O + IO_3^- \rightarrow I^- + 6OH^- \quad .. \quad .. \quad .. \quad (10)$$

Hence, the over-all reaction in this instance is

$$BH_2S_3^- + IO_3^- + 2OH^- \rightarrow BO_3^{3-} + 3S + I^- + 2H_2O \quad .. \quad .. \quad (11)$$

which is in accord with the observed stoicheiometry of $IO_3^-:NaBH_2S_3 = 1:1$.

Iodine as Oxidant

With iodine as the oxidant, an interesting observation, unobserved in the oxidation of $NaBH_2S_3$ with either chloramine T or iodate, has been made. It was noticed that when less than 250 mg of iodine per milligram of the sample were used, the stoicheiometry of $I_2:NaBH_2S_3 = 2:1$ did not hold (Table V). However, such difficulties are not encountered when at least 250 mg of iodine are added per milligram of sample.

In aqueous solutions of iodine, the most likely oxidising species are OI^-, I^+ and IO_3^- ions. If iodate is the oxidising species, then the stoicheiometry should be $I_2:NaBH_2S_3 = 1:1$, which is not the case. Taking OI^- as the likely oxidising species under the highly alkaline conditions, the reaction is probably explained by the following steps:

$$BH_2S_3^- + 7OH^- \rightarrow SH^- + 2S + 4H_2O + BO_3^{3-} + 4e^- \quad .. \quad .. \quad (4a)$$

$$2I_2 + 4OH^- \rightarrow 2OI^- + 2I^- + 2H_2O \quad .. \quad .. \quad .. \quad (12)$$

(which is a well known reaction of iodine in basic solution), followed by:

$$4e^- + 2H_2O + 2OI^- \rightarrow 2I^- + 4OH^- \quad .. \quad .. \quad .. \quad (13)$$

The over-all reaction (obtained by adding (4a), (12) and (13)) is

$$BH_2S_3^- + 2I_2 + 7OH^- \rightarrow SH^- + 2S + 4I^- + BO_3^{3-} + 4H_2O \quad .. \quad (14)$$

thus explaining the observed stoicheiometry of the reaction.

TABLE V

FAILURE OF EXPECTED 1:2 STOICHEIOMETRY FOR SULPHURATED SODIUM BOROHYDRIDE - IODINE REACTION WHEN THE PROPORTION OF $NaBH_2S_3$ TO I_2 IS LESS THAN 1:250 *m/m*

Amount of $NaBH_2S_3$ sample taken*/mmol	Actual amount of sample in the aliquot/mg	Amount of iodine added/mg	Amount of $NaBH_2S_3$ sample determined†/mmol
6·020	3·974	128	11·430
		256	10·280
		384	9·262
		512	8·028
		768	7·024
		896	6·502

* Actual amount of the sample ($NaBH_2S_3$) calculated from its mass.
† Calculated on the basis of the stoicheiometry $NaBH_2S_3 : I_2 = 1:2$.

Of the three oxidants suggested for use, potassium iodate is preferred as it requires smaller amounts of the oxidant and the stoicheiometry is still constant after 24 h of contact.

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An Infrared Spectroscopic Method for the Rapid Simultaneous Determination of Fat and Moisture in Meat and Meat Products

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A method is described for the simultaneous determination of fat and moisture in fresh meat and meat products by cold extraction of both of these constituents into a known volume of solvent consisting of trichloroethylene plus methanol, followed by measurement of the infrared reflectance of the solution at the wavelengths at which fat and water absorb radiation. Multiple attenuated total reflection spectroscopy (multiple ATR) is used to make the infrared measurements and the advantages of this technique over conventional transmission spectrophotometry are discussed.

The rapidity of the method (approximately 5 min per determination of both fat and water on a weighed sample) compensates for its slightly lower degree of precision in comparison with conventional methods, and makes it particularly suitable for use in process control.

Officially accepted methods for the determination of fat in meat and meat products[1,2] involve prolonged Soxhlet extraction of the fat from a well dried sample and are therefore unsuitable for use in process control in which the minimum time lapse between receiving the sample and producing the result is of major importance.

Most rapid methods reported are based on the extraction of fat from the sample into a non-polar solvent followed by measurement of a physical property of the solution, such as density,[3] refractive index[4] or dielectric constant,[5] which is proportional to the fat concentration. These techniques suffer from the disadvantage that water from the sample becomes dispersed in the non-polar solvent during extraction and has to be removed before measurement of the fat concentration can be made. Furthermore, the physical parameter measured is a change in property of the solvent and is, therefore, not specific for the solute (fat) to be determined. Hence, any other material which is extracted from the sample and which affects the particular physical property may alter the apparent concentration, thus tending to limit the range of applicability of this type of determination.

The method described here does not suffer from these disadvantages as the solvent dissolves the fat and water from the sample completely, forming a single homogeneous phase, and the physical property used to measure the fat concentration is the infrared absorption of the carbonyl groups in the glyceride molecules, which is, of course, specific for the solute rather than a property of the solvent. As the water is quantitatively extracted from the sample, measurement of the infrared absorption at a wavelength corresponding to the bending of the O–H bonds of hydroxyl groups in the water molecules yields a direct and simultaneous estimate of the moisture content of the solution.

The behaviour of mixtures of alcohols with chlorinated hydrocarbons as solvents for the extraction of lipids from animal tissue has been studied in considerable detail by Folch *et al.*,[6] Bligh and Dyer[7] and Winter.[8] In particular, chloroform - methanol mixture (2 + 1) has been widely used and is capable of dissolving small amounts of fat and water without separation of the phases. The solubilities of fat and water in a solvent of this type are interdependent in that the higher the concentration of fat the lower the amount of water required to cause phase separation, and *vice versa*. However, the water content of a sample of fresh meat is fixed approximately by the fat content according to the relationship

$$X_w = 0{\cdot}77\ (1-X_f) \qquad (1)$$

where X_w and X_f are the mass fractions of water and fat in the sample, and, for meat products, the value of X_w cannot be greater than that given by the above equation. Thus,

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with samples for which the concentration of fat in the extract is high, the amount of water to be extracted is correspondingly low, a fact that compensates for its decreased solubility in the fat solution. In the present work it was found that the most suitable solvent system for dissolving the amounts of fat and water typically present in a wide variety of fresh and processed meats consisted of the mixture trichloroethylene - methanol (54 + 46 *m/m*). Water and fat could be quantitatively extracted by maceration with 50 ml of this solvent in 2 min and phase separation did not occur provided that the amount of sample taken did not exceed 3 g.

The water and fat extracted from the meat sample contribute significantly to the final volume of the solution, which was shown by experiment to be the sum of the volumes of solvent (corrected for evaporation during maceration), water and fat, if densities of 10^3 and 910 kg m^{-3}, respectively, be assumed for the last two components. From equation (1), the maximum variation in the final volume of the solution when the fat content of the 3-g sample changes from 0 to 100 per cent. *m/m* is from 52·31 to 52·08 ml if the volume of solvent after maceration is 50·0 ml. The error introduced into the calculation of the masses of fat and water from the concentrations is, therefore, likely to be less than 0·5 per cent.

Multiple attenuated total reflection spectroscopy (multiple ATR) was used to measure the infrared absorbances of the solutions. This technique, which has been adequately described elsewhere,[9–11] has the advantage over conventional transmission measurements in that no accurately machined cell with fixed short path length is required, associated problems with interference fringes being thus eradicated. It is necessary only to ensure that the area of contact between the solution and the prism as well as the angle of incidence remain constant. Use of multiple ATR was preferred, therefore, as the apparatus required was more robust and a flow-through cell could be constructed with ease (see Fig. 1). Ideally, a double-pass plate of the type described by Harrick[9] could be used with the solution simply contained in a beaker. Finally, any extraneous particulate matter, which would cause scattering in transmission measurements, does not constitute a problem with multiple ATR because there is no propagation of the infrared beam in the absorbing solution.

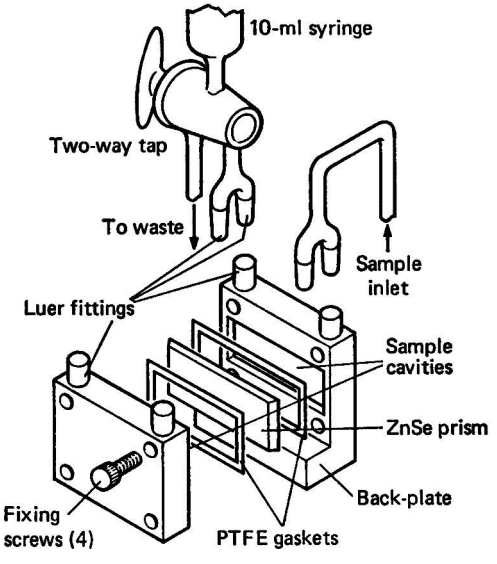

Fig. 1. Stainless-steel multiple ATR cell and filling arrangements. The cell is located on studs on the multiple ATR unit by means of PTFE-sleeved holes drilled into the base of the back-plate.

The percentage reflected intensity (*R*) is defined as the ratio of the light intensity of the sample beam passing through the multiple ATR unit to that of the reference beam, and is measured directly from the transmission scale of the spectrophotometer. It is adjusted to

100 per cent. at 5260 nm (at which wavelength neither fat nor water absorbs significantly) before each measurement is made on a sample; it is then convenient to express the absorbance (A_s) at any wavelength due to the presence of a solute as

$$A_s = \log_{10} R/R_s \quad .. \quad .. \quad .. \quad .. \quad .. \quad (2)$$

where R and R_s are the percentage reflected intensities of pure solvent and solution at that wavelength.

A_s can be used in conjunction with a calibration graph in order to determine the concentration, and hence the mass, of the extracted solute in a known volume of solution. The solutes, in this instance fat and water, have absorption maxima at 5730 and 6080 nm, respectively, in the solvent used.

Experimental

Apparatus

All infrared measurements were made by means of a Perkin-Elmer, Model 237, infrared spectrophotometer in conjunction with the multiple ATR unit, which was a modified version of the Beckman RIIC, Model TR25, with a facility for continuous adjustment of the angle of incidence of the infrared beam. The cell and filling arrangements are shown in Fig. 1. The multiple ATR prism was made of zinc selenide (Spectroscopic Accessory Company) so as to give 21 reflections at an angle of incidence of 45°. A comb-type attenuator was used in the reference beam of the spectrophotometer. A home-made dispenser calibrated to deliver 53·0 ml was used to measure out the solvent, and an MSE homogeniser served to macerate the meat samples.

Reagents

Mixed solvent (solvent A). This solvent was made up in 5-l batches by the addition of 2·70 l of trichloroethylene (BDH Chemicals Ltd, Purified grade) to 2·30 l of methanol (AnalaR grade).

Lard. Lard was used for calibration and was first purified by heating it to 60 °C under vacuum for 4 h, stirring it with anhydrous sodium sulphate and filtering it while hot through a glass-fibre filter-paper (Whatman GF/A). It was then stored in an air-tight jar at −22 °C.

Sample Preparation

Bulk meat samples were prepared in accordance with the British Standard method[12] and stored in air-tight glass jars at −22 °C.

Extraction of the Fat and Moisture

About 3 g of the prepared meat sample were accurately weighed into a vortex flask and 53·0 ml of solvent A were added from the dispenser. The mixture was macerated for 2 min by means of the homogeniser, operating at full speed, and filtered through a glass-fibre filter-paper (Whatman GF/A) into a 50-ml flask, which was then stoppered.

Infrared Measurements

The multiple ATR cell was rinsed through four times with 5-ml portions of the meat extract by means of the syringe (Fig. 1), and the percentage reflected intensity at 5260 nm set to 100 per cent. The intensities at 5730 and 6080 nm were then measured after manual adjustment of the wavelength drum to the appropriate position. The cell was then washed out with five 5-ml portions of solvent, the reflected intensity at 5260 nm was set to 100 per cent. and those at 5730 and 6080 nm were re-measured; the absorbances at these wavelengths were calculated from equation (2).

Calibration Procedure

The multiple ATR cell was filled in turn with pure solvent and solutions in this solvent of lard (2 per cent. m/V) or of water (4 per cent. m/V). The spectrum of each sample was recorded between 5000 and 7000 nm after the value of R at 5260 nm had been set to 100 per cent. (see Fig. 2). Solutions of water show a significant absorption at 5730 nm, the wave-

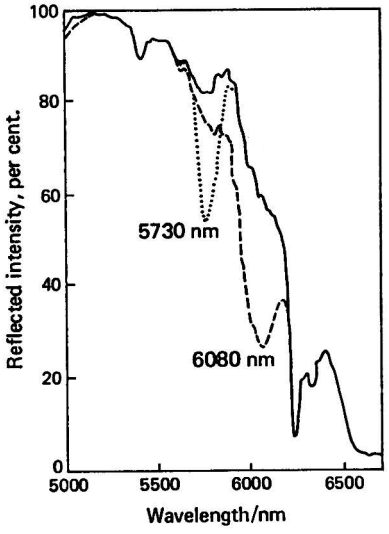

Fig. 2. Multiple ATR spectra of fat and water solutions in solvent A. Angle of incidence 45°; number of reflections 21; and slit width 500 μm. ——— Pure solvent; – – – – water solution 4 per cent. *m/V*; and ····· fat solution 2 per cent. *m/V*.

length at which fat has its absorption maximum. It was therefore necessary to establish a relationship between the absorbances due to water at 5730 nm and at 6080 nm, and to this end solutions of known amounts of water were made up in solvent A and the percentage reflected intensities were measured at these wavelengths as described above. Twelve solutions were made up, each containing sufficient pure lard and water in 50·0 ml of solvent A to give concentrations equivalent to extracts from a 3-g meat sample that had fat contents ranging from 13 to 37 per cent. *m/m*, and the equivalent water contents calculated from equation (1). The percentage reflected intensities were measured at 5730 and 6080 nm as before with the same apparatus and instrument settings as were to be used for the actual fat and water determinations. Calibration graphs for concentration against absorbance calculated from equation (2) were then constructed for fat and water.

Determination of Fat and Moisture

A sample of meat (about 3 g) was accurately weighed and the fat and water were extracted as described above. The absorbances at 5730 and 6080 nm were then measured and, from these values, the fat and moisture contents of the sample were calculated.

In the early experiments, the final volume of solution after extraction was determined by the addition to the solvent of a dye that did not interfere with the infrared readings. Measurement of the visible absorbance of this dye before and after extraction was used to calculate the final volume of solution from the equation:

$$\text{Final volume (ml)} = (A_b/A_a) \times 53{\cdot}0$$

where A_b and A_a are the absorbances of the solution before and after extraction. Oil Red O was suitable for this purpose as a very low concentration gave a convenient absorbance reading (at 523 nm) that obeyed the Beer - Lambert law, and, moreover, there was no interaction with extracted water or fat. It was shown by these experiments that, if the extraction conditions were maintained constant, the final volume of the solution was insensitive to the temperature changes that may occur in the typical laboratory. Under the conditions used here the final volume was always 53·0 ml and the continued inclusion of the dye in the solvent was found to be unnecessary.

Comparison with Standard Methods

The above procedure was compared with the British Standard reference methods for determination of fat[1] and moisture[2] in six aliquots taken from each bulk sample. Soxhlet extraction of the fat was carried out with light petroleum (boiling range 40–60 °C) instead of diethyl ether, which has been shown to give high values with some samples.[13]

Results

Calibration Equations

The relationship between the absorbances of water at 5730 nm (A'_w) and 6080 nm (A_w) was found by linear regression to be

$$A'_w = 0{\cdot}067\ A_w + 0{\cdot}0105$$

The correlation coefficient was 0·948 and the standard error of the estimate of A'_w from A_w was 0·000 56.

The value for A_f, the absorbance due to fat at 5730 nm, is therefore

$$A_f = A'_f - A'_w = A_f - 0{\cdot}067\ A_w - 0{\cdot}0105$$

where A'_f is the experimentally measured absorbance at 5730 nm.

The calibrations for absorbance against fat and water contents of the solutions were determined with weighed amounts of these substances and found to be

$$C_f = 12{\cdot}81\ A_f - 0{\cdot}153 \text{ and } C_w = 14{\cdot}77\ A_w - 0{\cdot}708$$

where C_f and C_w are the fat and water concentrations in grams per 100 ml of solution. The correlation coefficients and standard errors of the estimates of C_f from A_f were 0·9980 and 0·029, respectively, and corresponding values for C_w from A_w were 0·9980 and 0·022, respectively.

Comparison with Standard Methods

The fat and moisture contents as determined by infrared and standard methods are compared in Tables I and II. Each value is the mean of six determinations on the same bulk sample.

TABLE I

FAT CONTENTS OF MEAT AND MEAT PRODUCTS USING DIFFERENT ANALYTICAL METHODS

Bulk sample	Mean fat content, per cent. *m/m*, on "as received" sample		Best estimate of standard deviation	
	British Standard method	Infrared method	British Standard method	Infrared method
Pork luncheon meat (1) ..	31·0	30·4	0·36	0·66
Pork luncheon meat (2) ..	—	30·7	—	0·64
Corned beef	8·3	8·3	0·05	0·20
Beef sausage	30·5	30·8	0·44	0·50
Pork sausage	26·7	26·6	0·45	0·47
Crop steak	25·7	25·3	0·31	0·64
Minced pork	24·6	24·4	0·20	0·65

Discussion

Calibration

The gradients and intercepts of the calibration graphs are, of course, dependent on the instrumental conditions, which must be maintained constant during the measurements. Each laboratory is required to fix its own values for monochromator slit width, sensitivity, angle of incidence of the infrared beam on the multiple ATR prism as well as area of contact between solution and prism in order to optimise the signal to noise ratio of the instrument.

The saponification values of fats from land animals vary[14] from 194 to 197. Thus, the number of carbonyl groups per gram in meat fat is constant with a relative error of less than 1·5 per cent. irrespective of the animal source, and the calibration graph constructed with lard can be used for the determination of fat in beef or lamb as well as in pork.

Comparison of the Methods of Analysis

Tables I and II show the excellent agreement between the results obtained by the infrared and British Standard methods for the determination of fat and moisture. Coefficients of correlation between the two methods calculated from these values were 0·999 for the fat and 0·997 for the moisture. Student's *t*-values for the distribution about zero of the differences between the methods were 1·46 and 1·03 for fat and moisture, respectively, which values are well below the 5 per cent. significance level for six degrees of freedom, so that the two methods are equivalent within the limits of the experimental error.

TABLE II

MOISTURE CONTENTS OF MEAT AND MEAT PRODUCTS USING DIFFERENT ANALYTICAL METHODS

Bulk sample	Mean moisture content, per cent. *m/m*, on "as received" sample		Best estimate of standard deviation	
	British Standard method	Infrared method	British Standard method	Infrared method
Pork luncheon meat (1) ..	49·3	49·2	0·41	0·63
Pork luncheon meat (2) ..	—	49·7	—	0·90
Corned beef	61·1	60·8	0·15	0·36
Beef sausage	48·2	48·5	0·29	0·39
Pork sausage	51·7	51·3	0·50	0·78
Crop steak	56·6	57·0	0·16	0·46
Minced pork	58·4	58·6	0·30	0·50

It can also be seen from Tables I and II that the variance in replicate determinations on the same sample is higher for the infrared method than for the British Standard method. It is likely that some of this decrease in precision can be attributed to sampling errors, as the mass of meat taken for the infrared determination is limited to 3 g by the solubilities of fat and water in the solvent. An increase in sample size could be accommodated by an equivalent increase in volume of the solvent but difficulties would then arise with choice of a suitable method of maceration and increased cost of solvent.

The values for pork luncheon meat (2) in the tables were obtained by the infrared method after extraction of the fat and moisture for 4 min instead of the usual 2 min, account being taken of the difference in the final volume of solution that resulted from increased loss of the solvent by evaporation. The close agreement between these results and those for pork luncheon meat (1), obtained from the same sample by extraction for the usual 2 min, shows that no significant additional amounts of fat or moisture are extracted by maceration for longer periods. Samples of gelatin, glucose and starch, when macerated with the solvent for prolonged periods of time, exhibited the same absorbance at 5730 nm as that of the pure solvent. No significant contribution to this absorbance from meat components other than fat is therefore to be expected.

Rapidity of the Methods

The British Standard methods for fat and moisture determination require approximately 20 and 18 h, respectively, although perhaps during only one tenth of these periods is the attention of the operator needed. The infrared method, on the other hand, requires only about 5 min for both determinations, provided that calibration graphs are available and, although the operator is employed for the whole of this time, the saving in time is obvious. As the results become available so soon after receipt of the sample the method is ideal for use in process control, when speed is a most important factor.

Conclusion

The infrared method for the simultaneous determination of fat and moisture described above is extremely rapid in comparison with the alternative methods currently available and yields results that show excellent correlation with those obtained by the conventional reference procedures. The precision of the method described is, in general, lower than those obtained with the latter methods, but the short time (about 5 min) required to carry out the determinations makes the method attractive for use in process control.

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Analytical Methods Committee

REPORT PREPARED BY THE MEDICINAL ADDITIVES IN ANIMAL FEEDS SUB-COMMITTEE B

The Determination of Robenidine in Animal Feeds and Pre-mixes

The Analytical Methods Committee has received and approved for publication the following Report from its Medicinal Additives in Animal Feeds Sub-Committee B.

Report

The constitution of the Sub-Committee responsible for the preparation of this Report was: Dr. D. R. Williams (Chairman), Mr. G. Drewery, Mr. R. Fawcett (appointed May, 1974), Dr. K. Field, Mr. R. S. Hatfull, Dr. R. McEwan (appointed July, 1974), Mr. K. A. Rawlings (resigned July, 1974), Mr. G. H. Smith, Mr. R. C. Spalding (resigned April, 1974) and Mr. J. E. Stirrup (appointed January, 1974), with Mr. P. W. Shallis as Secretary and Mr. J. J. Wilson as Assistant Secretary.

Introduction

Robenidine [1,3-bis(4-chlorobenzylideneamino)guanidinium chloride] is a coccidiostat that is claimed to be effective against all the major species of *Eimeria* that infect chickens and turkeys. It is included in broiler and turkey feeds at a recommended level of 33 mg kg^{-1} and a pre-mix containing 6·6 per cent. of robenidine in a cereal meal carrier is commercially available. The pre-mix is currently marketed in the UK under the name of "Cycostat."

The Sub-Committee began its work by investigating methods made available by American Cyanamid Company (Princeton Laboratories) and issued by Product Research and Development Laboratories, Europe - Mid-East - African Division, Cyanamid International, for determining robenidine in animal feeds and pre-mixes. In the method proposed for determining robenidine in feeds the drug is extracted with acidified acetone and the extract is cleaned up on a column of aluminium oxide, from which the robenidine is eluted with acetonitrile containing 2-methoxyethanol and ammonia. The eluate is treated with ethanolic potassium hydroxide and the absorbances are read at 440 and 550 nm. The absorbances are corrected by subtracting the absorbances at 440 and 550 nm of the treated eluate after acidification, and the difference between the corrected absorbances is directly proportional to the robenidine concentration, which is determined by reference to a standard graph. For the determination of robenidine in a pre-mix the drug is extracted into methanolic sodium hydroxide and the absorbance of the extract is measured at 352 nm. A correction for background interference is made by subtracting the absorbance at 352 nm of the extract after acidification. The concentration of robenidine in the pre-mix is obtained from the corrected absorbance by reference to a standard graph.

Experimental

Animal Feeds

All members of the Sub-Committee carried out preliminary experiments to familiarise themselves with the method, and this work brought to light certain problems. There was a tendency to obtain low recoveries by the method and it was at first thought that the quality of the acetonitrile and of the aluminium oxide could be of particular importance. Subsequent work, however, showed that no difficulties were caused by the acetonitrile, provided that it had a low water content. In the method, as submitted to the Sub-Committee, the use of Woelm basic aluminium oxide, activity 1, was specified for the chromatography. Those members who used this material found it to be satisfactory on all occasions, but members who used apparently equivalent materials from other sources found that only some of the batches tried performed satisfactorily.

During further work carried out in order to find the reason for the low recoveries of robenidine by the method, it was discovered that the presence of water in the extract from

the feed at the chromatography stage was critical. In one laboratory it was found that if more than about 3 per cent. of water was present in the extract there was a tendency for some of the robenidine to be eluted from the column with the wash liquor during the clean-up stage. In order to overcome this problem the effects of drying the feed in an oven before extraction were investigated. It was found that drying for 15 h at 70 °C appeared to be satisfactory, but that when higher temperatures were used low recoveries of robenidine were obtained. In view of the risk of overheating during the drying of the feed it was considered that drying the extract might prove to be more convenient. All attempts to dry the extract with anhydrous sodium sulphate resulted in subsequent low recoveries of robenidine, but drying with molecular sieve was satisfactory, and this modification was incorporated in the method. The lower limit of the determination was found to be about 3 mg kg^{-1} of robenidine.

Difficulties in the determination of robenidine in turkey feeds containing grass meal were brought to the attention of the Sub-Committee, together with the suggestion that the use of acidic aluminium oxide, activity 1, for the chromatography, could overcome this problem. The Sub-Committee compared the use of acidic and basic aluminium oxides for the determination of robenidine in feeds, both in the presence and absence of grass meal. It was concluded that in the presence of grass meal the use of acidic aluminium oxide could have some advantage, but that in its absence the use of basic aluminium oxide was to be preferred.

A collaborative test of the recommended method was carried out in which a sample of an animal feed pre-medicated at a level of 33 mg kg^{-1} was distributed to the eight participating laboratories. The feed was medicated with a 0·72 per cent. pre-mix and the level of medication was based on the mean of experimentally determined values for the robenidine content of the pre-mix. Each laboratory analysed three separate sub-samples of the bulk sample they received: the results are shown in Table I.

TABLE I

DETERMINATION OF ROBENIDINE IN ANIMAL FEED BY THE RECOMMENDED METHOD

Laboratory	Robenidine found, mg kg^{-1}, in sub-sample 1	2	3	Mean value/mg kg^{-1}
A	32·7	31·8	31·8	32·10
B	30·8	29·2	33·2	31·07
C	32·0	32·4	32·0	32·13
D	33·9	35·0	33·9	34·27
E	33·2	33·1	—	33·15
F	33·0	33·0	31·0	32·33
G	29·2	28·3	30·2	29·23
H	27·2	28·0	29·2	28·13
Standard deviation/mg kg^{-1}				1·03
Coefficient of variation, per cent.				3·29
Critical difference among means				2·95
Significant difference for comparison with a constant				1·52

Results and discussion

The results obtained in the collaborative test are considered to be satisfactory. A statistical evaluation of the results was carried out and showed that the mean results from Laboratories B, G and H are significantly different from the value of 33 mg kg^{-1}. It also showed that the mean value for Laboratory B is significantly less than that for Laboratory D, that the mean value for Laboratory G is significantly less than those for Laboratories D, E and F, and that the mean value for Laboratory H is significantly less than those for Laboratories A, C, D, E and F. The recommended method is given in Appendix I.

It has been reported to the Sub-Committee that nitrofurazone (100 mg kg^{-1}), acinitrazole (500 mg kg^{-1}) and enheptin (500 mg kg^{-1}) present in a feed will interfere in the determination of robenidine by exhibiting a small apparent robenidine content. Gross interference is caused by the presence of dinitolmide in a feed, but it has been reported that this interference can be overcome by using acidic aluminium oxide de-activated with 1 per cent. *m/m* of water in place of the basic aluminium oxide specified.

Pre-mixes

All members of the Sub-Committee carried out some preliminary investigations of the method on a pre-mix containing 6·6 per cent. of robenidine. No serious problems were encountered in this work, although some minor modifications were incorporated in the method. Satisfactory results were obtained in all laboratories.

A collaborative test was arranged in which all laboratories carried out three separate determinations on a pre-mix containing 6·6 per cent. of robenidine.

Results

The results of a collaborative test on a pre-mix that was specially prepared to contain 6·6 per cent. of robenidine are given in Table II. The number of results available was not sufficient to permit a full statistical analysis, but the standard deviations were calculated.

TABLE II

DETERMINATION OF ROBENIDINE IN A PRE-MIX BY THE RECOMMENDED METHOD

Laboratory	Robenidine found, per cent., in sub-sample 1	2	3	Mean value, per cent.	Standard deviation, per cent.
A	6·54	6·54	6·58	6·55	0·0231
B	6·58	6·83	6·86	6·76	0·1537
D	6·6	6·4	6·3	6·43	0·1528
E	6·63	6·74	6·73	6·70	0·0608
F	6·99	6·83	6·83	6·88	0·0924
G	6·83	6·75	6·75	6·78	0·0462
H	6·50	6·42	6·50	6·47	0·0462

The differences between the standard deviations are not significant, although they are apparently large because of the small sample size. The recommended method is given in Appendix II.

Recommendation

The Sub-Committee recommends that the methods given in Appendixes I and II should be used appropriately for the determination of robenidine in animal feedingstuffs and pre-mixes.

APPENDIX I

Recommended Method for the Determination of Robenidine in Animal Feeds

Scope and Field of Application

The method is applicable to the determination of robenidine in poultry feeds from the normal levels of inclusion down to about 3 mg kg^{-1}. The presence of grass meal, nitrofurazone, acinitrazole, enheptin and dinitolmide in the feed might cause interference in the determination.

Principle

Robenidine is extracted from the feed with acidified acetone and the extract is dried with molecular sieve. A portion of the dried extract is cleaned up on a column of deactivated basic aluminium oxide and the robenidine is eluted with acetonitrile containing 2-methoxyethanol and ammonia. The eluate is treated with ethanolic potassium hydroxide and the absorbances of this solution are read at 440 and 550 nm. The treated eluate is acidified and the absorbances are again measured at 440 and 550 nm. The difference in absorbance at 440 nm less the difference at 550 nm is directly proportional to the robenidine concentration, which is determined by reference to a standard graph.

Reagents

Except when otherwise stated, analytical-reagent grade reagents were used.

Robenidine standard.

Acetone.

Acetonitrile.

Aluminium oxide. Weigh, to the nearest 0·5 g, 200 g of Woelm basic aluminium oxide, activity 1, and transfer it into a 500-ml conical flask. From a pipette run 2 ml of water down the inner wall of the flask, insert the stopper, and shake the flask vigorously for 2 min. Store the aluminium oxide in a sealed container and set aside overnight before use.

Ammonia solution, density 0·88 *g* ml^{-1}.

Ethanol.

Hydrochloric acid, density 1·19 *g* ml^{-1}.

Methanol.

2-Methoxyethanol.

Molecular sieve. Type 3A, 8–12-mesh beads.

Potassium hydroxide.

Trichloroacetic acid.

Robenidine standard solution. Weigh accurately, to the nearest 0·001 g, 0·1 g of robenidine standard into a 250-ml calibrated flask (see Procedure). Add some methanol to the contents of the flask, shake it on a mechanical shaker until the solid has dissolved and make the volume up to the mark with methanol (solution A).

1 ml of solution ≡ 400 μg of robenidine.

Extraction solvent. With a pipette place 8·3 ml of hydrochloric acid in a 1-l calibrated flask, and make the volume up to the mark with acetone. Prepare this solvent freshly each day.

Wash solvent. Place 100 ml of 2-methoxyethanol in a 1-l calibrated flask and make the volume up to the mark with acetonitrile.

Elution solvent. Place 40 ml of ammonia solution in a 1-l calibrated flask and make the volume up to the mark with wash solvent.

Ethanolic potassium hydroxide solution. Weigh, to the nearest 0·2 g, 3 g of potassium hydroxide and transfer it into a 100-ml calibrated flask. Dissolve it in ethanol and make the volume up to the mark with the same solvent.

Trichloroacetic acid solution. Weigh, to the nearest 0·1 g, 2 g of trichloroacetic acid and transfer it into a stoppered flask. Dissolve it in 4 ml of wash solvent.

Apparatus

Chromatographic columns. Glass tubes 250 mm long and of internal diameter 10·5 mm. Taps, if fitted, must be made of polytetrafluoroethylene.

Calibrated cylinder. Calibrate a glass cylinder with distilled water and mark it at 11 ml.

Mechanical shaker.

Spectrophotometer, with 20-mm cells.

Procedure

Caution. Robenidine solutions are light sensitive and must be shielded at all times by means such as the use of low actinic glassware or performing the relevant operations in a darkened room. Solution A can be used for about 1 week, whereas all other solutions should be prepared freshly each day.

Extraction

Weigh, to the nearest 0·1 g, 50 g of test sample into a 1-l flask. Add 8 ml of extraction solvent for each gram of sample taken, and insert a stopper in the flask. Shake the flask for 1 h on the mechanical shaker, and then set it aside for 5 min in order to allow the solids to settle. Filter the solution, by gravity, through a Whatman No. 42, or equivalent, filter-paper into a 100-ml calibrated flask. Collect approximately 100 ml of filtrate and add 5 g of molecular sieve. Shake the mixture for about 5 min, and then allow the sieve to settle.

Preparation of chromatographic columns

Compress a small plug of glass-wool into the lower end of a chromatographic column. Measure in the calibrated cylinder 11 ml of prepared aluminium oxide and transfer it to the column, ensuring the minimum exposure to the atmosphere. Tap the column to settle the aluminium oxide and open wide the column taps, if fitted.

Chromatography

With a pipette transfer 5 ml of the sample extract into the column, resting the pipette tip against the column wall. When the sample extract has been absorbed on to the aluminium oxide, add from a pipette 15 ml of wash solvent. After about 5 ml of wash solvent has been absorbed, stir, with a glass rod, the top 20 mm of the aluminium oxide column to remove air bubbles. Collect the column effluent in a beaker and, when the flow has ceased, discard the effluent. Place a 50-ml calibrated flask under the column, from a pipette run 20 ml of elution solvent into the column and collect all the eluate in the flask.

Determination

Swirl the collection flask to mix the contents and add from a pipette 1 ml of ethanolic potassium hydroxide (see Note 1). Shake the flask, transfer a portion of the solution to a 20-mm spectrophotometer cell and measure the absorbances against the elution solvent at 440 and 550 nm. Acidify the contents of the cell (see Note 2) with 0·5 ml of trichloroacetic acid solution. Stopper the cell, shake it and re-measure the absorbances at 440 and 550 nm.

Preparation of standard solutions

With a pipette place 5·0 ml of solution A into a 100-ml calibrated flask and dilute to the mark with extraction solvent. Mix well, transfer 5·0-, 10·0-, 20·0- and 40·0-ml portions of this solution to four separate 100-ml calibrated flasks, and dilute each to the mark with extraction solvent. These solutions are equivalent to 1, 2, 4 and 8 μg ml^{-1} of robenidine, respectively.

Preparation of standard graph

Set up four chromatographic columns as described above. By pipette transfer a 5-ml portion of each standard solution on to a separate chromatographic column and continue as described above under *Chromatography* and *Determination*. Prepare a standard graph by plotting absorbance against the concentration of robenidine in the standard solutions (see Note 3).

Calculation of Results

Determine the absorbance due to robenidine from the expression

$$A = B_{440} - B_{550} - (A_{440} - A_{550})$$

where

B_{440} = absorbance of the alkaline solution at 440 nm
B_{550} = absorbance of the alkaline solution at 550 nm
A_{440} = absorbance of the acidic solution at 440 nm
A_{550} = absorbance of the acidic solution at 550 nm.

Read the concentration of robenidine in the test solution equivalent to A from the standard graph.

Calculate the concentration of robenidine in the sample from the expression

$$\text{Robenidine, p.p.m.} = \frac{c \times y}{m}$$

where

y ml = volume of extraction solvent
m g = mass of sample taken
c μg ml^{-1} = concentration of robenidine in the test solution as determined from the standard graph.

NOTES—

1. After the contents of the collection flask have been made alkaline the four absorbance readings must be made within about 3 min.
2. If optical cells of small volume are used, acidification of a fixed volume of the solution in a separate container should be effected rather than acidification within the cell.
3. The standard graph is plotted in terms of the concentrations of the standard solutions and the gradient obtained is not an absolute extinction value. For this reason identical procedures are applied to the 5-ml portions of sample extract and standard solutions.

APPENDIX II

Recommended Method for the Determination of Robenidine in Pre-mixes

Scope and Field of Application

The method is generally applicable to the determination of robenidine in pre-mixes with inert or cereal carriers.

Principle

Robenidine is extracted from the pre-mix sample with a 0·05 M solution of sodium hydroxide in methanol. A portion of the extract is acidified with trichloroacetic acid and the absorbance at 352 nm of the acidified extract is subtracted from the absorbance at 352 nm of the alkaline extract to give the corrected absorbance. The concentration of robenidine in the sample is then calculated by reference to a standard graph.

Reagents

Robenidine standard.
Methanol.
Sodium hydroxide pellets.
Trichloroacetic acid solution, 50 g l^{-1} *in methanol.*
Sodium hydroxide solution in methanol, approximately 0·05 M. Dissolve 2·0 g of sodium hydroxide in 1000 ml of methanol. Clarify the solution by filtration through a hardened filter-paper.
Robenidine standard solution. Weigh, to the nearest 1 mg, 0·100 g of robenidine and transfer it into a 250-ml calibrated flask. Add approximately 200 ml of sodium hydroxide solution in methanol, shake the flask on a mechanical shaker for 15 min, dilute to volume with sodium hydroxide solution in methanol and mix well (solution A). By pipette transfer 10·0 ml of solution A into a 100-ml calibrated flask, and dilute to volume with methanol (solution B).

Apparatus

Spectrophotometer. With hydrogen light source and 10-mm silica cells.
Filter-paper. Hardened, fluted.

Procedure

Caution. Robenidine solutions are light sensitive and must be shielded at all times by means such as the use of low actinic glassware or performing the relevant operations in a darkened room.

Test portion

Weigh, to the nearest 1 mg, about 10 g of the pre-mix sample, transfer it into a suitably stoppered flask or bottle of capacity greater than 300 ml, and add exactly 250 ml of sodium hydroxide solution in methanol. Place the container on a mechanical shaker, shake it for 25 min and filter the mixture.

Dilution

By pipette transfer 5·0 ml of the clear filtrate into a 50-ml calibrated flask, dilute to volume with methanol and mix well. By pipette transfer 5·0 ml of this solution into another 50-ml calibrated flask, dilute to volume with methanol and mix well. By pipette transfer 15·0 ml of this solution into a 100-ml calibrated flask, dilute to volume with sodium hydroxide solution in methanol and mix well.

Measurement

Read the absorbance of this solution immediately after dilution to volume at 352 nm in a 10-mm silica cell against sodium hydroxide solution in methanol. Transfer approximately 10 ml of the solution into a small flask, add one drop of trichloroacetic acid solution and mix well (the faint yellow colour will disappear). Read the absorbance of the acidified solution at 352 nm in a 10-mm silica cell against sodium hydroxide solution in methanol. Take all readings as quickly as possible after placing the cell in the spectrophotometer. Determine

the corrected absorbance by subtracting the absorbance of the acidified solution from the absorbance of the alkaline solution.

Standard graph

By pipette transfer 5-, 10-, 15- and 20-ml portions of solution B into separate 100-ml calibrated flasks. Dilute each to volume with sodium hydroxide solution in methanol so as to produce solutions containing 2, 4, 6 and 8 μg ml^{-1} of robenidine, respectively, and, immediately after each separate dilution has been made, determine the corrected absorbance of the solution as described under *Measurement*. Construct a graph of concentration of robenidine in micrograms per millilitre against the corrected absorbance.

Calculation of Results

Calculate the robenidine content of the pre-mix from the expression

$$\text{Robenidine, per cent.} = \frac{A}{m} \times 16{\cdot}67$$

where

A μg ml^{-1} = concentration of robenidine in test portion as read from the calibration graph
m g = mass of sample taken.

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Analytical Methods Committee

REPORT PREPARED BY THE COMPLEXIMETRIC STANDARDS PANEL OF THE ANALYTICAL STANDARDS SUB-COMMITTEE

The Precise Standardisation of Disodium Dihydrogen Ethylenediaminetetraacetate Dihydrate by Spectrophotometric Titration against Pure Bismuth Metal

The Analytical Methods Committee has received and approved for publication the following Report from its Analytical Standards Sub-Committee.

Report

In 1968 the Analytical Methods Committee received a request from Commission V3 of the International Union of Pure and Applied Chemistry to consider the possibility of recommending suitable standards for use in complexiметric titrations. The Analytical Methods Committee passed this request to the Analytical Standards Sub-Committee, which had been set up in 1960 at the request of IUPAC to recommend standard substances for use in acid - base titrimetry. It was decided by the Sub-Committee that some priority should be given to work on complexiметric standards and, so as not to interfere with the programme of work on acid - base standards that was in hand, the Complexiметric Standards Panel was formed. The constitution of the Panel, which was responsible for the preparation of this Report, was: Professor E. Bishop (Chairman), Mr. H. Bennett, Mr. D. J. Bucknell, Mr. A. G. Hill, Dr. R. G. Monk, Dr. E. J. Newman (resigned May, 1970), Mr. H. N. Redman, Dr. J. M. Skinner, Dr. W. I. Stephen, Mr. W. Westwood, Dr. W. J. Williams and Dr. C. Woodward (resigned September, 1973), with Mr. P. W. Shallis as Secretary and Mr. J. J. Wilson as Assistant Secretary.

Introduction

The most widely used complexing agent is undoubtedly disodium dihydrogen ethylenediaminetetraacetate dihydrate (EDTA) and the Panel decided in the first instance to concentrate its efforts on the standardisation of this material for use in complexiметric titrations. The presence in EDTA of nitrilotriacetic acid is known to be a complicating factor, and for this reason a specially selected batch of EDTA known to contain less than 100 $\mu g\ g^{-1}$ of nitrilotriacetic acid has been used throughout the Panel's work.

Although the Panel had no difficulty in deciding upon the most appropriate complexing agent as a first consideration, the position with regard to the titration systems to be investigated was far less clear. EDTA has been recommended as the complexing agent for the titration of many metals, oxides and salts and several different indicators have been proposed for a visual or spectrophotometric detection of the end-point. The Panel decided at the outset to attempt to concentrate on visual detection of the end-point, as this was the system that was most widely used in practice.

Information was collected by the Panel on the use of mercury(II), lead, bismuth, cadmium, zinc, calcium and magnesium for the standardisation of EDTA. This survey showed the most commonly used indicators to be xylenol orange, dithizone, Eriochrome black T and Acid Alizarin black SN. The Panel, however, held reservations about the value of some of these indicators for use in the high-precision assay of EDTA. Of these indicators only dithizone was known to be commercially available in a pure state but this material suffered the disadvantage of being unstable in solution; the others are either mixed substances or were known to contain related active impurities.

It was decided that as a first stage of the work the availability of pure indicators should be investigated. A paper-chromatographic investigation of many of the commercially available indicators showed that all except a sample of catechol violet contained more than

one coloured component. Attempts were made to produce pure xylenol orange and semi-xylenol orange by the method proposed by Murakami, Yoshino and Harasawa,[1] but in all instances a mixed product was obtained. An unpublished method for the preparation of pure methylthymol blue was also investigated, but again a product containing no other coloured constituents could not be obtained.

As a result of this survey of indicators the Panel decided to begin its work on the precise assay of EDTA by investigating the titrations of bismuth with EDTA, using catechol violet as indicator, and of zinc and cadmium with EDTA, using dithizone as indicator. To maximise the sensitivity of equivalence point detection and to enable mathematical evaluation of the relevant equilibrium constants to be carried out under titration conditions it was decided to use the spectrophotometric titrations as far as was possible.

The work carried out on the titration of bismuth with EDTA, using catechol violet as the indicator, is described here. The recommended procedure for the precise standardisation of EDTA for use in the titration of bismuth is given in the Appendix. It is intended in due course to recommend other metals or pure compounds as standards. Although it is possible that EDTA solutions standardised by the recommended method may be usable for the titration of other cations, it must be emphasised that the Panel has not examined this aspect. It would be essential that the conditional formation constants of indicator and EDTA complexes be closely similar to those for bismuth, that the experimental conditions be identical and that the cation react with the nitrilotriacetic acid impurity in the EDTA.

The Precise Assay of EDTA with Use of Pure Bismuth Metal

The advantages of bismuth metal as a standard for the complexiметric assay of EDTA are: that it is a mono-nuclidic element and therefore of highly accurately known relative atomic mass; that the high equivalent mass of the metal minimises weighing errors; that the high stability of the bismuth - EDTA complex allows the titration to be carried out at an acidity of about 0·01 mol l^{-1} of nitric acid, at which acidity few other cations interfere; and that the metal is stable in air and requires no handling precautions to be taken in order to prevent oxidation. The occasional occurrence of blow-holes in the rod must be watched for, however, as is mentioned below.

Indicators

Initially, xylenol orange and semi-xylenol orange were considered as possible indicators for the titration of bismuth with EDTA but were abandoned owing to the non-availability of either compound in a pure state. Commercial xylenol orange is a mixture of xylenol orange, semi-xylenol orange and residual impurities derived from its preparation, mainly cresol red and iminodiacetic acid. For the assessment of the true equivalence point of the titration it was required that the conditional formation constants of the complexes of bismuth with EDTA should be determinable and for this reason a pure indicator was required.

A commercial sample of catechol violet (CV) that had been shown by paper and thin-layer chromatography to contain only one coloured constituent was available. This material was used in all further work after it had been kept over saturated sodium hydroxide solution under vacuum in order to remove most of the 15–20 per cent. of acetic acid remaining from the preparation process and then conditioned over moist magnesium nitrate hexahydrate (about 50 per cent. relative humidity).

The sample was titrated potentiometrically with sodium hydroxide solution (glass - saturated calomel cell) and was found to contain 93·1 $\pm$ 0·5 per cent. of catechol violet and 2·1 per cent. of acetic acid, the uncertainty being mainly due to the proximity of the two potential breaks, corresponding to the sulphonic acid group of the catechol violet and to acetic acid.

The water content of the catechol violet was found to be 5·1 per cent. by drying *in vacuo* over magnesium perchlorate. The conditioned sample was found to be stable to normal variations in atmospheric humidity.

Complexes of Bismuth and Catechol Violet

Catechol violet forms 1:1 and 2:1 metal - indicator complexes with many cations. Bismuth is typical in this respect and forms a blue Bi_2CV complex (absorbance maximum 605 to 610 nm) and a red BiCV complex (absorbance maximum about 540 nm). Conditional formation constants of these two species in 0·01 mol l^{-1} nitric acid were determined by spectro-

photometry and values for log K_1 (K_1 = [BiCV]/[Bi][CV]) and log K_2 (K_2 = [Bi_2CV]/[BiCV][Bi]) of 5·4 and 5·3, respectively, were obtained. In the presence of 1 mol l^{-1} of ammonium nitrate both of these values fell to 4·9.

When a solution of bismuth nitrate is titrated with EDTA, using catechol violet as the indicator, at a pH of about 2·0 (0·01 mol l^{-1} nitric acid), the colour changes from the blue of Bi_2CV to the yellow of the protonated catechol violet through a series of shades of violet, red and orange. The changes are due to the continuous decrease in [Bi_2CV] and the rise to a maximum of [BiCV], followed by decomposition of this complex by EDTA.

Standard Bismuth Metal

The sample of bismuth rod used in the work was analysed to determine gaseous elements by vacuum fusion and other impurities by mass spectrometry. Determination of oxygen was also achieved by fast neutron activation. Vacuum fusion gave a value of 4 μg g^{-1} and neutron activation not more than 22 μg g^{-1} of oxygen, while the sum of all other elements was 13 μg g^{-1}. It appears, therefore, that the metal was 99·997 to 99·998 per cent. pure. In all assays of EDTA a bismuth purity of exactly 100 per cent. was used for calculation purposes.

Some variability in the oxygen content was indicated by the neutron-activation determinations, this being occasioned by the presence of blow-holes in the metal. A sample taken in the region of a blow-hole and showing a dark, oxidised surface contained only 5 μg g^{-1} more oxygen than clean samples, so that it appears that blow-holes do not constitute a serious problem and that the colour of a film on metallic bismuth is a particularly sensitive indication of oxygen.

In the assay of EDTA using bismuth the effect of blow-holes was minimised by sawing the metal bar longitudinally and transversely so as to increase the probability of their detection. Any metal covered with an oxide film was cleaned in acid as described in the method.

EDTA

A selected batch of EDTA was used in the work and it contained less than 60 μg g^{-1} of nitrilotriacetic acid. It is known that nitrilotriacetic acid can be used to titrate bismuth with catechol violet as the indicator, so that any nitrilotriacetic acid present will be included and calculated as EDTA. The EDTA sample also contained a very small amount of a white inert material that was initially heterogeneously distributed and that gave a faintly turbid solution. The batch was therefore thoroughly homogenised before the comparative inter-laboratory determinations were carried out.

Practical Problems

Owing to the tendency of bismuth to hydrolyse, with precipitation of basic bismuth salts, it is necessary to maintain a high acidity. It is not, therefore, possible to carry out the whole of a bismuth - EDTA titration at a pH of 2·0 or more and, in fact, only about 0·2 per cent. of the EDTA can be titrated at such a low acidity. Most of the reactants must, therefore, be mixed in about 1 mol l^{-1} acid, under which conditions there is a risk of precipitation of free EDTA. It is possible to dissolve solid EDTA in the acidic bismuth solution, but the process is slow and heating is required. In practice, no difficulty has been experienced in adding the EDTA dissolved in ammonia solution to the bismuth solution provided that stirring has been adequate.

In any titration mass action and kinetic considerations make it desirable to maintain as high a concentration of reactants as possible. In the work carried out it was found that the solubility of the bismuth - EDTA complex limited the reactant concentrations to about 0·08 mol l^{-1}. Above this concentration, and in the presence of about a 0·2 per cent. excess of bismuth, precipitation occurred. The fact that the precipitate was the bismuth - EDTA complex and not a basic bismuth compound was shown by its dissolution on dilution with water.

Experimental Spectrophotometric Titrations

In order to determine the conditional formation constants of the relevant complexes under titration conditions and also the position of the equivalence point on the titration graph, spectrophotometric titrations were carried out in which absorbances were measured at 540

and 610 nm, the wavelength maxima of BiCV and Bi_2CV, respectively, after each increment of titrant. The concentrations of BiCV, Bi_2CV and free catechol violet were calculated and the results analysed by means of a least-squares computer program, whereby the three conditional formation constants and the percentage of EDTA in the sample were obtained.

Values in the range 4·15–4·35 were found for the logarithmic formation constants of BiCV and Bi_2CV, while values for log K_L, the conditional formation constant for the bismuth - EDTA complex, lay between 10·7 and 12·5. The latter constant depends critically on values obtained around the equivalence point and cannot be of high accuracy when, as in this instance, K_L is high and the concentrations of BiCV and Bi_2CV are very low. Nevertheless, the results give a clear indication that the complex is of satisfactorily high stability, log K_L being about 11; variations in this value have little effect on the equivalence point unless the value falls below about 10·5, when the region of the graph before and after equivalence becomes more rounded. As it is, virtually complete decomposition of both bismuth - catechol violet complexes occurs at less than a 0·02 per cent. excess of EDTA and the absorbances at 540 nm and 610 nm become constant. For this titration the graph of amount of titrant against absorbance at 540 nm is straight for an EDTA to bismuth ratio between 99·985 and 99·995 mol per cent. and the intersection of this line and the horizontal through the value of the absorbance at 540 nm with an excess of EDTA was found to occur between 100·000 and 100·002 mol per cent.

The low values for K_1 and K_2 can only be partially accounted for by the presence of 0·5 to 1·0 mol l^{-1} concentrations of sodium and ammonium nitrates in the solution and must also be associated in some manner with the bismuth - EDTA complex.

Results for a typical titration experiment are shown in Fig. 1, together with the values of the constants given by the least-squares treatment for this titration. The graphs plotted have been computed from these constants and the appropriate reagent concentrations.

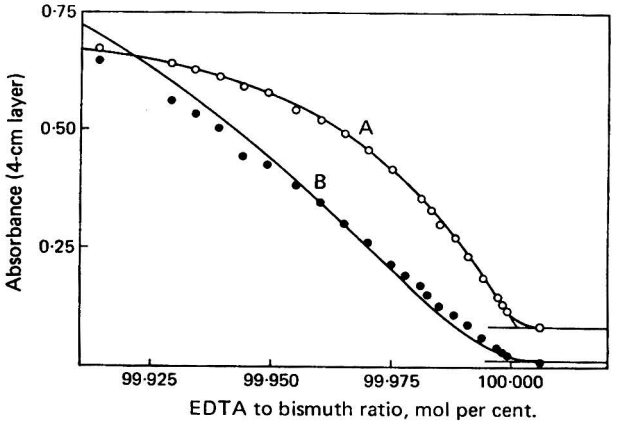

Fig. 1. Computed spectrophotometric titration graphs for EDTA and bismuth. Experimental data: pH, 2·0; A, 540 nm (BiCV maximum); B, 610 nm (Bi_2CV maximum); total bismuth = 0·095 930 mol l^{-1}; catechol violet concentration = 8·80 μmol l^{-1}. Least-squares stability constants: log K_1 = 4·38; log K_2 = 4·18; log K_L = 10·86.

Development of Recommended Method

Having established the fundamentals of the titration further experiments showed that some advantage was to be gained by increasing the pH to about 2·4 and measuring the absorbance at 560 nm. Under these conditions the final constant absorbance with excess of EDTA was reduced to rather less than half of its value at pH 2·0 and 540 nm and the slope of the linear portion of the graph slightly increased. The results agreed to within 0·002 per cent. of those given by the original procedure.

The use of a pH greater than about 2·5 is not recommended as on one occasion the attempted use of a pH of 3·0 gave rise to drifting absorbance readings and it appeared that hydrolysis

of bismuth was giving rise to slow reaction rates. Fig. 2 shows a typical titration graph obtained by use of the recommended method.

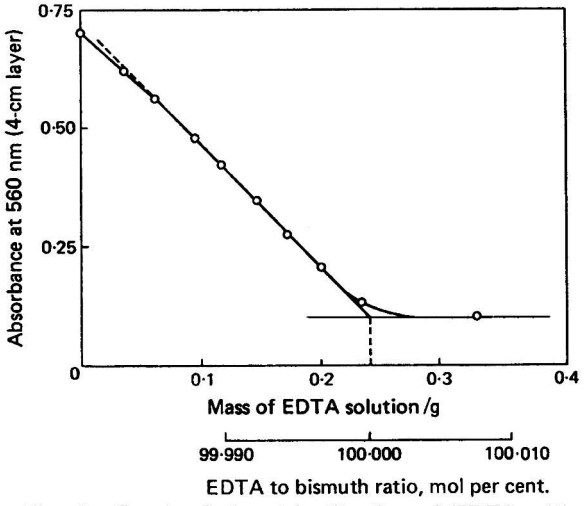

Fig. 2. Spectrophotometric titration of EDTA with bismuth by recommended method. Standard EDTA concentration = 0·003 713 g g^{-1}.

Results of collaborative assays

The results of collaborative assays of the homogenised sample of EDTA in four laboratories by the recommended method are shown in Table I.

TABLE I

RESULTS OF COLLABORATIVE ASSAYS

Laboratory		EDTA content, as $[CH_2N(CH_2COOH)(CH_2COONa)]_2.H_2O$, per cent.			Mean, per cent.	Relative standard deviation, per cent.
A		99·926,	99·928,	99·930	99·928	0·002
B		99·936,	99·934,	99·930,	99·932	0·004
		99·923,	99·933,	99·931,		
		99·929,	99·934,	99·934		
C		99·941,	99·942,	99·938	99·940	0·002
D	(a)	99·938,	99·930,	99·935,	99·933	0·004
		99·927,	99·935	99·928		
		(99·909),*	99·937,	99·930		
	(b)	99·937,	99·944,	99·935,	99·937	0·004
		99·933,	99·935,	99·939,		
		99·936				
D (a) + (b)					99·935	0·004
				Over-all mean	99·934	0·005

* Omitted from calculation of mean and standard deviation as a result of three statistical tests.[3–6]

The (b) set of results for Laboratory D were obtained by use of a modified procedure that was intended to minimise the risk of loss of EDTA during its addition to the bismuth solution. The difference between the means of sets (a) and (b) was rather below the borderline of significance as shown by the *t*-test ($1·9 < 2·2$) and they have therefore been combined to give a mean of 99·935 and a relative standard deviation of 0·004 per cent.

An analysis of variance of results from all laboratories gives a relative standard deviation of 0·004 per cent. within a laboratory and 0·010 per cent. between laboratories.

A final justification of the rejection of the single low value of 99·909 is the fact that the deviation of this value from the over-all mean is 0·025, which is equal to five standard deviations. The probability of a deviation of this magnitude is only $2·9 \times 10^{-7}$ for a single-sided test, or $8·9 \times 10^{-6}$ in 31 determinations.

APPENDIX

Recommended Method for the Precise Assay of EDTA by Spectrophotometric Titration with Bismuth Metal

Reagents

Bismuth metal rod, purity 99·999 per cent. With a small hacksaw fitted with a clean, fine-toothed blade make a 1–2 cm longitudinal cut down the axis of the rod. Then saw across the rod to give hemicylindrical pieces of metal 2–5 mm thick and of mass 0·4–1 g. (This method of cutting gives a good chance of exposing blow-holes.) If the metal has a dull oxide film or blow-holes, immerse it in 4 mol l^{-1} hydrochloric acid until it is clean (which should take only a few seconds), wash it once in 1 mol l^{-1} hydrochloric acid, then thoroughly with water and finally twice with redistilled acetone. Then dry it in a current of air. If the metal is clean and bright carry out the acetone wash only.

Nitric acid, 12 mol l^{-1}. Dilute three volumes of analytical-reagent grade nitric acid, sp. gr. 1·42, with one volume of water.

Ammonia solution, 3 mol l^{-1}.

Sulphamic acid solution. Dissolve 5 g of sulphamic acid in 100 ml of water. Do not keep this solution for more than 3 months.

Catechol violet solution. Dissolve 0·05 g of chromatographically pure catechol violet in 100 ml of water. Do not keep this solution for more than 1 month.

Standard Solutions

Standard solutions should be made up by volume or mass, according to the method of titration to be used. If mass titration is used a suitable burette can be made by drawing off a hexagonal polyethylene ampoule of 5–7-ml capacity to a jet delivering 0·01–0·02-ml drops.

Bismuth nitrate solution, 0·02 mol l^{-1}. Weigh, to the nearest 0·01 mg, about 0·4 g of bismuth metal. Dissolve it in 3·5 ml of 12 mol l^{-1} nitric acid in a covered 50-ml beaker and warm the solution just sufficiently to remove oxides of nitrogen without allowing it to boil. When the solution is colourless, or only a very pale yellow, dilute it to about 20 ml and add 0·2 ml of 5 per cent. sulphamic acid solution in excess of that required to destroy any residual colour. Allow the solution to cool, transfer it, with washings, to a 100-ml calibrated flask, and dilute to the mark.

EDTA solution, 0·01 mol l^{-1}. Weigh out about 0·37 g of the sample of EDTA to be assayed and dissolve it in about 20 ml of water containing 2 ml of 1 mol l^{-1} ammonia solution. Transfer the solution, with washings, to a 100-ml calibrated flask and dilute to the mark.

Apparatus

Balance and weights. These should be capable of weighing to 0·01 mg and should have been previously calibrated.

pH meter. The meter should be equipped with glass and calomel electrodes.

Spectrophotometer. This is used with 40-mm cells.

Transfer pipette. A polyethylene ampoule holding 20–30 ml, drawn off to a jet about 10 cm long (see Fig. 3).

Beakers. Tall-form, 250-ml beakers with cover glasses.

Weighing bottle. A necked sample bottle holding 10–15 ml and equipped with a platinum-wire (0·5 mm diameter) loop for handling (see Fig. 4).

Magnetic stirrer. This has followers to fit the weighing bottle and 250-ml beaker.

Microburettes. Volume 5 ml, graduated in 0·02-ml divisions, for volumetric titration, or polyethylene ampoule, as specified in the transfer pipette, for mass titration.

Procedure

Weigh, to the nearest 0·01 mg, about 2·0 g (see Note 1) of the pure bismuth metal and, with tweezers, transfer it to a tall-form, 250-ml beaker. Then check the balance reading without the sample in case any particles of the brittle metal have become detached and remain on the pan.

To the metal add 4·0 ml of 12 mol l^{-1} nitric acid per gram of bismuth (see Note 2) and cover the beaker with a cover-glass. When the reaction decreases, apply heat just sufficiently to complete dissolution of the metal and expel oxides of nitrogen, occasionally raising the cover

for a few moments to facilitate the latter process, but do not allow the solution to boil. When the solution is colourless or only a very pale yellow remove the beaker from the source of heat and wash down the cover-glass and sides of the beaker with sufficient water to bring the volume to 30–40 ml. Add 5 per cent. sulphamic acid solution, dropwise, until the solution is decolorised and then add a further 0·2 ml; allow the solution to cool.

Fig. 3. The polythene transfer pipette.

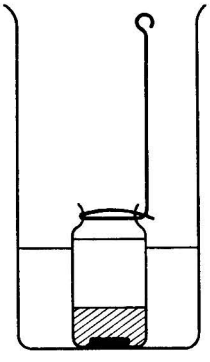

Fig. 4. The weighing bottle in position in the beaker.

Weigh, to the nearest 0·01 mg, an amount of the EDTA sample that is calculated to be in excess of that required to complex all of the bismuth but by not more than 10 mg, into the weighing bottle. Lower the bottle into the beaker containing the bismuth solution (see Fig. 4) and add 10 ml of 3 mol l^{-1} ammonia solution to the EDTA in the bottle, followed by the stirrer bar. Start the stirrer motor and continue stirring until the EDTA has dissolved. Transfer the EDTA solution to the bismuth solution by means of the polyethylene ampoule, and follow with a water wash of about 5 ml, swirling the contents of the beaker gently in order to ensure mixing. Transfer the solution backwards and forwards between the beaker and the bottle three or four times to ensure equilibration and then wash the bottle with water three or four times, using the polyethylene ampoule for all transfers and keeping its tip inside the beaker until this stage is complete. Next, lift the bottle out of the solution, wash it with water and remove it from the beaker.

Dilute the solution to about 100 ml, insert a stirrer follower and the glass and calomel electrodes and adjust the pH to 2·3–2·4, using 3 mol l^{-1} ammonia solution and, finally, 1 mol l^{-1} nitric acid. Add 3·0 ml of 0·05 per cent. catechol violet solution and titrate the solution with 0·02 mol l^{-1} bismuth nitrate solution until the yellow colour begins to darken; at this stage add about a further 0·2 ml of bismuth nitrate solution, after which the solution should become dark orange - brown in colour. Then dilute to about 120 ml, check the pH and re-adjust it to 2·3–2·4 if necessary.

Transfer a portion of the solution to a 40-mm spectrophotometric cell and measure its absorbance at 560 nm, which should be at least 0·6; if it is not, add more bismuth until this value is reached. Titrate the solution with 0·01 mol l^{-1} EDTA, adding about 0·02-ml increments and measuring the absorbance at 560 nm after each increment; after each measurement transfer the contents of the cell back into the beaker. Continue the titration until the absorbance falls below 0·25, at which level add about 0·5 ml more of 0·01 mol l^{-1} EDTA. Measure the absorbance, which is that of the indicator virtually free from BiCV and Bi_2CV, and plot a graph of the readings. (See Note 3.)

Draw the best straight line to fit the points in the absorbance range 0·25–0·60 and extend it to cut the horizontal line through the final absorbance value obtained in the presence of excess of EDTA, as in Fig. 2. Take the point of intersection as the equivalence point of the titration.

Calculation

All masses referred to below are the apparent mass in air. If the same weights are used for weighing both materials the only buoyancy correction necessary is that of EDTA against bismuth. The buoyancy effect on the material of the weights cancels out in the calculation.

Let concentration of bismuth solution	$= b$ g g^{-1} or b g ml^{-1}
concentration of EDTA solution	$= e$ g g^{-1} or e g ml^{-1}
mass of bismuth metal	$= B$ g
mass of EDTA	$= E$ g
mass or volume of bismuth solution	$= V_B$ g or ml
mass or volume of EDTA solution	$= V_E$ g or ml
percentage of EDTA (as disodium dihydrogen ethylenediaminetetraacetate dihydrate) in sample	$= A$
Relative atomic mass of bismuth	= 208·9806
Relative molecular mass of EDTA	= 372·241
Buoyancy correction of EDTA relative to bismuth	= 1·000 57
Number of gram-moles of bismuth	$= \frac{B + bV_B}{208{\cdot}9806}$
Number of gram-moles of EDTA	$= \frac{1{\cdot}000\ 57\ (E + eV_E)A}{100 \times 372{\cdot}241}$

The numbers of gram-moles of bismuth and EDTA are equal at the equivalence point, and therefore

$$A = \frac{178{\cdot}021\ (B + bV_B)}{E + eV_E}$$

NOTES—

1. All volumes are given for 2·0 g of bismuth. For other masses of the metal adjust the volumes proportionately and try to keep the final volume of solution within 10 per cent. of that calculated. It is desirable to maintain the reactant concentrations as high as possible, but the solubility of the bismuth - EDTA complex limits these to about 0·08 mol l^{-1}.
2. The amount of nitric acid specified is adequate to dissolve the bismuth and precipitation of the subnitrate does not occur on dilution unless acid has been lost by overheating and evaporation. The nitrate concentration in the final solution should be kept to a minimum as it lowers the stability of the already rather weak BiCV complex.
3. The graph of the absorbance at 560 nm against the volume of 0·01 mol l^{-1} EDTA is straight for only a short distance, corresponding to an EDTA to bismuth ratio of about 99·985–99·995 mol per cent.

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Book Reviews

ISOLATION AND IDENTIFICATION OF DRUGS IN PHARMACEUTICALS, BODY FLUIDS, AND POST-MORTEM MATERIAL. Volume 2. Edited by E. G. C. CLARKE, assisted by MILDRED LANG and K. G. MARRIOTT. Pp. xiv + 1258. London: The Pharmaceutical Press. 1975. Price £13·50.

Volume 2 of this valuable work sets out to be no more than the supplementary volume to the original text (published in 1969) that it is, and it is clearly the author's intention that the two volumes should be part of one whole book. This is readily apparent from the arrangement and presentation of the text, which follows the same general pattern in both volumes. The treatment is varied, however, and ranges from complete re-writing of whole chapters from Volume 1, to the supplementation of others, together with the introduction of substantial amounts of new material. Thus, the initial chapter on screening tests, which has been completely re-written, extends the range of tests that can be performed on urine, blood and stomach contents to include, among others, tests for paraquat, phenacetin, paracetamol and organophosphorus compounds. A complete new section on extraction procedures for urine, stomach contents and blood, together with tests involving combinations of thin-layer and gas - liquid chromatography with ultraviolet spectrophotometry and chemical examination of the extracts, adds greatly to the practical utility of the book.

The treatment of extraction methods in toxicology, although equally valuable, is very much an extension of and supplement to that in Volume 1, and in this sense is rather less satisfactory in that it is not entirely clear to the non-expert when the alternative methods given in Volume 2 might supersede or be preferable to those of Volume 1. Choice of methodology is, no doubt, often a matter of experience, but for this very reason the clearest possible guidance for the non-expert is important. Of the techniques described, useful and important extensions are included on gas chromatography and infrared spectroscopy, while entirely new chapters on mass spectrometry, alone and combined with gas chromatography, and the radioimmunoassay of drugs make valuable new additions. Drug metabolism similarly receives a considerably expanded treatment.

This updating of methodology is supplemented by considerable additions to the collection of monographs in Part 2, mainly concerned with new drugs. The indexes to analytical data, covering melting-point, paper, thin-layer and gas - liquid chromatography, the Marquis test, ultraviolet and infrared absorption spectra, which relate to these additions, similarly follow the same pattern as Volume 1. They, necessarily, suffer the disadvantage that they need to be examined in conjunction with the corresponding tables in Volume 1.

The general impression is that of a valuable text, usefully updated. The need for extensive cross-referencing between the two volumes presents certain disadvantages, which are no doubt outweighed by the value of the new material. It is to be hoped, however, that in any future revision the author will resist the temptation to produce yet another supplementary volume rather than a single unified text.

Notwithstanding these criticisms, Volume 2 is undoubtedly a must for all engaged in toxicological practice.

JOHN B. STENLAKE

GRADIENT LIQUID CHROMATOGRAPHY. By C. LITEANU and S. GOCAN. *Ellis Horwood Series in Analytical Chemistry*. Pp. xiv + 338. Chichester: Ellis Horwood Limited. Distributed by John Wiley and Sons, New York, London, Sydney and Toronto. 1974. Price £10·50.

The technique of chromatography is part of the group of procedures that are based on the differential migration of the components of a mixture in a system of two phases, one of which moves with respect to the other. It is always tacitly assumed that between the two phases there exists an equilibrium state for the distribution of the various solutes. Indeed, most of the papers dealing with the theoretical aspects of the separation of solutes by chromatography insist that this is the situation. In practice, it really makes little difference if the equilibrium is not truly established, as long as steady-state conditions obtain. There have been numerous papers which have discussed this point and many workers in this field have contributed to the gradual development of the currently held theories.

It is interesting to note that the problem of deciding, for a multi-component mobile phase, what is the mobile phase at a particular point in the solvent flow has led the authors to devote a chapter to the kinetics of eluent migration and to draw some conclusions and make some generalisations that they would find hard to substantiate from some of the references quoted. The general situation is somewhat confused, and while the authors have attempted to draw meaningful conclusion, its

would have been better had they presented their arguments in greater detail and hence, perhaps, proved more convincing.

Nevertheless, the authors are to be congratulated on the manner in which they have approached the fundamental problems of chromatography. It has certainly stimulated the reviewer into asking whether or not some firmly held views should be revised, and the first part of the book is an excellent piece of work, which should be read by every chromatographer.

When discussing the use of gradients it is apparent that the authors are preaching what they practice. For those who wish to use mobile phase gradient chromatography this book is a "must." The apparatus, etc., discussed may not be directly applicable to all types of solid - liquid chromatography but the essential philosophy certainly is.

I found the book a mixture of sound practical techniques and highly involved theories of minutiae of chromatographic separations. This combination will not please everyone, but overall I found it to be fairly good; it should find a place in many libraries. For many it may appear to be somewhat esoteric, but it contains so much "meat" that even for the most pragmatic worker it is worthwhile reading. The price is somewhat high, but as a reference book it is good value. L. S. BARK

METHODS OF BIOCHEMICAL ANALYSIS. Volume 22. Edited by DAVID GLICK. Pp viii + 567. New York, London, Sydney and Toronto: John Wiley & Sons. 1974. Price £13·50.

Several topics discussed in this volume have been dealt with in earlier volumes of the series but the rapid rate of advance makes further treatment necessary.

J. P. Felber of Lausanne considers the radioimmunoassay of polypeptide hormones and enzymes. The method was developed by Yalow and Berson in 1960 (see this series, Volume 12, 1964) originally for insulin determinations, but the scope of the technique has since been greatly extended.

The principle of the method is competition between labelled and unlabelled antigens for a common specific antibody, but the process is not carried out at antigen concentrations such that the antibody - antigen complex AgAb is precipitated. The primary reaction Ag + Ab$\rightleftharpoons$AgAb implies an equilibrium constant $K = [AgAb]/[Ag][Ab]$. In the radioimmunoassay, labelled antigen Ag* is added in tracer concentrations. The antibody concentration is constant and less than that of Ag*, which is also constant. Addition of unlabelled antigen sets up competition of Ag with Ag* for the sites in the antibody and this lowers the radioactivity of the complex. The assay is based on the percentage of total labelled antigen bound to the antibody as Ag*Ab. References are given to theoretical derivations. The labelled antigen is obtained by the interaction of tyrosine residues with iodine isotopes ^{131}I or ^{125}I, although the latter is preferred. Thus $Na^{125}I$ is oxidised (*e.g.*, by chloramine T) and positively charged iodine binds to the antigen.

When a hormone or an enzyme or antigen lacks a tyrosine residue (*e.g.*, bradykinin) tyrosine is introduced via the *N*-terminal arginyl group or (as in secretin) a histidine residue is iodinated. Methods for purifying antigens are described. Antisera are made by repeated small injections of immunogen into guinea pigs, rabbits or goats and, after incubation, separation by one of several methods is often needed. Detailed procedures are given for applying the method to a great range of problems in general biochemistry and clinical endocrinology. This is a long article (94 pages) with 312 references.

G. Brooker of Charlottesville, Virginia, describes newer developments in the determination of cyclic-AMP and other cyclic nucleotides, adenylate cyclase and phosphodiesterase. It was Rall and Sutherland who, in 1958, recognised that 3′,5′-adenosine monophosphate was a key intermediate and its role in kinase activation has since been widely studied. The analytical problems concerning cyclic-AMP and cyclic-GMP have already been discussed by Goldberg and O'Toole (this series, Volume 20, 1972) but progress remains rapid. Brooker, in stressing the importance and difficulty of analysis, notes that cyclic nucleotides comprise less than one millionth of the cell's contents. In the presence of phosphodiesterase, cyclic-AMP is labile and rapid fixation of tissues is of cardinal importance. Methods of freezing and cooling with minimal delay down to the temperature of liquid nitrogen are fully described; microwave irradiation is also discussed. Techniques for extraction and purification are reviewed and current methods of analysis are considered critically. Methods of cytochemical localisation are covered briefly. This article is a noteworthy technical contribution, which will be valuable to workers in this advancing field.

T. P. Singer (this series, Volume 4, 1957) reviewed the determination of succinate dehydrogenase activity and a method based on phenazine methosulphate (PMS) then described has been widely adopted, although some limitations and complexities have emerged. The same author now brings

the subject up to date and includes determinations of the activity of NADH, choline and α-glycerophosphate dehydrogenases. Succinate dehydrogenase is reversible in aerobic cells and the enzyme can be assayed in either direction but succinate oxidation is more rapid than fumarate reduction. The materials to be assayed differ quite widely and various electron acceptors are in common use but problems arise, *e.g.*, PMS has two reaction sites, namely the dehydrogenase and ubiquinone (Q–10, H_2) sites. Detailed procedures are given for the following assays: succinate-ubiquinone reductase, phenazine methosulphate, hexacyanoferrate(III), fumarate reductase, "reconstitution" activity and analysis for covalently-bound flavin. In addition, high and low relative molecular mass forms of NADH dehydrogenase are included with NADH systems involving ubiquinone or hexacyanoferrate(III) or piericidin binding. Choline dehydrogenase and α-glycerophosphate dehydrogenase in mitochondria are dealt with.

This excellent survey illustrates sophisticated methods of using spectrophotometry in measuring enzyme activities.

S. Grossman, G. Oestreicher and T. P. Singer of San Francisco write about determining the activity of phospholipases A, C and D. They use Hanahan's nomenclature in which phospholipases A_1 and A_2 hydrolyse the acyl groups on the 1- and 2-positions of a *syn*-3-phosphoglyceride (or the 2-position of a *syn*-3-plasmalogen), respectively. Phospholipase C hydrolyses the phosphoglyceride to the corresponding diglyceride and phospholipase D splits it into the corresponding phosphatidic acid and free nitrogenous base.

For each type of phospholipase, physical, colorimetric and radiochemical methods are described. The haemolysis of erythrocytes by the action of phospholipase A_2 has definite limitations but remains useful. Radiochemical methods are described rather fully and include an interesting procedure for the simultaneous assay of phospholipases A, C and D. Phospholipase B (lysophosphatidase) is not covered by the review because "no completely satisfactory method for this enzyme has been reported," although it might be possible to devise a radiochemical procedure.

K. P. M. Heirwegh of Leuven, Belgium, and six colleagues review recent advances in the separation and analysis of diazo-positive bile pigments like bilirubin and conjugated bilirubins. Diazo reagents that yield uncharged azo derivatives have made the separation of pigments and the recognition of structure much easier, and hold promise for analysis in clinical biochemistry. Direct-acting bilirubin forms azo derivatives by reaction with diazotised sulphanilic acid in an acidic aqueous medium. Total bilirubin reacts when the diazo coupling occurs in the presence of a reaction accelerator (water-miscible organic solvent or certain organic compounds). Diazotised sulphanilic acid is called a Type I reagent, yielding water-soluble azo derivatives, while diazotised ethyl anthranilate is a Type II reagent giving azo derivatives that are soluble in organic solvents.

After discussing the structures and physical properties of bile pigments, metabolic problems are surveyed. The diazo coupling itself and various analytical methods for its investigation are reviewed. Under the heading "Recommended procedures" quantitative procedures are given. There is also a discussion of the separation and determination of structure of azo derivatives by various techniques. This section includes sugar analysis and ring structure determinations by combined gas - liquid chromatography and mass spectrometry. The article is a lucid exposition by specialists.

W. T. Binnerts and H. A. Das, from The Netherlands, report on the determination of iodine in biological materials, a problem where the amounts range from 0·001 mg l^{-1} for inorganic iodine in blood to above 3 g kg^{-1} (dry mass) for seaweed, sponges or thyroid powder. Many difficulties have been met and discussed in a voluminous literature, one result being that several procedures require strict adherence to conditions that the user has to establish or verify himself. At present however, neutron-activation analysis is proving to be very satisfactory. When a chemical separation is necessary a known amount of the inactive element is added as a carrier. Details are given of the reactions, the irradiation process, mineralisation before or after irradiation and separations. Illustrative applications are cited.

As far back as 1934 Sandell and Kolthoff discovered that the reaction $2Ce(IV) + As(III) \rightarrow 2Ce(III) + As(V)$, as catalysed by iodide, is a first-order process provided that As(III) is present in large excess. The concentration of Ce(IV) is used as a yardstick, the decrease in log absorbance (at 420 nm) being proportional to the iodide concentration where the reaction time is kept constant. The cerium - arsenic reaction can be used for the simultaneous determination of different forms of iodine and can be adapted to automation. The analytical possibilities are displayed thoroughly with a final section on clinical analyses.

S. Jacobs, of the National Institute of Medical Research, London, has an essay on ultrafilter membranes in biochemistry. An ultrafilter has pores or interstices of molecular dimensions and

ultrafiltration can be defined as a process for retaining selected molecular species. In an interesting historical introduction Jacobs notes the work of Bechhold (1907) on colloids, of Elford (1934) on ultrafiltration applied to bacteria and virus preparations and the work of Graber and Loureiro (1939) on graded membranes.

Collodion solutions in diethyl ether - ethanol yield self-supporting membranes, whereas those prepared from acetic acid - collodion solutions are very fragile. The preparation and calibration of graded membranes is described and the determination of pore diameter is examined critically. The heteroporosity of graded ultrafilter membranes (Jacobs, *Filtr. Sep.*, 1972, **9**, 525) is important and as a criterion the ratio of maximum pore diameter to average (or effective) pore diameter is of service. Cellulose acetate membranes for zone electrophoresis are described and denitrated nitrocellulose membranes are also useful. Various commercial types of ultrafilters are available, including a hollow-fibre assembly. The review includes a substantial and informative section on applications of graded membranes to the ultrafiltration of proteins. Jacobs, however, concludes that "there appears to be no appreciable progress in the methods of controlling the fine structure of the filter or ultrafilter membranes."

E. Ribi, R. Parker and K. Milner, of Hamilton, Montana, describe microparticulate gel chromatography accelerated by centrifugal force and pressure. Microfine silica leaflets about 10–20 mm wide (obtained under the trade-name Quso) form a voluminous gel on stirring with an organic solvent (*e.g.*, 1 g per 20 ml, stirred magnetically for 7 min). When this gel is packed into short tubes by centrifugation a column rigid enough to be extruded is obtained. Microparticulate silica adjusted to contain 2, 4, 6 or 8 per cent. of water is now available commercially for a Centri-chrom apparatus that is used in analytical centrifugal chromatography. An alternative technique is to accelerate elution by pressure applied to the centrifugally packed column. Separations are monitored by spraying with suitable indicators or by charring extruded columns. The two procedures have been applied to the separation of aliphatic lipids, prostaglandins, steroids, cannabinoids, barbiturates, dapsones, pesticides, sugars and fractions from tubercle bacilli. The silica leaflets are much smaller than the particles used in column chromatography or thin-layer methods and provide a very large surface area of 400 $m^2 g^{-1}$. The new procedure will not displace other chromatographic techniques, but for some problems it allows better separations than is otherwise possible.

P. Douzou of Paris recounts the advantages of using sub-zero temperatures in biochemistry in a long (112 page) article blending background data, experimental techniques and applications with basic biochemical problems. A major aim of the work is to lower the velocity of biochemical processes enough to permit more penetrating studies of intermediates, despite the incidental hazards. The first section of the review summarises basic measurements on the main accessible parameters that vary widely in solvent mixtures (water plus weakly protic solvents) over a temperature range down to −105 °C, *i.e.*, molecular solvent volume, viscosity, dielectric contant and relative basicity or acidity. The next section deals with solvent and temperature effects on "biomolecules," especially helical macromolecules, protein and enzymes. The final section deals with methodology at sub-zero temperatures with special reference to spectrophotometry, fluorescence and optical rotatory dispersion. The recognition of intermediates is illustrated by studies on flavoproteins, α-chymotrypsin and haemoproteins. There is also a discussion of control of peroxidatic reactions and the temporal resolution of enzymic processes. Kinetic studies by fast techniques are considered in terms of a "slow" temperature jump and stopped-flow procedures.

This is a very good volume in an established series. Each of the nine contributions covers much ground with authority and is well referenced. Production is excellent with only a few misprints, none of which is very important.

R. A. MORTON

CHIMIE ANALYTIQUE QUANTITATIVE. Volume I. MÉTHODES CHIMIQUES ET PHYSICO-CHIMIQUES. Volume II. MÉTHODES SÉLECTIONNÉES D'ANALYSE CHIMIQUE DES ELÉMENTS. By GASTON CHARLOT. Volume I: Pp. x + 325. Volume II: Pp. viii + 571 + xxiv. Paris: Masson et Cie. 1974.

While these two volumes are meant to be complementary to one another, each is a very worthwhile volume.

The first volume, which deals with the techniques, is divided into four main parts, dealing with reactions in solution, methods of measurement, electrochemical methods and miscellaneous methods.

The part concerned with reaction in solutions covers acid - base, redox, complexation, precipitation, solvent extraction, ion exchange and chromatography. Although this is covered in approximately 120 pages, it is not superficial. The various sections are informative and contain sufficient

information to give not only the basic points but also good illustrative examples.

The author is to be congratulated on the way he has covered a large number of techniques without presenting a mere catalogue.

The second of the two volumes deals with the various methods available for the quantitative analysis of the elements; they are considered alphabetically and range from aluminium to zirconium. Here the author has given a catalogue, but it is very useful and comprehensive. There are literally hundreds of references given, to original papers, reviews, etc., and all at the appropriate places. These references are well selected and indicate to the reader the exact reference that will enable him to obtain more information.

This volume will certainly serve as one of the reference sources in the reviewer's laboratory. It is one of the best volumes I have encountered for some time.

For students, these books are highly recommended as a set. For the practising chemist, whose general techniques are up-to-date, the second volume will perhaps be of more value; but it would be useful to have both in order to be able to refresh the memory on some theoretical points whenever necessary.

The fact that the books are in French should not deter even the most ardent anglophile—the author has a crisp scientific style that greatly helps in the understanding of the text.

L. S. BARK

CHROMATOGRAPHIC METHODS. By R. STOCK and C. B. F. RICE. Third Edition. Pp. viii + 383. *Science Paperbacks No.* 39. London: Chapman and Hall and Science Paperbacks. 1974. Price: hardback £5·25, paperback £2·90.

This book is intended to provide a comprehensive account of the various chromatographic methods in current use. In this third edition the accounts of gas and gel chromatography in the second edition have been expanded and high-performance liquid chromatography is also included. While one can always find items to criticise when reviewing any literature, it would have been interesting to find at least a few particular industrial applications in place of the "model" experiments.

Nevertheless, "Chromatographic Methods" is a good introduction to the subject and should serve as a useful textbook for both undergraduates and those who are beginning to employ these techniques. The text is easy to follow, explanations and experimental detail being especially clear.

The chromatographic techniques covered are liquid-phase chromatography (adsorption, partition, ion-exchange and gel filtration); paper chromatography; zone electrophoresis; gas chromatography; and thin-layer chromatography.

Bearing in mind that the book covers such a wide range of techniques (each worthy of a volume of its own), there is a worthwhile range of detail in the various chapters and no impression of skimpiness is received.

The price of the paperback edition is sufficiently reasonable for institutions to make more than one copy available for student reading.

D. SIMPSON

LIQUID SCINTILLATION COUNTING. VOLUME 3. PROCEEDINGS OF A SYMPOSIUM ON LIQUID SCINTILLATION COUNTING ORGANISED BY THE SOCIETY FOR ANALYTICAL CHEMISTRY, BRIGHTON, ENGLAND, SEPTEMBER 3–6, 1973. Edited by M. A. CROOK and P. JOHNSON. Pp. 309. London, New York and Rheine: Heyden & Son Ltd. 1974. Price £10; $27·50; DM82.

This volume records the Proceedings of an International Symposium on Liquid Scintillation Counting and is the third in the series. It is divided into five sections corresponding to the Plenary Sessions of the Symposium, the first chapter in each being the Plenary Lecture of the session.

The perennial problems of quench correction and sample preparation occupy much of the book and the statement of one of the authors that these will "soon belong to history" seems optimistic.

The first section commences with a general account, by D. L. Horrocks, of applications of the method to counting different types of radiations. In a later chapter, the same author discusses the counting of some radionuclides used in radioimmunoassay. This section also includes a chapter on recent advances in photomultiplier tubes and one on an intercomparison of materials for counting vials.

The next section deals with quench correction techniques. In the initial chapter on standardisation, Elizabeth Mueller stresses the importance of reproducing the characteristics of the sample in the standard with which it is compared. The difficulty of achieving this in some instances is

illustrated by the quotation "we found it necessary in assaying muscle tissue in various solvent systems to prepare a set of standards for each system, using not only the same solvent and the same kind of muscle from an unlabelled animal, but a piece of muscle that been washed until it had approximately the same colour as the sample to be assayed." P. E. Stanley considers the change of background count with quenching and calculates the correct channels ratio by an iterative procedure. He also uses a Monte Carlo technique for studying the factors that influence pulse shape in quenched samples.

One of the problems of quench correction is that colour quenching and chemical quenching require different channels ratio correction curves. Both Mrs. Mueller and P. E. Stanley refer to a recent technique, "lesser pulse height analysis," which overcomes this difficulty. F. E. L. ten Haaf also deals with this problem and shows, by using a simplified model calculation, that by summing the logarithms of the two photomultiplier tube outputs the response is similar for both types of quenching.

The remaining chapters in this section deal with colour quenching for a series of carotenoids, a practical device for obtaining quench correction curves and a computer program for processing data.

The next two sections of the book cover sample preparation, commencing with a general review of homogeneous counting by B. E. Gordon. He emphasises the value of the double ratio method as a check on homogeneity, the dangers of adsorption in counting vials and the advantages of combustion in the preparation of organic samples. A comparison of results obtained by various solubilisation methods and combustion of tissue samples is given by J. G. Dent and P. Johnson, while B. Scales gives an account of a survey of measurements of carbon-14 in biological samples carried out by various methods. He considers the results of the latter to be "very disturbing." In the present state of the art, this type of study is of great value.

The problems of quench correction make the combustion method of sample preparation, when the counting species is water or carbon dioxide, very desirable, particularly if it can be made automatic. This is the subject of four papers. Three of these papers give detailed accounts of different systems, a commercial adaptation of the Peterson automated tube furnace, a semi-automatic in-vial combustion process and an automated oxygen-flask system. The fourth paper, by R. G. Cooper, describes experiments carried out with a number of commercial automatic oxidisers. These experiments were generally terminated because of teething troubles with the equipment and the author concludes that manual-flask combustion, with equal financial involvement in supporting equipment, would require a very good instrument to better it.

The final section of the book is headed "Special Topics" and includes accounts of the applications of Čerenkov counting, the determination of carbon-14 in circulating blood, counting iron-55, an in-vial oxidation process for the determination of plutonium-241 in smear samples and the determination of urease by measuring the evolution of carbon-14. There is also an interesting chapter on the use of liquid scintillation counting spectrometers for bioluminescence assays—a stop-gap use as the instrumental requirements differ from those of liquid scintillation counting. One application mentioned is the determination of bacterial growth in 10 min by detection of ATP.

A note at the beginning of the book draws attention to the fact that some of the authors have a commercial interest in the technique. This is fair comment, particularly in a few instances where the connection is not obvious from the address of the author.

The book is well produced and there are few misprints of any consequence. The legend of Fig. 5 on page 9 is incomplete, while on page 240 tellurium-204 should be thallium-204.

The price is only slightly higher than that of the previous volume, in spite of inflation. The book is a useful addition to the liquid scintillation counting library. D. I. Coomber

Practical Electrophoresis. By G. J. Moody and J. D. R. Thomas. *Merrow Technical Library, MTL/PS/5.* Pp. viii + 104. Watford: Merrow Publishing Co. Ltd. 1975. Price £2·50; $8·30.

This text gives an excellent introduction to the various ways in which electrophoresis can be performed. It is especially suitable for use in post-academic courses, where a large amount of theory is not required, although for those who wish to study the theory behind the technique in detail, up-to-date references are given. For students and other workers who are about to take an interest in electrophoresis the text summarises the most important points simply and clearly; the list of companies making equipment for electrophoresis will be particularly appreciated.

I recommend the book because it gives, as the title suggests, practical information.

Frans M. Everaerts

LECTURES AND COURSES

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Determination of Aluminium, Calcium, Manganese and Titanium in Ferrosilicon Alloys by Atomic-absorption Spectrophotometry

A method for the determination of aluminium, calcium, titanium and manganese in ferrosilicon alloys is described. After elimination of silicon by the addition of hydrofluoric acid and subsequent dissolution of the residue, the solution is ready to be analysed by means of atomic-absorption spectrophotometry. The proposed method is free of interferences from the most common elements used in the production of ferrosilicon alloys, and is suitable for the determination of concentrations of aluminium and titanium down to 0·1 per cent., manganese down to 0·01 per cent. and calcium down to 0·02 per cent. These limits can be reduced by varying either the dilution of the sample solution or the amount of sample taken.

M. DAMIANI, M. G. DEL MONTE TAMBA and F. BIANCHI
Centro Sperimentale Metallurgico S.p.A., Via di Castel Romano, 00129 Rome, Italy.

Analyst, 1975, **100**, 643–647.

The Gas-chromatographic Determination of Selenium in Steel with 4-Chloro-1,2-diaminobenzene

A simple and practical method is described for the determination of small amounts of selenium in steel. A diluted mixture of equal volumes of hydrochloric and nitric acids is used to dissolve the steel sample, the selenium is completely converted into the quadrivalent state in the presence of perchloric acid and the large amount of iron(III) is masked with phosphoric acid. No loss of selenium has been encountered when using this treatment. Selenium(IV) reacts with 4-chloro-1,2-diaminobenzene to form 5-chloropiaselenol, which is extracted into toluene and determined by means of a gas chromatograph equipped with a thermal conductivity detector. When samples of 0·1 g are used, the standard deviation at the 0·1–0·3 per cent. of selenium level is 0·002–0·003 per cent.

M. AKIBA, Y. SHIMOISHI and K. TÔEI
Department of Chemistry, Faculty of Science, Okayama University, Tsushima, Okayama-shi, Japan.

Analyst, 1975, **100**, 648–654.

Simple Titrimetric Methods for the Assay of Sulphurated Sodium Borohydride

Back-titration procedures involving the use of oxidants such as chloramine T, potassium iodate and iodine are described for the accurate assay of sulphurated sodium borohydride. Quantitative reactions are completed within 1 h at room temperature for the concentrations and proportions described. The methods are simple, reproducible and accurate within the limits described.

V. M. SADAGOPA RAMANUJAM and NORMAN M. TRIEFF
Department of Preventive Medicine and Community Health, University of Texas Medical Branch, Galveston, Texas 77550, U.S.A.

Analyst, 1975, **100**, 655–660.

APPOINTMENTS VACANT

An Infrared Spectroscopic Method for the Rapid Simultaneous Determination of Fat and Moisture in Meat and Meat Products

A method is described for the simultaneous determination of fat and moisture in fresh meat and meat products by cold extraction of both of these constituents into a known volume of solvent consisting of trichloroethylene plus methanol, followed by measurement of the infrared reflectance of the solution at the wavelengths at which fat and water absorb radiation. Multiple attenuated total reflection spectroscopy (multiple ATR) is used to make the infrared measurements and the advantages of this technique over conventional transmission spectrophotometry are discussed.

The rapidity of the method (approximately 5 min per determination of both fat and water on a weighed sample) compensates for its slightly lower degree of precision in comparison with conventional methods, and makes it particularly suitable for use in process control.

P. J. FARNELL

Procter Department of Food and Leather Science, The University of Leeds, Leeds, LS2 9JT.

Analyst, 1975, **100**, 661–667.

The Determination of Robenidine in Animal Feeds and Pre-mixes

Report prepared by the Medicinal Additives in Animal Feeds Sub-Committee B.

ANALYTICAL METHODS COMMITTEE

The Chemical Society, Burlington House, London, W1V 0BN.

Analyst, 1975, **100**, 668–674.

The Precise Standardisation of Disodium Dihydrogen Ethylenediaminetetraacetate Dihydrate by Spectrophotometric Titration against Pure Bismuth Metal

Report prepared by the Complexiметric Standards Panel of the Analytical Standards Sub-Committee.

ANALYTICAL METHODS COMMITTEE

The Chemical Society, Burlington House, London, W1V 0BN.

Analyst, 1975, **100**, 675–682.

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