The Analyst

The Analytical Journal
of The Chemical Society

A monthly international publication dealing with all branches of analytical chemistry

Volume 101 No 1202 Pages 321–408 May 1976

THE ANALYST

THE ANALYTICAL JOURNAL OF THE CHEMICAL SOCIETY

Published by The Chemical Society

Editorial: The Director of Publications, The Chemical Society, Burlington House, London, W1V 0BN. Telephone 01-734 9864. Telex No. 268001.

Advertisements: J. Arthur Cook, 9 Lloyd Square, London, WC1X 9BA. Telephone 01-837 6315.

Subscriptions (non-members): The Chemical Society Publications Sales Office, Blackhorse Road, Letchworth, Herts., SG6 1HN.

Volume 101 No 1202 May 1976

Reprints of Review Papers

Reprints of the following Review Papers published in *The Analyst* since 1965 are available from the Publications Sales Officer, The Chemical Society, Blackhorse Road, Letchworth, Herts., SG6 1HN (not through Trade Agents).

The price per reprint is £1; orders for six or more reprints of the same or different Reviews are subject to a discount of 25%. The appropriate remittance, made out to The Chemical Society, should accompany any order.

"Activation Analysis," by R. F. Coleman and T. B. Pierce (January, 1967).
"Techniques in Gas Chromatography. Part I. Choice of Solid Supports," by F. J. Palframan and E. A. Walker (February, 1967).
"Heterocyclic Azo Dyestuffs in Analytical Chemistry," by R. G. Anderson and G. Nickless (April, 1967).
"Determination of Residues of Organophosphorus Pesticides in Food," by D. C. Abbott and H. Egan (August, 1967).
"Radioactive Tracer Methods in Inorganic Trace Analysis: Recent Advances," by J. W. McMillan (September, 1967).
"Gamma-activation Analysis," by C. A. Baker (October, 1967).
"Precipitation from Homogeneous Solution," by P. F. S. Cartwright, E. J. Newman and D. W. Wilson (November, 1967).
"Industrial Gas Analysis," by (the late) H. N. Wilson and G. M. S. Duff (December, 1967).
"The Application of Atomic-absorption Spectrophotometry to the Analysis of Iron and Steel," by P. H. Scholes (April, 1968).
"Inorganic Ion Exchange in Organic and Aqueous - Organic Solvents," by G. J. Moody and J. D. R. Thomas (September, 1968).
"Radiometric Methods for the Determination of Fluorine," by J. K. Foreman (June, 1969).
"Techniques in Gas Chromatography. Part II. Developments in the van Deemter Rate Theory of Column Performance," by E. A. Walker and J. F. Palframan (August, 1969).
"Techniques in Gas Chromatography. Part III. Choice of Detectors," by T. A. Gough and E. A. Walker (January, 1970).
"Laser Raman Spectroscopy," by P. J. Hendra and C. J. Vear (April, 1970).
"Ion-selective Membrane Electrodes," by Ernö Pungor and Klára Tóth (July, 1970).
"X-ray Fluorescence Analysis," by K. G. Carr-Brion and K. W. Payne (December, 1970).
"Mass Spectrometry for the Analysis of Organic Compounds," by A. E. Williams and H. E. Stagg (January, 1971).
"The Application of Non-flame Atom Cells in Atomic-absorption and Atomic-fluorescence Spectroscopy," by G. F. Kirkbright (September, 1971).
"Liquid Scintillation Counting as an Analytical Tool," by J. A. B. Gibson and A. E. Lally (October, 1971).
"The Determination of Some 1,4-Benzodiazepines and Their Metabolites in Body Fluids," by J. M. Clifford and W. Franklin Smyth (May, 1974).
"Atomic-fluorescence Spectrometry as an Analytical Technique," by R. F. Browner (October, 1974).
"The Use of Precipitate Based Silicone Rubber Ion-selective Electrodes and Silicone Rubber Based Graphite Voltammetric Electrodes in Continuous Analysis," by Zs. Féher, G. Nagy, K. Tóth and E. Pungor (November, 1974).
"The Examination of Meat Products with Special Reference to the Assessment of the Meat Content," by D. Pearson (February, 1975).
"Chemiluminescence in Gas Analysis and Flame-emission Spectrophotometry," by J. H. Glover (July, 1975).
"The Analytical Role of Ion-selective and Gas-sensing Electrodes in Enzymology," by G. J. Moody and J. D. R. Thomas (September, 1975).
"Thiazolylazo Dyes and Their Applications in Analytical Chemistry," by Håvard R. Hovind (November, 1975).
"Sample Preparation in the Micro-determination of Organic Compounds in Plasma or Urine," by Eric Reid (January, 1976).
"Recent Advances in the Ring Oven Technique," by Herbert Weisz (March, 1976).
"The Radioimmunoassay of Drugs," by J. Landon and A. C. Moffat (April, 1976).
"Analysis and Assay of Polyene Antifungal Antibiotics," by A. H. Thomas (May, 1976).

Summaries of Papers in this Issue

Analysis and Assay of Polyene Antifungal Antibiotics

A Review

Summary of Contents

Reprints of this Review paper can be obtained from The Publications Sales Officer, The Chemical Society, Blackhorse Road, Letchworth, Herts., SG6 1HN, at £1 per copy (with a 25% discount for six or more copies), post free.

A remittance for the correct amount, made out to The Chemical Society, should accompany every order; these reprints are not available through Trade Agents.

A. H. THOMAS

Division of Antibiotics, National Institute for Biological Standards and Control, Hampstead, London, NW3 6RB.

Analyst, 1976, **101**, 321–340.

The Determination of Arsenic(III) and Total Arsenic by Atomic-absorption Spectroscopy

In the atomic-absorption determination of arsenic by the hydride evolution method with sodium borohydride, maintenance of the pH between 4 and 5 permits the selective determination of arsenic(III) in mixtures of arsenic(III) with arsenic(V). Total arsenic can be determined separately by evolution from 5 M hydrochloric acid. A dissolution technique has been developed for herbage and the method applied to the analysis of water and orchard leaves.

J. AGGETT and A. C. ASPELL

Chemistry Department, University of Auckland, Auckland, New Zealand.

Analyst, 1976, **101**, 341–347.

The Determination of the Precious Metals by Flameless Atomic-absorption Spectrophotometry

The optimum conditions for the determination of palladium, platinum, rhodium, ruthenium, iridium and osmium by carbon rod atomic-absorption spectrophotometry are described and data for sensitivity, detection limit and reproducibility are given. With the exception of osmium the sensitivity and detection limits are such that the working range of the atomic-absorption technique can be extended downwards by an order of magnitude or more when compared with that with an air - acetylene flame.

The effect of mutual interference is investigated and methods of reducing it are proposed.

G. L. EVERETT

Johnson Matthey Chemicals Ltd., Royston, Hertfordshire.

Analyst, 1976, **101**, 348–355.

The Development of Remote Spectrographic Heads for Metallurgical Analysis and Their Application to Product Inspection Analysis

Direct-reading optical-emission spectroscopy has been widely adopted in the iron and steel industry as a rapid analytical technique for process control and for other laboratory analyses. The conventional spark stand, however, severely restricts the size and shape of the sample that can be accommodated on the instrument and thus limits the more general use of the technique for on-site analysis outside the laboratory. This disadvantage has now been overcome by using a fibre-optic light-guide to link a mobile excitation head to a conventional spectrometer.

Parallel but independent work has been carried out in the authors' laboratories, first on photographic and later on direct-reading spectrometers. At the Corby laboratories, further work has resulted in the manufacture and application of an inspection analyser that incorporates a 5-m guide. Typical calibration graphs, reproducibilities and examples of material identification are given, and possible applications are discussed.

A. D. AMBROSE

Research Centre, British Steel Corporation, Tubes Division, Corby, Northamptonshire, NN17 1UA.

and J. D. HOBSON

Dunford Hadfields Ltd., East Hecla Works, Sheffield, S9 1TZ.

Analyst, 1976, **101**, 356–366.

Detection and Determination of of Polynuclear Aromatic Hydrocarbons by Luminescence Spectrometry Utilising the Shpol'skii Effect at 77 K

Part II. An Evaluation of Excitation Sources, Sample Cells and Detection Systems

A comparison has been made of the use of a xenon arc continuum source, a mercury-vapour lamp and a fixed-wavelength helium - cadmium laser source for excitation in the production of quasi-linear luminescence emission spectra of some polynuclear aromatic hydrocarbons in n-alkane solvents at 77 K. The performance of a new design of cryostat sample cell for work at 77 K has been evaluated and compared with that of a commercially available Dewar-flask sample cell. The use of d.c. integration, photon counting and of repetitive optical scanning in conjunction with a signal averager has been investigated for registration of the luminescence obtained from PAH compounds by use of the Shpol'skii effect.

B. S. CAUSEY, G. F. KIRKBRIGHT and C. G. de LIMA

Department of Chemistry, Imperial College of Science and Technology, London, SW7 2AY.

Analyst, 1976, **101**, 367–378.

Determination of Dicumenyl Peroxide by Gas Chromatography

A method is described for the determination of small amounts of dicumenyl peroxide in liquid samples or powdered materials. Dicumenyl peroxide is isolated from the latter by extraction.

P. HUDEC, B. NOVOTNÁ and J. PETRŮJ

Research Institute of Macromolecular Chemistry, Tkalcovská 2, Brno, Czechoslovakia.

Analyst, 1976, **101**, 379–380.

MAY 1976 Vol. 101 No. 1202

The Analyst

Analysis and Assay of Polyene Antifungal Antibiotics

A Review*

A. H. Thomas

Division of Antibiotics, National Institute for Biological Standards and Control, Hampstead, London, NW3 6RB

Summary of Contents

Introduction

In this review, the chemical and biological properties of the polyene antifungal antibiotics are examined in order to show how these properties can be used for the analysis and assay of the antibiotics. The biosynthesis, production and stability of the antibiotics have also been included as this information is useful in anticipating the occurrence of likely impurities that would affect the analysis and assay. The use of the antibiotics has been considered, as this should determine the method of assay used. Previous reviews have dealt with the chemistry,[1–3] production,[4] biology[5,6] and therapeutic use[7] of the polyene antifungal antibiotics.

Chemical and Biological Properties

Numerous antifungal antibiotics have been isolated from soil actinomycetes, predominantly of the genus *Streptomyces*. The antibiotics exhibited a characteristic ultraviolet absorption spectrum, which suggested that the chromophores were unsaturated straight-chain polyenes.[8,9] The number of consecutive conjugated double bonds in the chromophore formed the basis of the classification of these antibiotics as tetraenes, pentaenes, hexaenes and heptaenes.[2] The stereochemistry of the chromophore can be deduced from the infrared and ultraviolet absorption spectra. A chromophore that has all-*trans* bonds exhibits a spectrum in which the peak at the longest wavelength, which is also the narrowest, is either the strongest in the group or only slightly weaker than the second peak. On the evidence of their ultraviolet absorption spectra, stability to stereoisomerising reagents and presence of *trans*-ethylenic peaks in the 10.1–10.6 μm region of the infrared spectrum, Oroshnik and Mebane[2] reasoned that the chromophores of the tetraenes, pentaenes, methylpentaenes and hexaenes had all-*trans* configurations. The spectra of the heptaenes were more complicated to interpret, as they were degraded in both wavelength maxima and fine structure, indicating the presence of stereoisomers that possibly contain more than one *cis* bond. The peak due to *cis* bonds is found at a wavelength of $1/\sqrt{2}$ of the maximum wavelength, but it was not prominent in the *cis*-heptaenes, which indicated that the *cis* bond was adjacent to the terminal double bonds. The *cis* peak is a measure of the strain in the chromophore due to the *cis* configuration, which is greatest when the *cis* bond is centrally placed. During the isolation of some heptaenes, wavelength maxima were found to vary from 401 to 405 nm. The original *cis*-heptaenes readily underwent light-induced stereoisomerisation to give products with an increased wavelength maximum where the peak was more prominent. The hindrance-free spectra and stability of the maximum wavelength of candidin and amphotericin B indicated that they were produced and isolated in the all-*trans* configuration. The spectra of three representative polyene antifungal antibiotics are shown in Fig. 1.

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

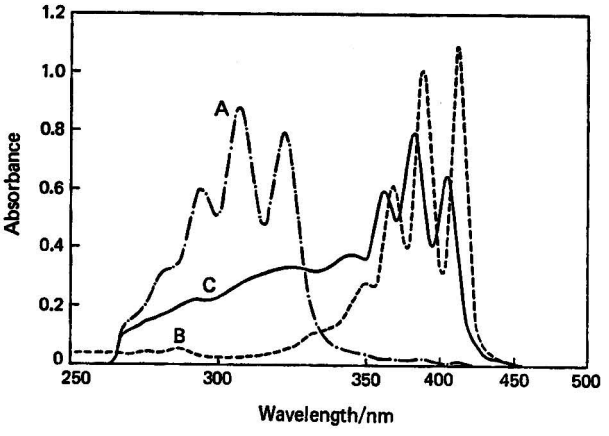

Fig. 1. Typical spectra of polyene antifungal antibiotics. Solution in dimethylformamide (1-cm cell). A, nystatin (tetraene), 20 μg ml^{-1}; B, trichomycin (heptaene), 15 μg ml^{-1}; and C, amphotericin B (heptaene), 7 μg ml^{-1}.

A sharp peak in the infrared spectrum at about 5.83 μm, indicative of an unconjugated lactone ring, is a feature common to all polyene antifungal antibiotics. The size varies from the 26-atom ring of natamycin[10,11] to the 38-atom ring of nystatin[12,13] and amphotericin B[14,15]; in the last two molecules, the lactone bond is between carbon atoms 1 and 37.

Most of the polyenes contain carbon, hydrogen, oxygen and nitrogen. The nitrogen atom is present as a primary amino group and acid hydrolysis results in the liberation of an amino-sugar, mycosamine (3-amino-3,6-dideoxy-D-mannose). An isomer of mycosamine, perosamine (4-amino-4,6-dideoxy-D-mannose), was isolated from perimycin. Mycosamine in the pyranose form is glycosidically linked to the hydroxyl at carbon atom 19 of the lactone ring of amphotericin B. In addition, most polyenes, with the exception of some of the methylpentaenes, possess a carboxyl group that is present as a zwitterion, indicated by a strong peak at 6.35–6.50 μm. Most of the polyenes behave as amphoteric compounds with a characteristic isoelectric point (*e.g.*, natamycin, pH 6.5).[16] They are soluble in acids and bases but they are not usually stable at the pH required to dissolve them.

Another feature of the polyenes is the large number of free hydroxyl groups in the lactone ring: candidin[17] has seven, while amphotericin B[14,15] and nystatin[12,13] have eight each. The presence of the hydroxyl groups and zwitterion is responsible for the comparative insolubility of these compounds in organic solvents. Another reason put forward for the exceptional insolubility and relatively high stability of amphotericin B is the existence of a six-membered ketal ring structure,[15] joining carbon atoms 13 and 17, and such a structure is also thought to be present in crystalline nystatin.[12]

The heptaenes are sub-divided on the basis of the amino-sugar released by acid hydrolysis and the aromatic amine released by alkaline retroaldol cleavage[18] (Table I).

Complete mass spectra of the pertrimethylsilylated derivatives of natamycin, nystatin and amphotericin B have been reported[26] and the structures are shown in Fig. 2. The stereochemistry of the 14 asymmetric centres and the configuration of the glycosidic link of amphotericin B have been determined by X-ray single-crystal analysis of *N*-iodoacetylamphotericin B.[14,27]

The solubility and the colour of the pure antibiotic is determined by the chromophore. Colour is increased from buff to dark yellow and the limited water solubility is decreased with increasing hydrophobic nature of the chromophore as the number of the double bonds is increased. The antibiotics are soluble in dimethylformamide, dimethyl sulphoxide and aqueous solutions of alcohols. Infrared studies showed the presence of strong intermolecular hydrogen bonds, involving not only the hydroxyl groups but also the carboxyl and the amino groups of amphotericin B in dimethyl sulphoxide solution. The zwitterionic character of the antibiotic is also disrupted by the solvent molecules, which produces exceptional solubility in

TABLE I

CLASSIFICATION OF THE HEPTAENE ANTIBIOTICS

Group	Amino-sugar	Aromatic amine	Antibiotic
1	Mycosamine	Absent	Amphotericin B[14,15] Candidin[17] Mycoheptin[19]
2	Mycosamine	*p*-Aminoacetophenone	Candicidin[18] Hamycin[20] Heptafungin A[21] Levorin A and B[22] Trichomycin A[23]
3	Mycosamine	*N*-Methyl-*p*-aminoacetophenone	Aureofungin[24]
4	Perosamine	*N*-Methyl-*p*-aminoacetophenone	Perimycin[25]

dimethylformamide and dimethyl sulphoxide.[28] Antibiotic solutions can be carefully diluted with water, as they are not visibly precipitated at concentrations of less than 50 μg ml^{-1}, although they exist as micellar suspensions in aqueous media.[29,30] The ultraviolet absorption spectra of these micelles or colloidal dispersions exhibit a peculiar type of spectral "degrada-

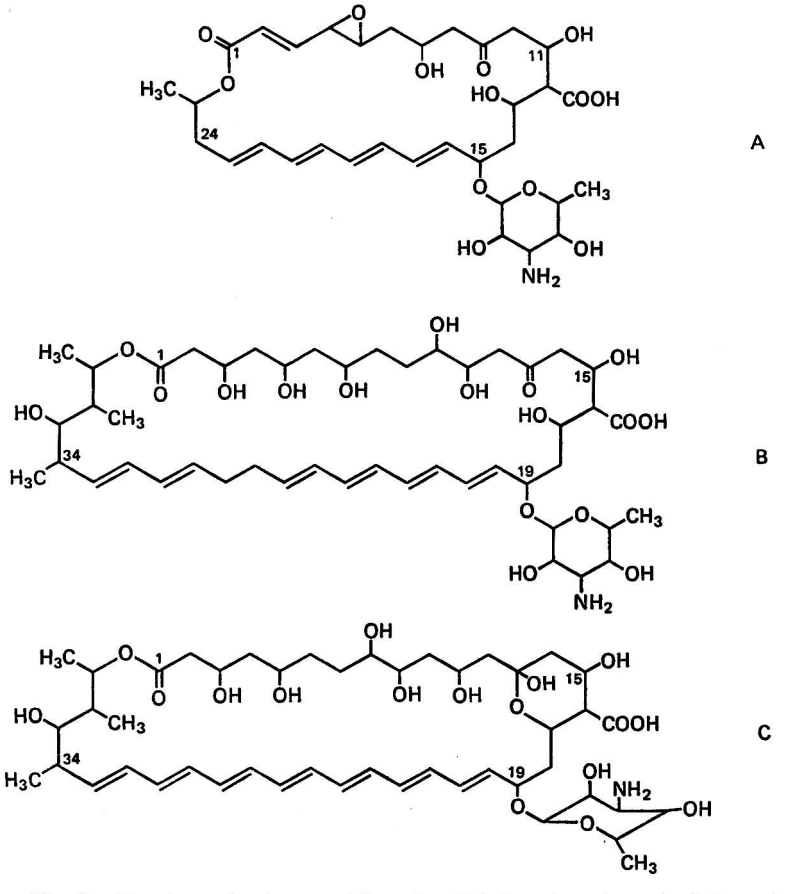

Fig. 2. Structure of polyene antifungal antibiotics: A, natamycin (tetraene), relative molecular mass 665.7, references 10 and 11; B, nystatin A1 (tetraene), relative molecular mass 926.1, references 12 and 13; and C, amphotericin B (heptaene), relative molecular mass 924.1, references 14 and 15.

tion" similar to the effects of severe steric hindrance, which has caused more than one investigator some perplexity.[2]

Both the flexible polyhydroxyl group and the rigid double-bond system are necessary for biological activity. Amphotericin B can be considered to have two chains. The chain that contains the polyene chromophore is completely hydrophobic, whereas the chain that contains the large number of hydroxyl groups has a hydrophilic and hydrophobic face, rendering the chain amphipathic. One end of the molecule is very polar, containing both mycosamine and the carboxyl group; the other end is completely non-polar except for one very conspicuous hydroxyl group. Amphotericin B molecules can be packed together to form a cylindrical structure with the interior lined by the hydroxyl groups of the amphipathic face. The exterior of the cylinder is completely non-polar. Thus, aqueous pores can be made in thin lipid membranes, which may account for the biological action of amphotericin and other polyene antibiotics that results in the leakage of potassium ions from susceptible cells.[31,32]

The polyene antibiotics possess both fungicidal and fungistatic properties against a wide range of fungi, but there are great differences between the sensitivities of different species of fungi to the different polyenes. The incidence of resistance to these antibiotics is rare both in clinical practice and in the laboratory. In addition to their antifungal activities, many polyenes also inhibit algae and some protozoa.

Nystatin and natamycin (pimaricin) are administered orally; as they are poorly absorbed, relatively large doses can be tolerated although they are ineffective in the treatment of systemic mycosis. Natamycin is given by inhalation for respiratory infections. Nystatin and natamycin are used topically but both are too toxic for parenteral use. Amphotericin B is used orally for systemic therapy, although it is poorly absorbed, and is also used topically. Parenteral administration of amphotericin B is effected by using a bile salt complex (Fungizone), which forms a colloidal suspension when mixed with water. Owing to the toxicity of this preparation, intravenous therapy should be used only in life-threatening infections.[6,33] The bile salt complex is considered to be more toxic than the parent antibiotic,[6,34] although it is not clear whether the increased toxicity is due to the bile salt or the increased availability of the "solubilised" amphotericin B. Toxicity tests in animals did not confirm that the bile salt complex was more toxic than amphotericin B.[29] Less toxic derivatives of amphotericin B that still retained antifungal activities have been prepared. Substitution of a proton on the amino group of mycosamine by acetylation resulted in a loss of antifungal activity,[28] whereas glycosylation did not.[35] Esterification of the carboxyl group gave more soluble compounds, the methyl ester retaining the antifungal activity of the parent antibiotic.[36,37] Animal tests showed that the toxicity of the methyl ester of amphotericin B was less than that of the parent compound.[37–39] The increased solubility of the more finely dispersed colloidal suspension of the methyl ester did not increase its toxicity and therefore the role of the bile salt in the toxicity of the amphotericin B complex should be re-investigated, as an increase in the availability of amphotericin B *per se* does not result in an increase in toxicity. Candicidin has been found to be less effective than nystatin for eliminating fungi from the intestinal tract; as with other polyenes, no absorption could be detected.[40] A method for detecting the very low level absorption of the polyene antibiotics is required; experiments with carbon-14 labelled polyenes would be helpful. Following the observation that the oral administration of candicidin resulted in a reduction in the size of the prostate gland of dogs,[41] candicidin has been used with moderate success for the treatment of human benign hypertrophied prostate glands.[42] Also, some polyenes produced a hypocholesterolemic effect in small laboratory animals,[43,44] although this property has not yet been exploited in man.

The polyenes can be used to prevent fungal contamination in tissue culture or as food preservatives (*e.g.*, natamycin on the rind of hard cheese and nystatin on the surface of bananas),[45] or nystatin can be used as a growth promoter in animal feeds.

Some biological and chemical properties of selected polyenes, derivatives and common impurities are shown in Tables II–IV. The wide range of figures quoted reflect the different methods used in the measurement of biological activity and toxicity and the variation in the purity and homogeneity of polyenes used.

Production

There are many examples of complex mixtures of antifungal substances being obtained from micro-organisms that produce the polyene antifungal antibiotics. Non-polyene antifungal

TABLE II

CHEMICAL PROPERTIES OF POLYENE ANTIFUNGAL ANTIBIOTICS AND CO-PRODUCED NON-POLYENE ANTIFUNGAL ANTIBIOTICS

Chromophore group	Antibiotic	$\lambda_{max.}$/nm			$E^{1\%}_{1cm}$ at $\lambda_{max.}$			α_D(solvent)*	Reference
Tetraene	Amphotericin A	291.0	304.0	318.5	580	870	800	+136 (DMF) +163 (C_5H_5N)	2
	Natamycin (pimaricin)	289.5	302.5	317.5	710	1 100	1 020	+258 (DMF) +180 (DMSO)	2, 16
	Nystatin	291.0	304.0	318.5	570	850	780	+ 12 (DMF) + 21 (C_5H_5N)	2
Heptaene	Amphotericin B	363.0	382.0	406.0	980	1 670	1 890	+238 (DMF)	2
	Candidin	364.0	383.5	407.5	985	1 730	1 910	+363 (DMF)	2
	Mycoheptin	362.0	382.0	406.0	940	1 600	1 800		19
	Candicidin	360.0	379.0	401.0	—	—	—		2
	Hamycin	363.0	383.0	406.0	—	962	918	+216 (C_5H_5N)	2
	Heptafungin	360.0	380.0	402.0	—	660	—		21
	Levorin A	356.0	379.0	398.0	702	1 000	798		22
	Levorin B	362.0	381.0	404.0	620	1 000	947		22
	Trichomycin	361.5	382.0	404.0	625	863	750		2
	Trichomycin A	363.0	384.5	407.0	—	—	—		23
	Fungimycin (perimycin)	361.0	383.0	406.0	—	—	—		2
	Aureofungin	359.0	379.0	402.0	—	—	—	+83.7 (DMF)	46
Non-polyene antibiotics	Antimycin A	225.0	321.0	—	625	116	—		47
	Cycloheximide	287.0	—	—	130	—	—		48

* DMF = dimethylformamide; DMSO = dimethyl sulphoxide; C_5H_5N = pyridine.

antibiotics may also be co-produced with the polyene antibiotic. Strains of *Streptomyces noursei* produced cycloheximide and nystatin.[63] Antimycin has been demonstrated as a contaminant of the aromatic heptaene ascosin[64] and levoristatin, which is co-produced with levorin by strains of *Actinomyces levoris*,[65] has been identified as an antimycin A complex.[66] Antimycin A and cycloheximide have been isolated from strains of *Streptomyces griseus*,[67,68] which are used for the production of candicidin, so there is a possibility that either of these non-polyene antibiotics could contaminate candicidin.

TABLE III

TOXICITY RANGE OF POLYENE ANTIFUNGAL ANTIBIOTICS, THEIR PREPARATIONS AND CO-PRODUCED NON-POLYENE ANTIFUNGAL ANTIBIOTICS TO MICE

Antibiotic	LD_{50}/mg kg^{-1} in mice				Reference
	Oral	Intraperitoneal	Subcutaneous	Intravenous	
Amphotericin A	—	450	—	—	49
Natamycin	1 500	250*	5 000*	5–10	5, 50
Nystatin	2 300–8 000	8–150	—	3	7, 37, 51
Amphotericin B	>8 000	280–1 640	—	4–11.3	28, 29, 37, 49
Candidin	>100	7–36	30	1.5	52, 53
Candicidin	98–400	2.1–7	—	—	7, 37
Candicidin A	—	47–65	277	—	53, 54
Candicidin B	—	53	159	—	53
Hamycin	100–300	8–18	—	6–9	6, 7
Levorin	—	9–40	—	43	5
Trichomycin	300	2.2	17	2.2	55
Fungimycin	>500	—	—	250	56
Amphotericin B + sodium deoxycholate (Fungizone)	—	88	—	4–4.5	29, 39
Amphotericin B methyl ester	—	1 320	—	75–106	28, 37, 39
Antimycin A	55	1.7–7.6	25	0.9	57
Cycloheximide	375	—	150–160	150–160	58

* Rats.

TABLE IV

ANTIFUNGAL ACTIVITY RANGE OF POLYENE ANTIBIOTICS AND CO-PRODUCED NON-POLYENE ANTIBIOTICS

Antibiotic	Minimum inhibitory concentration/μg ml^{-1}				Reference
	Candida albicans	*Cryptococcus neoformans*	*Saccharomyces cerevisiae*	*Trichophyton rubrum*	
Amphotericin A	4.7	3.1	2.4	4.7	59
Natamycin	3.0–6.0	3.0–10.0	0.9–15.0	3.0–15.0	5
Nystatin	1.2–3.1	1.6	0.8–3.1	6.3–14.0	5
Amphotericin B	0.05–3.7	0.1–0.6	0.05–0.5	0.5–30.0	5, 28, 29, 60
Candidin	0.6	—	0.5	—	5
Mycoheptin	0.62	0.62	0.55	—	19
Candicidin	0.02–0.5	—	0.01–0.03	500	3, 5, 21
Hamycin	0.01	0.005	0.012–0.015	20.0	5
Heptafungin	0.5–1.0	—	—	—	21
Levorin A	0.2	0.05	0.05	5.0	22
Levorin B	0.2	0.025	0.05	—	22
Trichomycin	0.03–0.7	0.17	0.03–0.25	—	5
Fungimycin	0.06–0.1	0.05	0.07	—	5
Aureofungin	0.01–0.4	0.01–0.05	0.1–0.4	0.4–1.0	5
Amphotericin B methyl ester	0.05–0.5	0.1–0.5	0.05–0.5	0.5–1.0	28, 60
Antimycin A	—	—	40.0	—	61
Cycloheximide	>1 000.0	>0.24	10.0	>1 000.0	62

Mixtures of closely related polyenes with the same chromophore have been reported in nystatin (A1 and A2),[69] candidin (candidin, candidinin, candidoin),[17] candihexin (I and II),[70] candicidin (A, B and C),[53] heptafungin (A and B),[21] levorin (A and B)[22] and trichomycin (A and B).[71] The formation of amphotericin A and amphotericin B from *Streptomyces nodosus*[72] is an example of the co-production of a tetraene and a heptaene. Similarly, a pentaene antifungal antibiotic is produced with mycoheptin.[19] The hexaene complex candihexin produced by a mutant strain of the candidin-producing *Streptomyces viridoflavus* contained two heptaenes identified as candidin components.[70] Numerous non-polyene antibacterial antibiotics have been isolated along with the polyene antibiotics, but these are less likely to affect the analysis and assay of the polyene antibiotics although they may affect toxicity. The same polyene has been recovered from micro-organisms isolated from widely separated areas. In 1955, a Streptomyces strain taken from a soil sample in Natal, named *Streptomyces natalenis*, produced the antibiotic natamycin.[50] Four years later, the antibiotic tennecetin was isolated from a Streptomyces strain collected in Chattanooga, Tennessee,[73] which was shown to be identical with natamycin.[74] The antibiotics candicidin, hamycin, levorin and trichomycin have very similar properties and the evidence to date suggests that they consist of the same major component with variations in their minor components.[75–77] Tetramycin, claimed to be a new tetraene antibiotic, has been isolated from a strain of *Streptomyces noursei*,[78] a micro-organism usually associated with the production of the tetraene nystatin.[63]

The proposal to classify the streptomycetes species based on the use of morphological and physiological criteria, including the identity of antibiotics produced by different strains,[79] cannot be completely realised until the definitive differentiation of many of the polyene antibiotics has been completed.

Because of the co-production of antibiotics by cultures producing the polyenes, strain selection and special extraction procedures have to be employed in order to obtain high titres of the required antibiotic. Strains have been selected for the production of nystatin that have lost the ability to produce cycloheximide and related antibiotics.[80] Most polyene antibiotic-producing cultures are grown under submerged aerobic conditions at temperatures ranging from 26 to 34 °C in a nutrient broth containing one or more nitrogen sources, a carbon-containing metabolisable energy source and inorganic salts.[4,81,82] Nitrogen sources included yeast products, meat products, soyabean meal and casein hydrolysates. Among the amino-acids examined experimentally, asparagine had a stimulating effect on the synthesis of amphotericin B[83] and candicidin.[84] Energy sources used include carbohydrates, sugars,

alcohols and animal and vegetable oils. Alteration of the carbohydrate composition of the medium in *Streptomyces nodusus* fermentations resulted in a significant change in the ratio of amphotericin A to amphotericin B.[85] The addition of glucose during the latter stages of fermentation extended the period of synthesis of candicidin, provided that the medium was maintained at pH 8, resulting in yields of 4 g l^{-1}.[82] Optimum yields of amphotericin B (4·5 g l^{-1}) were obtained with glucose as the sole carbon source.[83] Increased titres of amphotericin B, candicidin and nystatin have been recorded by the addition to the media of nickel, zinc, cobalt, iron and magnesium.[49,82,85]

Fermentation is usually stopped after 90–168 h, depending on the polyene being produced. Most of the polyene antibiotic is found associated with the mycelium, an exception being candihexin, for which about 80% of the antibiotic was found in the supernatant broth.[70] The first step in the recovery process is the removal of the mycelium from the fermented medium and the antibiotic is then extracted from the mycelium with a suitable solvent.

Amphotericin A and B are extracted with propan-2-ol at pH 10.5; the extract is neutralised and concentrated *in vacuo*, forming a precipitate which is washed with water and acetone before drying. This crude material is slurried with a 2% *m/V* solution of calcium chloride in methanol and the amphotericin A dissolves. The insoluble residue is extracted with acidic dimethylformamide, the extract is diluted with methanol, the pH is adjusted to 5 and the subsequent addition of water results in the precipitation of amphotericin B.[49] On a pilot scale, amphotericin B has been purified by countercurrent distribution with a system composed of chloroform - methanol containing 3% *m/V* of calcium chloride - water - butan-1-ol (20 + 20 + 10 + 1).[83]

Levorin is recovered by adjusting the whole culture fluid to about pH 5.6, adding diatomite and filtering. The residue is washed several times with methylene chloride to remove levoristatin (antimycin A) and other impurities and then extracted several times with solutions of propan-2-ol and acetone. The use of solvents of low boiling-point allows the levorin to be concentrated at 35–40 °C, reducing thermal inactivation to a minimum. The levorin precipitates on concentration and the precipitate is washed with methylene chloride prior to drying.[85]

Candicidin was originally purified by chromatography of a chloroform suspension of candicidin on a cellulose powder column.[86] Purification of the crude eluate from the column, containing about 20% *m/V* of candicidin, is achieved by solution and precipitation from a pyridine - acetic acid - water solvent system followed by thorough washing with acetone to give a preparation of 70% purity. Countercurrent distribution (pyridine - ethyl acetate - water, 3.5 + 6.5 + 8.3) was used to obtain preparations of 90% purity, final purification being achieved by repeated precipitation from pyridine - acetic acid - water mixtures. Pure candicidin has been crystallised as needles or rosettes from aqueous tetrahydrofuran.[87]

Chromatography on an alumina column was used to fractionate trichomycin. Fraction A was eluted with Sorensen's phosphate buffer (pH 8), fraction B with a mixture of 20% *V/V* pyridine and 5% *V/V* hydrochloric acid (95 + 5) and fraction C with pyridine - butan-1-ol - water (3 + 4 + 7).[88] Extraction of trichomycin with a specially developed solvent mixture (pyridine - dioxan - water - butyl acetate, 30 + 40 + 52 + 18) resulted in samples of high potency but uncertain purity.[89] The main fraction of trichomycin has been purified by countercurrent distribution [chloroform - methanol - borate buffer (pH 8.4), 2 + 2 + 1] and shown to consist of two similar components, A and B.[71]

The use of substrates labelled with carbon-14 has shown that propionate and acetate are the precursors of the macrolide ring of amphotericin B[83] and nystatin.[90] The aromatic moiety of candicidin is synthesised from glucose through the shikimic acid pathway to *p*-aminobenzoic acid, which is then incorporated into candicidin.[91] Studies with candihexin suggest that the biologically active candihexins A and B are formed from the biologically inactive candihexins E and F, which lack mycosamine. Glycosylation was thought to occur during cell secretion of the antibiotic, and it is likely that the formation of candicidin is similar.[92]

The purity of most polyene antibiotics will be dependent on the strain of antibiotic-producing micro-organism, the fermentation conditions and the extraction procedures used. The antibiotic could contain a non-polyene antibiotic, an unwanted polyene antibiotic, inactive precursors and degradation products. The active impurities could interfere with a biological assay and influence toxicity, while inactive impurities could interfere with a spectrophotometric assay.

Chemical Methods of Analysis and Assay

Chemical analysis is used to establish the identity and purity of polyene antibiotics. A polyene can be assigned to a specific chromophore group, *e.g.*, tetraene or heptaene, by its ultraviolet spectrum. The heptaenes are differentiated by the presence of either mycosamine or perosamine as the amino-sugar and often by the presence of an aminophenyl moiety (either *p*-aminoacetophenone or *N*-methyl-*p*-aminoacetophenone). The amino-sugar can be identified by chromatography after acid hydrolysis of the polyene.[20,22] The aminophenyl moieties are characterised after alkaline hydrolysis of the polyene by chromatography and ultraviolet absorption (*p*-aminoacetophenone, $\lambda_{max.}$ 318 nm; *N*-methyl-*p*-aminoacetophenone, $\lambda_{max.}$ 330 nm).[20,22] A limit test for *p*-aminoacetophenone is included in the monograph for candicidin in the British Pharmacopoeia.[93]

Numerous paper and thin-layer chromatographic systems have been used to check the identity and homogeneity of the polyene antibiotics; some are given in Tables V and VI. The considerable variation between the results of different groups is a reflection of the variation in the purity of the antibiotic and the experimental conditions used. Tailing is frequently encountered with both methods, possibly owing to the heterogenous nature of many of the polyenes, to degradation of the antibiotic during development, or to the unsuitability of the chromatographic methods for such insoluble compounds, and it is possible that the polyene may complex with divalent metals present in the stationary phase. Chromatographic methods are rarely described in detail, and only one literature reference mentions the temperature at which the R_F values were determined.[91] No mention is ever made of precautions taken against photo-inactivation or oxidation, yet both may accelerate decomposition of the polyenes. Even the solvent used for preparing the polyene solution for chromatography can influence the result. Nystatin dissolved in either methanol or dimethylformamide was degraded to give two spots on a thin-layer chromatogram but only one spot when dissolved in a mixture of methanol and dimethylformamide (1 + 3).[94] For identification purposes, the methods used for detecting the polyenes do not have to be very sensitive, but sensitive methods are needed in order to assess purity. Spray reagents described for use in thin-layer chromatography are not very satisfactory; a load of 10–100 μg was necessary to show the main component on a thin-layer chromatogram,[94,95] whereas a load of 2.5–10 μg gave good resolution of components when detected by ultraviolet absorption[96] or biological activity. Detection of active components is usually made with a strain of *Saccharomyces cerevisiae*. Care should be taken that residues of the developing solvents do not inhibit the growth of the yeast. A direct spectrodensitometric method has been developed to quantify the components of candihexin and candidin after separation by thin-layer chromatography.[96]

Chromatographic systems are satisfactory for distinguishing polyenes with different chromophore groups, *e.g.*, amphotericins A and B[59,83] or a co-produced pentaene in mycoheptin.[19] The tetraene natamycin was differentiated from the tetraenes amphotericin A and nystatin by the use of two separate chromatographic systems.[50] However, the aromatic heptaenes ascosin, candicidin, hamycin, levorin and trichomycin have never been convincingly separated from each other by chromatographic methods.[75,76,100,101] Thin-layer chromatography has been used to check the purity of polyene antibiotic derivatives,[28,35,37] and has also been used to show that de-esterification of the methyl ester of amphotericin B does not occur in mice.[104] Inactive components in candihexin complex lacking mycosamine, being less polar than the active candihexins, were demonstrated by thin-layer chromatography.[92,96] Contamination of ascosin with antimycin A was detected by developing an acetone extract of ascosin on a silica-gel plate with chloroform - acetone (9 + 1).[64] The absence of levoristatin (antimycin A) in levorin is confirmed by thin-layer chromatography on an alumina plate at pH 4 using water-saturated butanol as solvent. Levoristatin, if present, is revealed as a bluish lilac spot at R_F 0.6 when examined under an ultraviolet lamp.[105]

An attempt to identify polyenes by paper electrophoresis was unsuccessful; no single system differentiated all of the polyenes examined and no indication was given of the stability of the polyenes under the conditions of the experiment.[106]

Column chromatography has been used for the purification of polyene antibiotics but as a batch elution procedure it is unsuitable for use as an analytical method. High-performance liquid chromatography using a bonded non-polar stationary phase and a polar mobile phase (water - methanol - tetrahydrofuran, 420 + 90 + 45–90) allowed the separation of the components in different classes of polyenes[107] by varying the proportion of tetrahydrofuran in the

TABLE V

PAPER-CHROMATOGRAPHIC SYSTEMS FOR 10 ANTIFUNGAL ANTIBIOTICS

Solvent system (*V/V*)	R_F values of antibiotics										Reference
	Ascosin	Candicidin	Trichomycin	Nystatin	Antimycin A	Cycloheximide	Levorin A	Aureofungin	Hamycin	Candidin	
Water-saturated butan-1-ol*	0.33	0.44	0.35	0.25	0.93	0.90	—	—	—	—	97
Butan-1-ol - acetic acid - water (2 + 1 + 1)*	0.88	0.86	0.85	0.76	0.98	0.96	—	—	—	—	97
Butan-1-ol - pyridine - water (1 + 0.6 + 1)*	0.79	0.80	0.76	0.73	0.97	0.96	—	—	—	—	97
Acetone - water (1 + 1)*	0.43, 0.73	0.38, 0.69	0.69	0.82	0.86	0.91	—	—	—	—	97
Butan-1-ol - McIlvaines buffer (pH 6.5)	0.00, 0.55	0.00, 0.52	0.00, 0.51	—	—	—	—	—	—	0.34	98
Methanol - ammonia - water (20 + 1 + 4)	0.00, 0.61, 0.92	0.00, 0.44, 0.66	0.00, 0.43, 0.65	—	—	—	—	—	—	0.49	98
Butan-1-ol - acetic acid - water (20 + 1 + 25)	0.00, 0.42, 1.0	0.00, 0.44	0.00, 0.40	—	—	—	—	—	—	0.12	98
Butan-1-ol - pyridine - water (4 + 1 + 5)	—	0.39	0.54	—	—	—	—	—	—	—	99
Butan-1-ol - pyridine - water (6 + 1 + 7)	—	0.28	0.32	—	—	—	—	—	—	—	99
Butan-1-ol - ethanol - water (4 + 2 + 1)	—	0.53	0.48	—	—	—	—	—	—	—	99
Butan-1-ol - ethanol - water (3 + 3 + 2)	—	0.89	0.84	—	—	—	—	—	—	—	99
Butan-1-ol - pyridine - water (6 + 4 + 5)*	—	0.71	—	—	—	—	0.71	—	—	—	100
Methanol - 25% ammonia - water (20 + 1 + 4)*	—	0.55	—	—	—	—	0.55	—	—	—	100
Ethyl acetate - pyridine - water (4 + 3 + 2)*	—	0.66	—	—	—	—	0.66	—	—	—	100
Water-saturated butan-1-ol*	—	—	0.26	—	—	—	—	—	0.26	—	101
Butan-1-ol - pyridine - water (1 + 0.6 + 1)*	—	—	0.75	—	—	—	—	—	0.75	—	101
Acetone - water (1 + 1)*	—	—	0.78	—	—	—	—	—	0.77	—	101
Methanol - ammonia - water (20 + 1 + 4)	—	—	0.42, 0.67	—	—	—	—	—	0.44, 0.67	—	101
Butan-2-ol - ethanol - water (2 + 2 + 1)	—	0.60	0.59	—	—	—	—	0.69	—	—	46
Amyl alcohol - methanol - water (10 + 5 + 4)	—	—	0.49	—	—	—	—	0.66	—	—	46
Ethyl acetate - pyridine - water (12 + 5 + 14)*	—	—	0.17	—	—	—	—	—	0.08–0.22†	—	20
Butan-1-ol - acetic acid - water (20 + 1 + 25)*	—	—	0.34	—	—	—	—	—	0.22–0.43†	—	20
Propan-1-ol - water (4 + 1)*	—	—	0.41	—	—	—	—	—	0.38	—	20
Propan-2-ol - water (3 + 1)*	—	—	0.88	—	—	—	—	—	0.87–0.88†	—	20
Butan-1-ol - acetic acid - water (20 + 1 + 25)*	—	—	0.60	—	—	—	—	0.63	0.59	—	24
Propan-1-ol - water (4 + 1)*	—	—	0.58	—	—	—	—	0.59	0.56	—	24
Ethyl acetate - pyridine - water (12 + 5 + 14)	—	—	0.14, 0.17	—	—	—	—	0.18, 0.42	0.17	—	24
Butan-1-ol - methanol - water (2 + 2 + 3)*	—	—	0.94	—	—	—	—	0.96	0.95	—	24
Butan-1-ol - methanol - water (4 + 1 + 2)*	—	—	0.32	—	—	—	—	0.30, 0.67	0.31, 0.64	—	24
Butan-1-ol - pyridine - water*	—	—	0.28	—	—	—	—	0.26, 0.64	0.27, 0.61	—	24
Ethyl acetate - pyridine - 25% acetic acid (5 + 5 + 4)*	—	—	0.72	—	—	—	—	0.71	0.69	—	24

* Whatman No. 1 paper specified.
† R_F varied with purity of sample.

TABLE VI

THIN-LAYER CHROMATOGRAPHIC SYSTEMS FOR 13 ANTIFUNGAL ANTIBIOTICS

In all systems silica gel G was used as the stationary phase.

Solvent system (*V*/*V*)	Detection*	R_F values of antibiotics: Natamycin	Nystatin	Amphotericin A	Amphotericin B	Trichomycin	Antimycin A	Candicidin	Reference
Ethanol - ammonia - water (8 + 1 + 1)†	A	0.34	0.18	0.33	—	0.45	0.72	—	102
Butan-1-ol - acetic acid - water (3 + 1 + 1)†	A	0.34	0.18	0.33	—	0.17	—	—	102
Methanol - propan-2-ol - acetic acid (90 + 10 + 1)‡	B	0.40	0.54	—	0.18	—	—	—	103
Methanol - acetone - acetic acid (8 + 1 + 1)‡	B	0.54	0.66	—	0.45	—	—	—	103
Ethanol - ammonia - dioxan - water (8 + 1 + 1+ 1)‡	C and D	0.18	0.28	0.19	0.19	0.30	—	—	95
Butan-1-ol - pyridine - water (3 + 2 + 1)‡	C and D	0.55	0.65	0.56	0.32	0.58	—	—	95
Butan-1-ol - ammonia - methanol - water (20 + 1 + 2 + 4)	B	—	—	—	0.07	0.13	—	0.13	21
Chloroform - methanol - borate buffer (pH 8.3) (7 + 5 + 1)	B	—	—	—	0.60	0.49, 0.67	—	0.49, 0.67	21
Butan-1-ol - ethanol - acetone - 32% ammonia (2 + 5 + 1 + 3)	E	—	—	—	0.41	—	—	0.55	37
Chloroform - 95% ethanol - water (50 + 50 + 8)	E	—	—	—	—	—	—	0.34, 0.74, 0.95	91

Solvent system (*V*/*V*)	Detection*	R_F values of antibiotics: Mycoheptin	Heptafungin	Hamycin	Levorin	Candihexin	Candidin	Reference
Butan-1-ol - ammonia - methanol - water (20 + 1 + 2 + 4)	B	0.06	0.10	0.13	0.13	—	—	21
Chloroform - methanol - borate buffer (pH 8.3) (7 + 5 + 1)	B	0.18	0.55	0.49, 0.67	0.49, 0.67	—	—	21
Butan-1-ol - ethanol - acetone - 32% ammonia (2 + 5 + 1 + 3)	E	—	0.42	—	—	—	—	37
Lower phase of chloroform - methanol - 20% ammonia (2 + 2 + 1)	E	—	—	—	—	0.26,0.29,0.36, 0.39,0.43	—	96
	E	—	—	—	—		0.27,0.30,0.33, 0.37,0.40	92

* Method of detection—
A: 10% $KMnO_4$ and 0.2% bromophenol blue.
B: 0.2% *p*-dimethylaminobenzaldehyde in H_2SO_4 containing $FeCl_3$.
C: 5% $KMnO_4$.
D: Orthophosphoric acid, heat for 5 min at 100 °C.
E: Spectrodensitometric.
† Buffered with phosphate (pH 8).
‡ Activated for 1 h at 110 °C.

mobile phase. Nystatin was shown to consist of three components, two tetraenes and one heptaene. Candidin was resolved into at least five components, two trace components were detected in amphotericin B and candicidin was separated into five components. Candicidin was differentiated from hamycin and trichomycin and there was an indication that hamycin and trichomycin differed although there were similarities in their minor components.

Pyrolysis gas chromatography[77] can differentiate nystatin and amphotericin B from each other and from candicidin, levorin and trichomycin. Small differences in the pyrograms of samples of levorin were explained as showing quantitative differences in the composition of the samples. Gas chromatography of the products of chemical degradation of candicidin, levorin and trichomycin indicated that differences do exist between these antibiotics.[108]

The successful purification of trichomycin by countercurrent distribution[71,109] led to the use of this technique as an analytical method for identifying and examining the polyene antibiotics. A list of the systems used and results obtained is given in Table VII. In an attempt to compare the results of different workers, the partition coefficient, K, has been calculated using the equation

$$K = \frac{N_{max.}}{N_T - N_{max.}}$$

where $N_{max.}$ is the tube number containing the maximum concentration of antibiotic and N_T is the total number of transfers made.

Many polyenes have been shown to be complex mixtures when subjected to countercurrent distribution. Levorin was separated into two main components, levorin A and B, and levorin A was further fractionated into three components. Three components have been

TABLE VII

PARTITION COEFFICIENTS OF POLYENE ANTIFUNGAL ANTIBIOTICS OBTAINED IN COUNTERCURRENT DISTRIBUTION SYSTEMS

Solvent system (*V*/*V*)	N_T*	Partition coefficient, *K*†	Reference
Chloroform - methanol - borate buffer (pH 8.2–8.4) (2 + 2 + 1)	240	Trichomycin A 0.411; trichomycin B 0.739	71
	200	Amphotericin B 2.70; candidin 3.44; mycoheptin 4.26	19
	300	Candicidin 0.78; levorin A 0.74	22
	49	Levorin A 0.90; levorin B 5.1	22
	200	Trichomycin (major fraction) 0.61; trichomycin (minor fraction) 1.31	76
	200	Candicidin 0.61; levorin 0.61; hamycin 0.61	76
	600	Candicidin 1.02; levorin A2 1.07	100
	80	Levorin A1 0.60; levorin A2 1.07; levorin A3 1.37	100
	360	Candidoin 1.81; candididin 2.55; candidin 4.16	17
Chloroform - methanol - sodium acetate (pH 8.4) (2 + 2 + 1)	240	Trichomycin A 0.47	109
	200	Hamycin 1.10	20
	200	Aureofungin 0.72	24
Chloroform - methanol - water (2 + 2 + 1)	300	Candicidin 0.38; heptafungin A 0.77	21
Chloroform - methanol - citrate + phosphate buffer (pH 6.25) (2 + 2 + 1)	550	Candihexin 1.19; 1.37; 1.85; 2.18; 2.50; 3.37	70
n-Amyl alcohol - isoamyl alcohol - citrate + phosphate buffer (pH 5) (12 + 17 + 29)	200	Nystatin A1 4.6; nystatin A2 16.8	69
Pyridine - ethyl acetate - water (3.5 + 6.5 + 8.3)	49	Levorin A 1.00; levorin B 0.46	22

* N_T = total number of transfers; $N_{max.}$ = number of tube containing maximum concentration.
† Calculated from $N_{max.}/(N_T - N_{max.})$.

demonstrated in both candidin[17] and in heptafungin complex; the major component heptafungin A accounted for about 80% of the total and heptafungin B had the same partition coefficient as trichomycin B.[21] The separation of candihexin complex was markedly improved by reducing the pH of the partitioning system from 8.3 to 6.25, which revealed four hexaene, two heptaene and two non-polyene antifungal components.[70] On the basis of countercurrent distribution, the main active fractions of candicidin, hamycin, levorin and trichomycin were considered to be the same, but there were differences between minor, biologically inactive, components.[76] On re-running the main fractions of the above antibiotics, the distribution curves plotted on the basis of antifungal activity and absorption at 380 nm did not match. The tubes of maximum antifungal activity and maximum absorption varied by six and nine tubes, depending on the antibiotic, which suggests that more than one component may have been present. Reproducible differences between the partition coefficients of candicidin and levorin A have been reported, *e.g.*, candicidin 0.785, levorin A 0.744,[22] and candicidin 1.02, levorin A (complex) 0.98, levorin A2 1.07.[100]

It has been suggested that the heptaenes can be differentiated by the partition coefficients in two solvent systems[22] (see Table VIII).

TABLE VIII

PARTITION COEFFICIENTS OF HEPTAENE ANTIBIOTICS

Solvent system	Partition coefficient of heptaene		
	Group 1*	Group 2*	Group 4*
Pyridine - ethyl acetate - water (3.5 + 6.5 + 8.3)	0.5	1.0	2.0
Chloroform - methanol - borate buffer (pH 8.3) (2 + 2 + 1)	4.0	1.0	0.1

* See Table I.

The group 2 heptaenes could be further divided into three sub-groups on the basis of their distribution in chloroform - methanol - borate buffer (pH 8.3) (2 + 2 + 1), giving *K* values of about 1.0 for candicidin, trichomycin A and levorin A, 1.5 for trichomycin B and 5.1 for levorin B. *K* values obtained in other laboratories do not accord with this classification, possibly

because the experimental conditions varied, *e.g.*, differences in phase ratio [upper: lower, 1:1 (ref. 71) or 1:2 (ref. 76)], temperatures where quoted (11 °C;[109] 17, 19 and 25 °C[71]) and the purity of the samples, all variables which can affect the partition coefficient. In addition, the antibiotic may have degraded during the separation, which can take between 10 h (ref. 71) and 15 h (ref. 76) for 220 and 200 transfers, respectively. Countercurrent distribution has yielded useful information on the composition of the polyene antibiotics, but it would be even more useful if workers reported the experimental conditions and tried to standardise these conditions and the calculation of the partition coefficients. Monitoring the separation on the basis of absorption and biological activity would reveal the presence of both active and inactive components, while increasing the efficiency of countercurrent distributions would provide a powerful tool for the analysis of these complex substances. An apparatus for increasing the number of transfers without increasing the running time, under controlled temperature, has been built on the concept of the coil planet centrifuge.[110]

Chemical assays for the polyene antibiotics include titrimetric, colorimetric and spectrophotometric methods and a non-aqueous titration for nystatin has been described. Optimum conditions were obtained for both potentiometric and visual end-point titrations by using a solution of 5–50 mg of nystatin in 15 ml of acetic acid - dioxan (1 + 14), titrated with 0.01 N perchloric acid.[111] The yellow colour produced when nystatin is hydrolysed with sodium hydroxide[112] has formed the basis of a colorimetric assay for nystatin[113] and amphotericin B,[114] interference by extraneous matter being minimised by extracting the pigment with chloroform. This procedure resulted in an assay which correlated well with the microbiological assay and which was considered suitable for monitoring the stability of the antibiotics, including amphotericin B in intravenous infusions[115] provided that the infusion was kept in darkness. A quantitative method for the determination of natamycin, nystatin and amphotericin B, based on the formation of a blue colour in a strongly acidic medium, has been described in detail.[103] Samples were evaluated by comparison with a standard graph; the method was shown to be precise when tested on the intact antibiotic.

Spectrophotometric assay of the polyene antibiotics involves determining the net absorbance of the central peak by subtracting the average absorbance of the minima on each side of the peak itself. The calculated specific absorption of the sample is compared against that of a standard of known purity. The antibiotic concentration can also be calculated from the $E_{1cm}^{1\%}$ value of the pure antibiotic. Spectrophotometric methods are useful as they give a presumptive identification of the antibiotic. They are less sensitive than biological assays but more specific and sensitive than colorimetric methods, especially when measuring antibiotic concentrations in body fluids and tissues. A correction for non-specific absorption in the near-ultraviolet region is required and for this reason the method is unsuitable for the determination of tetraene antibiotics in biological specimens. A spectrophotometric method has been developed to measure amphotericin B in whole blood and plasma,[116] in which amphotericin B was extracted with butanol in order to reduce the high background absorbance that was due partly to trace amounts of haemoglobin and to recover any antibiotic that may be associated with cholesterol in the cellular fraction of whole blood. Spectrophotometric assays usually give higher values than do biological assays.[117] These differences may be due to degradation, which results in loss of activity without loss of the characteristic light absorption, or to the presence of an inactive aglycone as found in candihexin complex.[92] A good correlation is apparent between the specific absorption at 380 nm and potency for samples of levorin.[77] During stability studies on nystatin solutions, the correlation between biological activity and absorption at 360 and 320 nm disappeared for solutions over 24 h old.[118] A spectrophotometric method for the measurement of nystatin in the presence of its degradation products, in which irrelevant absorption was corrected for by the application of orthogonal functions, has been described[119]; unfortunately, there was no concurrent biological evaluation of the nystatin samples. The requirements of the FDA[120] include a limit test for the photodegradation of nystatin, which is determined spectrophotometrically. A qualitative assay based on the enhanced fluorescence of amphotericin B in acidic solution and claimed to be sensitive and linear in the range 0.1–10 μmol l^{-1} has been developed to measure the binding of amphotericin B to yeast cells.[121]

Chemical assays are convenient methods for evaluating the polyene antibiotics; however, they do not supply information about antifungal activity and are best used when the composition of the sample is known.

Biological Assay

Biological assay is the accepted criterion by which the polyene antibiotics and their preparations are assessed. The usual methods are diffusion and turbidimetric assays and details of some of the established methods are given in Table IX. The basis of the biological assay is to determine the potency of the sample under test by comparison with a standard of defined activity.

International standards exist for amphotericin B[131] and nystatin,[132] and national standards for candicidin are available in the USA and UK and for trichomycin in Japan. There is a need for international reference preparations of candicidin and trichomycin, but as there is only limited information on the relative compositions of these and other related antibiotics such as hamycin and levorin, more information is required before reference samples of these antibiotics can be established.[133] Ideally, the composition of the sample and the standard should be identical but, because of the heterogeneity of most polyenes, especially the aromatic heptaenes, this is unlikely to be achieved, so it may be difficult to obtain statistically valid assays with parallel dose-responses. Similarly, batches of these antibiotics currently available may have relative compositions that are completely different from that of the material used to establish the standard. The minimum potency requirement of the FDA and USP for nystatin has been increased from 2 000 U mg^{-1} (ref. 120) to 4 400 U mg^{-1} (ref. 134) and the present international standard for nystatin established in 1963 may no longer be representative of material in current use. An indication of the interference that may be encountered as a result of heterogeneity can be seen by reference to the minimum inhibitory concentrations (Table IV). The presence of a heptaene in a sample of nystatin may inflate the assay result and even a less active component will have an additive effect, *e.g.*, amphotericin A in amphotericin B. By using a strain of *Candida tropicalis* that was more sensitive to amphotericin B than to amphotericin A, a turbidimetric assay was developed for the assay of amphotericin B in the presence of amphotericin A.[135] Similarly, a strain of *Candida albicans* that was not susceptible to cycloheximide was chosen for the assay of nystatin fermentation broth likely to contain cycloheximide.[122] For other samples of nystatin, including products that contain tetracycline (Mysteclin), *Saccharomyces cerevisiae* is used, as tetracycline reduces the activity of nystatin against *Candida albicans*.[122] The alkaline diluent that is used to enhance diffusion in the diffusion assay for amphotericin B also inactivated amphotericin A.[126] Amphotericin A can be assayed in the presence of amphotericin B by using a mixed standard that approximates to the expected composition of the sample and by the selectivity of the diffusion process and the test organism *Rhodotorula glutinis*.[122]

An assay based on the inhibition of the formation of blastospores of *Candida tropicalis* has been described for ascosin, candicidin and nystatin.[136] Inhibition of respiration of sensitive yeast cultures has been used to assay nystatin or amphotericin B; the amount of carbon dioxide produced by the yeast was inversely proportional to the antibiotic concentration.[137] This assay was adopted for use with an AutoAnalyzer,[126] the amount of carbon dioxide being determined by the degree of decolorisation of an alkaline phenolphthalein solution. The incorporation of a continuous dilution system and a sample module of large capacity has further automated the method.[138] Conductimetric measurements showed that the leakage of intracellular constituents from yeast cells treated with amphotericin B or nystatin was proportional to the concentration of the antibiotic[139] and the use of potassium-sensitive electrodes[140,141] to monitor the loss of potassium ions from susceptible cells treated with polyene antibiotics should allow this effect to be used for the rapid assay of polyene antibiotics. A modified agar diffusion assay has been used for trichomycin, in which the antibiotic solution was placed on the surface of seeded agar in a test-tube. The concentration of the antibiotic was proportional to the distance between the liquid - agar interface and the depth where growth was visible.[142]

It is usual to use freshly prepared suspensions of test organisms for the assay of polyene antibiotics. *Candida tropicalis* grown in liquid inoculum medium can be used for up to 2 weeks when stored as a suspension at 4 °C.[126] *Saccharomyces cerevisiae* (ATCC 9763) suspensions in a 0.5% *m/V* solution of peptone in water could be kept frozen at −70 °C for up to 12 months with little change in the dose-response to amphotericin B, and unfrozen suspensions could be used for 2 weeks if kept at 4 °C.[143] Suspensions of *Saccharomyces cerevisiae* (ATCC 2601) were not recommended for storage at −70 °C, though a saline suspension was usable during 4 weeks when stored at 4 °C. When suspended in 0.012 M phosphate buffer and stored

TABLE IX

ASSAY METHODS FOR THE POLYENE ANTIBIOTICS

Test organism	Method*	Medium†	Antibiotic solution‡		Amount range§/ml^{-1}	Incubation		Reference
			Antibiotic	Solution		Temperature/°C	Time/h	
Rhodotorula glutinis (Squibb 2358)	D	3	Amphotericin A	1 mg ml^{-1} DMSO, dilute with 80% *V/V* DMSO	10–40 μg	24	24–36	122
Saccharomyces cerevisiae (A.T.C.C. 9763)	D	5	Natamycin	All dilutions with 50% *V/V* methanol	12.5–50 μg	30	24	16, 123
Saccharomyces cerevisiae (N.C.Y.C. 87)	D	1	Nystatin	75 mg in 50 ml of DMF, dilute 10 ml to 200 ml with a solution containing 9.56% *m/V* of KH_2PO_4 and 11.5% *V/V* of 1 N KOH	25–100 U	35–37	16	124
Saccharomyces cerevisiae (A.T.C.C. 2601)	D	1		1 000 U ml^{-1}, DMF, dilute to 256 U ml^{-1} DMF, dilute with 10% *m/V* potassium phosphate buffer (pH 6.0)	12.8–31.2 U	30	16–18	125
Candida albicans (Squibb 1539)	D	4		1 000 U ml^{-1} DMSO, dilute with 80% *V/V* DMSO	25–200 U	37	18–20	126
Candida tropicalis (A.T.C.C. 13803)	T	10		1 000 U ml^{-1} DMSO, dilute with medium 10	3.25–8 U	30	3–4	126
Saccharomyces cerevisiae (N.C.Y.C. 87)	D	1	Amphotericin B	60 mg per 100 ml DMF, dilute 10 ml to 100 ml with DMF, dilute with potassium phosphate buffer (pH 10.5) containing 8% *V/V* DMF	1–4 U	35–37	16	124
Saccharomyces cerevisiae (A.T.C.C. 9763)	D	1		1 mg ml^{-1} DMSO, dilute to 31.2 μg ml^{-1} DMSO, dilute with potassium phosphate buffer (pH 10.5)	0.64–1.56 "μg"	30	16–18	125
Saccharomyces cerevisiae (A.T.C.C. 9763)	D	1		20 μg ml^{-1} DMSO, dilute with potassium phosphate buffer (pH 10.5), keeping the concentration of DMSO at 5% *V/V*	0.5–1.96 "μg"	37	16–18	126
Paecilomyces varioti (MSSC 5605 NIAID)	D	1		10 "mg" ml^{-1} DMSO, dilute 1 to 10 with 60% *V/V* DMSO, then dilute 1 to 40 with 50% *V/V* propan-2-ol, dilute with potassium phosphate buffer (pH 10.5)	0.01–0.15 "μg"	30	24	127
Candida tropicalis (A.T.C.C. 13803)	T	10		DMSO, dilute to 30 μg ml^{-1} with 25% *V/V* DMSO, dilute to 1 μg ml^{-1} with alkaline medium 10	0.03–0.1 "μg"	30	3–4	126
Saccharomyces cerevisiae (N.C.Y.C. 87)	D	2	Candicidin	25 mg in 30 ml DMSO containing 0.1% *m/V* butylated hydroxyanisole, leave for 20 min, dilute with potassium phosphate buffer (pH 6)	0.1–2.0 U	30–32	16	128
Saccharomyces cerevisiae (A.T.C.C. 9763)	T	9		1 mg ml^{-1} DMSO, dilute with distilled water	0.03–0.12 "μg"			125
Paecilomyces varioti (MSSC 5605 NIAID)	D	1	Hamycin	2 500 U ml^{-1} in alkalinised 50% *V/V* propan-2-ol, dilute with potassium phosphate buffer (pH 10.5)	1–10 U	30	24	127
Curvularia lunata‖ (Wakker) Boedijn	D	8		60% *V/V* ethanol, dilute with an aqueous solution of 0.02% *m/V* Tween 80	1–6 μg	27	18–20	129
Torula utilis¶	D	6	Levorin	1 mg ml^{-1} DMSO, dilute with phosphate buffer (pH 7)	30–80 U	—	—	105
Candida albicans Yu 1200	D	7	Trichomycin	2 mg ml^{-1} solution containing 50% *V/V* acetone and 0.008% *m/V* NaOH, dilute with 25% *V/V* propan-2-ol	25–100 U	37	—	130

* D = diffusion assay; T = turbidimetric assay.

† Number refers to medium given in Table X.

‡ Dilute with solvent to assay levels; DMSO = dimethyl sulphoxide; DMF = dimethylformamide.

§ Amount/ml^{-1} stated by mass (μg), units of activity (U) or micrograms of activity ("μg").

‖ Now called *Cochliobolus lunarus*.

¶ Now called *Candida utilis*.

at 4 °C the culture proved satisfactory for the assay of nystatin for 1 year.[144] *Paecilomyces varioti* has been used for the assay of amphotericin B and hamycin in biological fluids.[126,127] Inoculated agar slants have to be incubated for 1 week at 30 °C in order to obtain the mature spores which are necessary as inoculum to obtain the high sensitivity required for the assay. Media used for the assay of polyene antifungal antibiotics are listed in Table X.

Most assay methods require the polyene antibiotic initially to be dissolved in a polar solvent (dimethylformamide or dimethyl sulphoxide). This solution is then further diluted in an aqueous buffer, which results in the formation of a micellar suspension which does not diffuse freely. This effect probably accounts for the poor diffusion of amphotericin B in agar and the poor dose-response observed with many polyene diffusion assay methods. In a search for a suitable diluent for amphotericin A and B, methanol was rejected as being too volatile to pipette accurately, propan-1- and -2-ol were toxic to the test organism, a colloidal suspension prepared in aqueous sodium deoxycholate foamed and dimethyl sulphoxide was viscous. A mixture of 30% *V*/*V* of dimethyl sulphoxide plus 40% *V*/*V* of methanol in water maintained amphotericin A and B in a true solution at a concentration of 0.1 mg ml^{-1} but not at 1.0 mg ml^{-1}.[135] Aqueous diluents containing 80% *V*/*V* of dimethyl sulphoxide are satisfactory for diffusion assays, while higher concentrations are inhibitory. The activity of nystatin in an agar diffusion assay was dependent on the solvent used, the activity decreasing in the order propylene glycol, methanol, 40% *V*/*V* aqueous dimethyl sulphoxide and water.[145]

TABLE X

MEDIA USED FOR THE ASSAY OF THE POLYENE ANTIFUNGAL ANTIBIOTICS

Concentrations of ingredients are given in grams per litre.

	Number of medium									
Ingredient	1	2	3	4	5	6	7	8	9	10
Peptone	9.4	9.4	5.0	6.0	—	10.0	10.0	—	10.0	5.0
Yeast extract	4.7	4.7	3.0	3.0	2.5	1.5	—	5.0	—	1.5
Malt extract	—	—	3.0	5.0	—	—	—	—	—	—
Beef extract	2.4	2.4	—	1.5	—	—	—	—	—	1.5
Dextrose	10.0	12.0	133.0	21.0	10.0	10.0	5.0	10.0	20.0	11.0
NaCl	10.0	10.0	—	10.0	—	10.0	—	10.0	—	3.5
KCl	—	—	10.0	—	—	30.0	0.5	—	—	—
KH_2PO_4	—	—	—	—	8.5	—	—	0.1	—	3.68
K_2HPO_4	—	—	—	—	—	—	—	—	—	1.32
Na_2HPO_4	—	—	—	—	—	3.0	—	—	—	—
$MgSO_4$	—	—	—	—	—	—	0.5	0.05	—	—
$NaNO_3$	—	—	—	—	—	—	2.0	—	—	—
NaOH	—	—	—	—	1.5	—	—	—	—	—
Glycerol	—	—	—	—	—	—	—	10 ml	—	—
Beef infusion	—	—	—	—	—	500 ml	—	—	—	—
Agar	23.5	23.5	18.0	15.0	15.0	16.0	15.0	Not stated	—	—
pH	6.0–6.2	6.0–6.2	Not stated	5.0–5.5	7.0	6.2–6.4	7.8–8.0	7.0	5.6–5.7	6.0

The polyenes react with susceptible sterol-containing cell membranes, causing the leakage of intracellular constituents; compounds that compete with the membrane sterol for the antibiotic, such as most sterols,[141] digitonin[126] and serum, will reduce its activity.[145] Cholesterol and protein are reported to be involved in the binding of levorin to serum lipoproteins, but only cholesterol is associated with the binding of amphotericin B.[146] The inhibition of glycolysis due to the loss of potassium ions from yeast cells treated with nystatin is prevented at neutral pH by the presence of ammonium or potassium ions,[147] and care should be taken in the respirometric assay that the pH is low enough to abolish this interference.

In nystatin diffusion assays, an increase in the concentration of monovalent cations resulted in an increase in zone size.[117] Increasing the sodium chloride content of nystatin assay agar from 1 to 4% *m*/*V* produced an increase in the size of zones of inhibition, but at the expense of decreased growth. In the absence of added sodium chloride there were no zones

of inhibition even at a concentration of nystatin of 200 U ml^{-1}.[126] The monovalent cations were thought to reduce the non-specific absorption of nystatin in the medium.

Special extraction procedures have to be used for the biological assay of preparations that contain polyene antibiotics. Amphotericin B and candicidin products are extracted with dimethyl sulphoxide[148] and nystatin products are blended with dimethylformamide to extract the active ingredient.[120] Diethyl ether is used to dissolve the ointment base of amphotericin B ointment, and hexane is used for the same purpose with candicidin ointment. The assay of nystatin animal feeds is complicated by the very low concentration of antibiotic present. Modifications to earlier methods[126,149] have included the replacement of *Candida alicans* by *Saccharomyces cerevisiae* as the test organism and dilution of the methanolic extract with 10% phosphate buffer instead of 50% aqueous dimethyl sulphoxide. The animal feed was first extracted with methanol, a portion of which was inactivated by heating and used as the blank for the preparation of the standard. A collaborative study showed the procedure to be satisfactory and it was recommended as the official method.[150]

The assay of antibiotics in biological specimens involves the measurement of low concentrations in small samples and requires methods of high sensitivity. An allowance has to be made for the natural antifungal activity of the biological specimen,[126] which can be effected by using, as a diluent for the standard, blood or serum of the same type and species as the samples to be assayed. A more accurate method is to determine the pre-dose antifungal activity of the individual specimen, but such an approach is not always practicable. When samples from patients infected with pathogenic fungi are to be assayed, the sample and stock standard solution can be pasteurised by heating them at 56 °C for 30 min.[126] In a microturbidimetric assay[126] for amphotericin B, the samples are diluted with 95% ethanol in order to precipitate the proteins and extract the antibiotic. The ethanolic solutions of standard and sample are dispensed into the compartments of a polystyrene tray and evaporated to dryness. Inoculated assay broth is then added to each compartment, the tray incubated and the opacity of each mixture read. The method for nystatin is similar except that the ethanol extraction is omitted so as to prevent loss of sensitivity because of dilution and drying. Corrections had to be made for the intrinsic colour and turbidity of most body fluids. The sensitivity of the method was 0.01 μg ml^{-1} for amphotericin B and 1 U ml^{-1} for nystatin. A sensitive diffusion assay for hamycin and amphotericin B in serum at the 0.01–0.02 μg ml^{-1} level has been described.[127] The severest limitation to the method resulted from the presence of natural antifungal agents in various sera, which invalidated the assay.

Stability

There are general statements in the literature describing the polyenes as a group of unstable compounds which in solution are decomposed by exposure to air, heat and light.[2,3] In the dry state and in the absence of heat and light, the polyenes are stable.[16,151] Most polyenes in aqueous conditions exist as micellar suspensions, which may confer some protection by shielding the labile sites of the molecule. The methyl ester of amphotericin B formed a finer colloidal suspension than amphotericin B in water and this suspension was less stable than a suspension of the parent material.[151] Similarly, an aqueous solution of natamycin was less stable than an aqueous suspension.[16]

Nystatin and amphotericin B solutions in phosphate - citrate buffer were stable between pH 5 and 7,[152] while natamycin was stable between pH 5 and 9.[16] Nystatin lost its activity more readily at extremes of pH than did amphotericin B or natamycin,[103] and the loss of nystatin activity proceeded at a greater rate than did the loss of absorption at the wavelength maxima.[152] The hydrochloride salt of amphotericin B methyl ester is a solid and in aqueous solution was found to be less stable at pH 4–4.5 than at pH 6–6.5.[151] At an alkaline pH, the resistance of natamycin solution to oxidation was decreased.[16]

The methylpentaene filipin in methanolic solution underwent autoxidation, the decrease in activity being correlated with the decay of the ultraviolet spectra due to the loss of a double bond, leading to conversion of the chromophore into a tetraene epoxide; extended autoxidation resulted in the formation of polymeric materials.[153] Very dilute methanolic solutions (0.001% m/V) were stable for prolonged periods but concentrated solutions in the absence of a nitrogen atmosphere were unstable even in the dark at 4 °C. The addition of small amounts of the antioxidant butylated hydroxyanisole had a stabilising effect.[153] Similarly, the oxidative inactivation of natamycin was accompanied by marked changes in the ultraviolet

spectrum and disappearance of the maxima, which was not the case when photo-inactivation occurred.[16] Nystatin solution was oxidised in the presence of air and loss of biological activity proceeded at twice the rate of loss of absorption maxima.[154] The inactivation of nystatin solution was increased at increased temperature and by ultraviolet irradiation, while the presence of an antioxidant reduced the rate of inactivation.[155] The oxidation of nystatin powder was increased in the presence of water, while iron(III) ions accelerated the degradation, which could be prevented by the addition of a chelating agent.[156] The products of nystatin oxidation were organic acids (mainly succinic acid); acetone and acetaldehyde were present if the oxidation occurred in the presence of water.[157] Acid hydrolysis of nystatin resulted in the elimination of mycosamine and the formation of a pentaene chromophore.[13] Reducing agents reacted irreversibly with amphotericin B, destroying its biological activity.[158]

Exposure of aqueous solutions of natamycin to ultraviolet irradiation resulted in the loss of biological activity, but the absorption spectrum did not disappear. Inactivation was thought to be due to a change in the configuration of the chromophore from *trans* to *cis*; chlorophyll protected natamycin from irradiation[159] and oxidation of natamycin was prevented by chlorophyll or ascorbic acid.[159] Ultraviolet irradiation of the methylpentaenes caused oxidative degradation, with the formation of an intermediate tetraene and finally a triene. Nystatin, when irradiated, yielded a mixture of two acidic compounds and a neutral triene.[160] The heptaenes trichomycin, hamycin and aureofungin lost their polyenic character and biological activity when exposed to ultraviolet irradiation, the reaction resembling a retroaldol cleavage accompanied by polymerisation. The aromatic compound *p*-aminoacetophenone, found as a degradation product of the irradiation of hamycin, was shown to accelerate the decomposition.[161] The change in absorption spectrum caused by the ultraviolet irradiation of the heptaene antibiotic DJ 400 was thought to be the result of isomerisation of the chromophore with one *cis* double bond to one with an all-*trans* configuration.

Biological assay of intravenous infusions of amphotericin B in dextrose solution showed that a loss of activity of 26% occurred when the infusions were exposed to light at room temperature over a 3-d period, but a colorimetric assay did not detect any loss of activity.[115] When the infusions were protected from light, there was no reduction in activity. No loss in activity was noted when infusions were exposed to normal lighting conditions over a period of 4–8 h, the time required to administer the drug.[162,163] The addition of sodium and chloride ions resulted in a turbid solution which lost 25% of its activity within 4 h owing to the colloidal aggregation of amphotericin B[163]; this was not commented upon when infusions were prepared with dextrose solution that contained 0.2% *m*/*V* of sodium chloride.[162]

Conclusions

Satisfactory qualitative and quantitative analytical methods have still to be developed that will permit the definition of the composition of the polyene antibiotic complexes and hence a comparison of the relative compositions of different antibiotics and different batches of the same antibiotic. This will be especially important if the heptaene antibiotics are used for their hypocholesterolaemic effect, which may be due to a particular component in the complex. The most promising methods available are high-performance liquid chromatography and countercurrent distribution. A satisfactory thin-layer chromatographic separation for the polyenes is still not available as a simple procedure for detecting active and inactive compounds. A separate assay to measure hypocholesterolaemic activity may be necessary if it is found not to be related to antifungal activity. The choice of diluent used to prepare the antibiotic solutions for assay is critical, as it affects the assay, and the choice of more suitable diluents could improve the dose-response of the bioassay. A rapid and accurate method of measuring the antibiotic concentration in blood is still required in order to monitor patients receiving the drug parenterally. The results of the chemical assessment of antibiotic stability should be treated with caution unless they are correlated with biological activity, as there is evidence that biological activity and chemical measurement do not correlate for degraded polyene antibiotics.

References

1. Vining, L. C., *Hindustan Antibiot. Bull.*, 1960, **3**, 37.
2. Oroshnik, W., and Mebane, A. D., *Fortschr. Chem. Org. NatStoffe*, 1963, **21**, 17.
3. Dutcher, J. D., "Kirk-Othmer Encyclopedia of Chemical Technology," Second Edition, Volume 16, John Wiley and Sons, New York, p. 133.

4. Perlman, D., *Prog. Ind. Microbiol.*, 1967, **6**, 3.
5. Mechlinski, W., Laskin, A. I., and Lechevalier, H. A., *Editors*, "Handbook of Microbiology," Volume III, CRC Press, Cleveland, Ohio, 1973, p. 93.
6. Hamilton-Miller, J. M. T., *Bact. Rev.*, 1973, **37**, 166.
7. Drouhet, E., "Systemic Mycoses," Ciba Foundation Symposium, J. and A. Churchill Ltd., London, 1968, p. 206.
8. Oroshnik, W., Vining, L. C., Mebane, A. D., and Taber, W. A., *Science, N.Y.*, 1955, **121**, 147.
9. Pledger, R. A., and Lechevalier, H., *Antibiot. A.*, 1955–56, 249.
10. Golding, B. T., Rickards, R. W., Meyer, W. E., Patrick, J. B., and Barber, M., *Tetrahedron Lett.*, 1966, **30**, 3551.
11. Ceder, O., and Hansson, R. W., *Tetrahedron*, 1967, **23**, 3753.
12. Chong, C. N., and Rickards, R. W., *Tetrahedron Lett.*, 1970, **59**, 5145.
13. Borowski, E., Zielinski, J., Falkowski, L., Ziminski, T., Galik, J., Kolodziejczyk, P., Jereczek, E., Gdulewicz, M., Shenin, Y., and Kotienko, T., *Tetrahedron Lett.*, 1971, **8**, 685.
14. Mechlinski, W., Schaffner, C. P., Ganis, P., and Avitabile, G., *Tetrahedron Lett.*, 1970, **44** 3873.
15. Borowski, E., Zielinski, J., Ziminski, T., Falkowski, L., Kolodziejczyk, P., Golik, J., Jereczek, E., and Adlercreutz, H., *Tetrahedron Lett.*, 1970, **45**, 3909.
16. Raab, W. P., "Natamycin (Pimaricin), Its Properties and Possibilities in Medicine," Georg Thieme Verlag, Stuttgart, 1972, p. 12.
17. Borowski, E., Falkowski, L., Golik, J., Zielinski, J., Ziminski, T., Mechlinski W., Jereczek, E., Kolodziejczyk, P., Adlercreutz, H., Schaffner, C. P., and Neelakanton, S., *Tetrahedron Lett.*, 1971, **22**, 1987.
18. Borowski, E., and Schaffner, C. P., "Abstracts of Communications, Vth International Congress on Biochemistry, Moscow, Aug. 10–16, 1961," Pergamon Press, Oxford and London, 1961, p. 3.
19. Borowski, E., Malyshkina, M., Kotienko, T., and Soloviev, S., *Chemotherapia* 1964–65, **9**, 359.
20. Deshapande, G. R., Kerur, D. R., and Narasimhachari, N., *Hindustan Antibiot. Bull.*, 1966, **8**, 185.
21. Kalasz, H., Szell, V., Gyimesi, J., Magyar, K., Horvath, I., and Szabo, I., *Acta Microbiol. Acad. Sci. Hung.*, 1972, **19**, 111.
22. Borowski, E., Malyshkina, M., Soloviev, S., and Ziminski, T., *Chemotherapia*, 1965–66, **10**, 176.
23. Nakano, H., *J. Antibiot.*, 1961, **14A**, 72.
24. Deshapande, G. R., and Narasimhachari, N., *Hindustan Antibiot. Bull.*, 1966, **9**, 76.
25. Lee, C. H., and Schaffner, C. P., *Tetrahedron*, 1969, **25**, 2229.
26. Haegele, K. D., and Desiderio, D. M., *Biomed. Mass Spect.*, 1974, **1**, 20.
27. Ganis, P., Avitabile, G., Mechlinski, W., and Schaffner, C. P., *J. Am. Chem. Soc.*, 1971, **93**, 4560.
28. Mechlinski, W., and Schaffner, C. P., *J. Antibiot.*, 1972, **25**, 256.
29. Bartner, E., Zines, H., Moe, R. A., and Kulesza, J. S., *Antibiot. A.*, 1957–58, 53.
30. Lampen, J. O., Morgan, E. R., Slocum, A., and Arnow, P., *J. Bact.*, 1959, **78**, 282.
31. Finkelstein, A., and Holz, R., "Membranes," Volume 2, Marcel Dekker, New York, 1973, p. 377.
32. De Kruijff, B., and Demel, R. A., *Biochim. Biophys. Acta*, 1974, **339**, 57.
33. Bennett, J. E., *New Engl. J. Med.*, 1974, **290**, 30.
34. Garrod, L. P., and O'Grady, F., "Antibiotics and Chemotherapy," Third Edition, E. and S. Livingstone, London, 1971, p. 235.
35. Falkowski, L., Golik, J., Kolodziejczyk, P., Pawlak, J., Zielinski, J., Ziminski, T., and Borowski, E., *J. Antibiot.*, 1975, **28**, 244.
36. Schaffner, C. P., and Mechlinski, W., *J. Antibiot.*, 1972, **25**, 259.
37. Bruzzese, T., Cambieri, M., and Recusani, F., *J. Pharm. Sci.*, 1975, **64**, 462.
38. Bonner, D. P., Mechlinski, W., and Schaffner, C. P., *J. Antibiot.*, 1972, **25**, 261.
39. Keim, G. R., Poutsiaka, J. W., Kirpan, J., and Keysser, C. H., *Science, N.Y.*, 1973, **179**, 584.
40. Bodey, G. P., Adachi, L., and Jones, V., *Curr. Ther. Res.*, 1974, **16**, 207.
41. Gordon, H. W., and Schaffner, C. P., *Proc. Nat. Acad. Sci.*, 1968, **60**, 1201.
42. Keshin, J. G., *Int. Surg.*, 1973, **58**, 116.
43. Schaffner, C. P., and Gordon, H. W., *Proc. Nat. Acad. Sci. U.S.A.*, 1968, **61**, 36.
44. Parekh, A. C., Creno, R. J., and Dave, C. V., *Res. Commun. Chem. Path. Pharmac.*, 1974, **9**, 307.
45. *Tech. Rep. Ser. Wld Hlth Org.*, 1969, No. 430.
46. Bhate, D. S., and Acharya, S. P., *Hindustan Antibiot. Bull.*, 1964, **6**, 170.
47. "The Merck Index," Eighth Edition, Merck & Co. Inc., Rahway, N.J., 1968, p. 62.
48. Kornfeld, E. C., Jones, R. G., and Parke, T. V., *J. Am. Chem. Soc.*, 1949, **71**, 150.
49. Sternberg, T. H., Wright, E. T., and Oura, M., *Antibiot. A.*, 1955–56, 566.
50. Struyk, A. P., Hoette, I., Drost, G., Waisvisz, J. M., van Eek, T., and Hoogerheide, J. C., *Antibiot. A.*, 1957–58, 878.
51. Seneca, H., *Antibiot. A.*, 1955–56, 697.
52. Taber, W. A., Vining, L. C., and Waksman, S. A., *Antibiot. Chemother.*, 1954, **4**, 455.
53. Lechevalier, H. A., Acker, R. F., Corke, C. T., Haenseler, C. M., and Waksman, S. A., *Mycologia*, 1953, **45**, 155.
54. Kligman, A. M., and Lewis, F. S., *Proc. Soc. Exp. Biol. Med.*, 1953, **82**, 399.
55. Waksman, S. A., and Lechevalier, H. A., "The Actinomycetes," Volume III, Baillière, Tindall and Cox Ltd., London, 1962, p. 397.
56. Oswald, E. J., Reedy, R. J., and Randall, W. A., *Antibiot. A.*, 1955–56, 236.
57. Vezina, C., *Antimicrob. Ag. Chemother.*, 1966, 757.
58. Waksman, S. A., and Lechevalier, H. A., "The Actinomycetes," Volume III, Baillière, Tindall and Cox Ltd., London, 1962, p. 248.
59. Gold, W., Stout, H. A., Pagano, J. F., and Donovick, R., *Antibiot. A.*, 1955–56, 579.
60. Howarth, W. R., Tewari, R. P., and Solotorovsky, M., *Antimicrob. Ag. Chemother.*, 1975, **7**, 58.

61. Ahmad, K., Schneider, H. G., and Strong, F. M., *Archs Biochem.*, 1950, **28**, 281.
62. Whiffen, A. J., *J. Bact.*, 1948, **56**, 283.
63. Hazen, E. R., and Brown, R., *Proc. Soc. Exp. Biol. Med.*, 1951, **76**, 93.
64. Kinsky, S. C., Gronau, G. R., and Weber, M. M., *Molec. Pharmac.*, 1965, **1**, 190.
65. Kuljbakh, V. O., Sokolov, L. B., Sveshnikov, J. F., Kuznetsova, O. A., Malinovskaja, G. N., Raigorodskaja, V. J., and Kholodova, G. V., *Br. Pat.*, 1 070 799, 1967.
66. Shenin, Y. D., Venediktov, V. V., Onoprienko, V. V., and Egorenkova, A. N., *Antibiotiki*, 1973, **18**, 483.
67. Karasawa, K., Tanaka, N., Yonehara, H., and Umezawa, H., *J. Gen. Appl. Microbiol.*, 1959, **5**, 13.
68. Whiffen, A. J., Bohonos, N., and Emerson, R. L., *J. Bact.*, 1946, **52**, 610.
69. Shenin, Y. D., Kotenko, T. V., and Exemplyarov, O. N., *Antibiotiki*, 1968, **13**, 387.
70. Martin, J. F., and McDaniel, L. E., *J. Antibiot.*, 1974, **27**, 610.
71. Hattori, K., Nakano, H., Seki, M., and Hirata, Y., *J. Antibiot.*, 1956, **9A**, 176.
72. Vandeputte, J., Wachtel, J. L., and Stiller, E. T., *Antibiot. A.*, 1955–56, 587.
73. Burns, J., and Holtman, D. F., *Antibiot. Chemother.*, 1959, **9**, 398.
74. Divekar, P. V., Bloomer, J. L., Eastham, J. F., Holtman, D. F., and Shirley, D. A., *Antibiot. Chemother.*, 1961, **11**, 377.
75. Khokhlova, Y. M., Korenyako, A. I., Nikitina, N. I., Puchnina, A. V., and Blinovno, Z., *Allg. Mikrobiol.*, 1963, **3**, 195.
76. Kalasz, H., Gyimesi, J., Uskert, A., Magyar, K., and Horvath, I., *Acta Chim. Acad. Sci. Hung.*, 1967, **51**, 431.
77. Burrows, H. J., and Calam, D. H., *J. Chromat.*, 1970, **53**, 566.
78. Dornberger, K., Fugner, R., Bradler, G., and Thrum, H., *J. Antibiot.*, 1971, **24**, 172.
79. Pridham, T. G., *Antimicrob. Ag. Chemother.*, 1963, 104.
80. Vanek, Z., Spizek J., Puza M., Vondracek, M., and Dolesilova, L., *Abstr. Int. Congr. Biochem.*, 1964, 445.
81. Zurabova, E. R., and Romankova, A. G., *Br. Pat.*, 1 254 212, 1971.
82. Liu, C.-M., McDaniel, L. E., and Schaffner, C. P., *Antimicrob. Ag. Chemother.*, 1975, **7**, 196.
83. Linke, H. A. B., Mechlinski, W., and Schaffner, C. P., *J. Antibiot.*, 1974, **27**, 155.
84. Brewer, G. A., and Frazier, W. R., *Antimicrob. Ag. Chemother.*, 1961, 212.
85. Musilikova, M., *Folia Microbiol.*, 1961, **6**, 175.
86. Siminoff, P., *U.S. Pat.*, 2 872 373, 1959.
87. Waksman, S. A., Lechevalier, H. A., and Schaffner, C. P., *Bull. Wld Hlth Org.*, 1965, **33**, 219.
88. Hosoya, S., Hamamura, N., and Ogata, S., *J. Antibiot.*, 1955, **8A**, 48.
89. Hosoya, S., *J. Antibiot.*, 1956, **9A**, 129.
90. Birch, A. J., Holzapfel, C. W., Rickards, R. W., Djerassi, C., Suzuki, M., Westley, J., Dutcher, J. D., and Thomas, R., *Tetrahedron Lett.*, 1964, **23**, 1485.
91. Liu, C.-M., McDaniel, L. E., and Schaffner, C. P., *J. Antibiot.*, 1972, **25**, 116.
92. Martin, J. F., and McDaniel, L. E., *Antimicrob. Ag. Chemother.*, 1975, **7**, 208.
93. "British Pharmacopoeia 1973, Addendum 1975," HM Stationery Office, London, 1975, p. 10.
94. Nussbaumer, P. A., *Pharm. Acta Helv.*, 1968, **43**, 462.
95. Ochab, S., *Dissnes Pharm. Pharmac.*, 1970, **22**, 351.
96. Martin, J. F., and McDaniel, L. E., *J. Chromat.*, 1975, **104**, 151.
97. Ammann, A., and Gottlieb, D., *Appl. Microbiol.*, 1955, **3**, 181.
98. Vining L. C., Taber, W. A., and Gregory, F. J., *Antibiot. A.*, 1954–55, 980.
99. Hattori, K., Ohashi, M., Aoyama, K., and Mizuno, T., *J. Antibiot.*, 1955, **8B**, 89.
100. Bosshardt, R., and Bickel, H., *Experientia*, 1968, **24**, 422.
101. Divekar, P. V., Vora, V. C., and Khan, A. W., *J. Antibiot.*, 1966, **11A**, 63.
102. Ikekawa, T., Iwami, F., Akita, E., and Umezama, H., *J. Antibiot.*, 1963, **16A**, 56.
103. Dryon, L., *J. Pharm. Belg.*, 1966, **21**, 433.
104. Monji, N., Bonner, D. P., Hashimoto, Y., and Schaffner, C. P., *J. Antibiot.*, 1975, **28**, 317.
105. Inter-Republic Specifications, Levorin, IRS-42, No. 3595–68, Ministry of Health of the USSR, Moscow, 1968.
106. Ochab, S., *Dissnes Pharm. Pharmac.*, 1971, **23**, 205.
107. Mechlinski, W., and Schaffner, C. P., *J. Chromat.*, 1974, **99**, 619.
108. Calam, D. H., *J. Chromat. Sci.*, 1974, **12**, 613.
109. Nakano, H., *J. Antibiot.*, 1961, **14A**, 68.
110. Ito, Y., and Bowman, R. L., *J. Chromat. Sci.*, 1973, **11**, 284.
111. Mazor, L., and Papay, K. M., *Z. Analyt. Chem.*, 1961, **184**, 272.
112. Unterman, W. H., *Revue Chim., Bucharest*, 1961, **12**, 415; *Analyt. Abstr.*, 1962, **9**, 847.
113. Chang, J. C., Honig, A. B., Warren, A. T., and Levine, S., *J. Pharm. Sci.*, 1963, **52**, 536.
114. Chang, J. C., Honig, A. B., Warren, A. T., and Levine, S., *J. Pharm. Sci.*, 1963, **52**, 603.
115. Gallelli, J. F., *Drug Intell.*, 1967, **1**, 102.
116. Herd, P. A., and Martin, H. F., *Clin. Biochem.*, 1974, **7**, 359.
117. Horvath, I., and Koczka, I., *Nature, Lond.*, 1964, **203**, 1305.
118. Venables, P., and Russell, A. D., *Antimicrob. Ag. Chemother.*, 1975, **7**, 121.
119. Amer, M. M., Wahbi, A. M., and Habeeb, A. A., *J. Pharm. Pharmac.*, 1975, **27**, 377.
120. "Code of Federal Regulations," Title 21 (1973), Part 148K, US Government Printing Office, Washington, D.C., 1973, p. 486.
121. Kotler-Brajtburg, J., Medoff, G., Schessinger, D., and Kobayashi, G. S., *Antimicrob. Ag. Chemother.*, 1974, **6**, 770.
122. Gerke, J. R., Levin, J. D., and Pagano, J. F., *in* Kavanagh, F., *Editor*, "Analytical Microbiology," Academic Press, New York and London, 1963, p. 387.

123. Platt, T. B., and Isaacson, D. M., *in* Laskin, A. I., and Lechevalier, H. A., *Editors*, "Handbook of Microbiology," Volume III, CRC Press, Cleveland, Ohio, 1973, p. 1017.
124. "British Pharmacopoeia 1973," HM Stationery Office, London, 1973, p. A102.
125. "Code of Federal Regulations," Title 21 (1973), Part 141.101, US Government Printing Office, Washington, D.C., 1973, p. 14.
126. Platt, T. B., Levin, J. D., Gentile, J., and Leitz, M. A., *in* Kavanagh, F., *Editor*, "Analytical Microbiology," Volume II, Academic Press, New York and London, 1972, p. 147.
127. Shadomy, S., McCoy, J. A., and Schwartz, S. I., *Appl. Microbiol.*, 1969, **17**, 497.
128. "British Pharmacopoeia 1973, Addendum 1975," HM Stationery Office, London, 1975, p. A8.
129. Maniar, A. C., and Mavdikar, S., *Hindustan Antibiot. Bull.*, 1962, **4**, 168.
130. "Minimum Requirements of Antibiotic Products," Ministry of Health and Welfare, Japanese Government, Tokyo, 1959, p. 409.
131. Lightbown, J. W., de Rossi, P., and Isaacson, P., *Bull. Wld Hlth Org.*, 1972, **47**, 343.
132. Lightbown, J. W., Kogut, M., and Uemura, K., *Bull. Wld Hlth Org.*, 1963, **29**, 87.
133. *Tech. Rep. Ser. Wld Hlth Org.*, 1968, No. 384, 12.
134. "United States Pharmacopeia," Nineteenth Revision, United States Pharmacopeial Convention Inc., Rockville, Md., 1974, p. 348.
135. Gerke, J. R., and Madigan, M. E., *Antibiot. Chemother.*, 1961, **11**, 227.
136. Tarbert, J. E., and Sternberg, T. H., *Mycologia*, 1954, **46**, 263.
137. Gerke, J. R., Gentile, J., Stander, H., and Pagano, J. F., *Antibiot. A.*, 1960, p. 563.
138. Platt, T. B., Weisblatt, H., and Guevrekian, L., *Ann. N.Y. Acad. Sci.*, 1968, **153**, 571.
139. Isaacson, D. M., and Platt, T. B., *Bact. Proc.*, 1968, **68**, 1.
140. Hammond, S. M., Lambert, P. A., and Kliger, B. N., *J. Gen. Microbiol.*, 1974, **81**, 325.
141. Gale, E. F., *J. Gen. Microbiol.*, 1974, **80**, 451.
142. Tsubura, E., *J. Antibiot.*, 1954, **7B**, 201.
143. Salt, D. T., Arret, B., and Wilner, J., *J. Pharm. Sci.*, 1973, **62**, 2040.
144. Tanguay, A. E., and Bogert, A. B., *Appl. Microbiol.*, 1974, **27**, 1175.
145. Wawrzkiewicz, K., *Polskie Archwm Wet.*, 1973, **16**, 85.
146. Klimov, A. N., and Nikiforova, A. A., *Experientia*, 1973, **29**, 661.
147. Marini, F., Arnow, P., and Lampen, J. O., *J. Gen. Microbiol.*, 1961, **24**, 51.
148. "Code of Federal Regulations," Title 21 (1973), Part 148 b and 148 v, US Government Printing Office, Washington, D.C., 1973, pp. 415 and 540.
149. Platt, T. B., Levin, J. D., and Massey, M. A., *J. Ass. Off. Analyt. Chem.*, 1969, **46**, 675.
150. Platt, T. B., and Itkin, A. G., *J. Ass. Off. Analyt. Chem.*, 1974, **57**, 536.
151. Bonner, D. P., Mechlinski, W., and Schaffner, C. P., *J. Antibiot.*, 1975, **28**, 132.
152. Hamilton-Miller, J. M. T., *J. Pharm. Pharmac.*, 1973, **25**, 401.
153. Rickards, R. W., Smith, R. M., and Golding, B. T., *J. Antibiot.*, 1970, **23**, 603.
154. Lokshin, G. B., Zhdanovich, Y. V., and Kuzovkov, A. D., *Antibiotiki*, 1966, **11**, 590.
155. Belenkii, B. G., Solovev, S. N., Filippova, A. I., Nevinskii, A. A., Mamtsis, A. M., and Kogan, S. B., *Antibiotiki*, 1966, **11**, 112.
156. Bashkovich, A. P., Shenin, Y. D., Kotenko, T. V., Raigorodskaya, V. Y., Karpenko, M. P., Krasnobaeva, N. A., Vasileva, N. G., Bogdanova, N. P., and Ekzemoplyarov, O. N., *Khim.-Farm. Zh.*, 1967, **1**, 31.
157. Zhdanovich, Y. V., Lokshin, G. B., and Kuzovkov, A. D., *Khim.-Farm. Zh.*, 1967, **1**, 42.
158. Weiss, M. R., and Levine, H. B., Sabouraudia, 1972, **10**, 132.
159. Dekker, J., and Ark, P. A., *Antibiot. Chemother.*, 1959, **9**, 327.
160. Narasimhachari, N., Dhawale, D. A., Joshi, V. B., and Gopalkrishnan, K. S., *Hindustan Antibiot. Bull.*, 1967, **10**, 290.
161. Narasimhachari, N., Deshpande, G. R., and Patil, P. L., *Hindustan Antibiot. Bull.*, 1967, **10**, 1.
162. Shadomy, S., Brummer, D. L., and Ingroff, A. V., *Am. Rev. Resp. Dis.*, 1973, **107**, 303.
163. Block, E. R., and Bennett, J. E., *Antimicrob. Ag. Chemother.*, 1973, **4**, 648.

Received *November 11th*, 1975
Accepted *December 16th*, 1975

Analyst, May, 1976, Vol. 101, pp. 341–347

The Determination of Arsenic(III) and Total Arsenic by Atomic-absorption Spectroscopy

J. Aggett and A. C. Aspell

Chemistry Department, University of Auckland, Auckland, New Zealand

In the atomic-absorption determination of arsenic by the hydride evolution method with sodium borohydride, maintenance of the pH between 4 and 5 permits the selective determination of arsenic(III) in mixtures of arsenic(III) with arsenic(V). Total arsenic can be determined separately by evolution from 5 M hydrochloric acid. A dissolution technique has been developed for herbage and the method applied to the analysis of water and orchard leaves.

As there is considerable evidence to suggest that the toxicity and physiological behaviour of arsenic are dependent on its valence state and in particular that arsenic(III) compounds are more toxic than arsenic(V) compounds,[1] it seems prudent to develop sensitive methods for the determination of arsenic in its different valence states. At present, the most satisfactory methods for the determination of low levels of arsenic are based on the evolution of arsine, *e.g.*, the Gutzeit, silver diethyldithiocarbamate and atomic-absorption spectrophotometric methods. Standard procedures for the former two methods, and earlier proposed atomic-absorption methods, are based on the use of zinc - hydrochloric acid as hydride generator. In the first instance it appeared that these methods should be capable of differentiating between arsenic(III) and arsenic(V), as recipes invariably seem to state that arsenic(V) must be reduced to arsenic(III) with tin(II) - hydrochloric acid prior to arsine generation. However, it has been our experience with the silver diethyldithiocarbamate method that zinc - hydrochloric acid generates arsine from arsenic(V) just as readily as from arsenic(III), which is not surprising as it is a stronger reducing agent than tin(II).

In 1973, Schmidt and Royer[2] reported the use of solid sodium borohydride - hydrochloric acid for the generation of arsine in the atomic-absorption spectrophotometric procedure. Among the claimed advantages of sodium borohydride was the fact that preliminary reduction of arsenic(V) to arsenic(III) was not necessary, which implies that this procedure also measures the total arsenic concentration of the sample.

Now, a breakdown of the process of formation of arsine from arsenic(V) suggests that there are two steps in the reaction:

$$\mathrm{As(V)} \xrightarrow{\text{Reduction}} \mathrm{As(III)} \quad .. \quad .. \quad .. \quad .. \quad (1)$$

$$\mathrm{As(III)} \xrightarrow[\text{transfer}]{\text{Hydride}} \mathrm{AsH_3} \quad .. \quad .. \quad .. \quad .. \quad (2)$$

Also, the fact that the use of both zinc and sodium borohydride in hydrochloric acid media does not differentiate between arsenic(V) and arsenic(III) suggests that under these rather acidic conditions reaction (1) proceeds at a rate similar to, or faster than, reaction (2).

However, the redox potential of the arsenic(V) - arsenic(III) couple is a function of pH (Fig. 1) and, as the rates of redox reactions that involve multi-electron transfer are often slow, it appeared that it might be possible to differentiate between arsenic(III) and arsenic(V) provided that reaction (1) is slower than reaction (2) at higher pH. This paper reports a study on the effect of pH on the evolution of arsine from aqueous solutions of both arsenic(V) and arsenic(III) and its application to the determination of specific forms of arsenic at trace levels.

Sodium borohydride was selected as generating agent because it is effective as a hydride transfer reagent over a wider range of acidity than zinc - hydrochloric acid. It was used as an aqueous solution rather than as a solid in order to avoid problems that might have arisen from the employment of a fast heterogeneous reaction. Atomic-absorption spectroscopy was chosen as the measurement technique as it appears to be the most satisfactory

in terms of sensitivity and precision at low levels and is more compatible with a rapid arsine generation technique than are the Gutzeit and silver diethyldithiocarbamate methods.

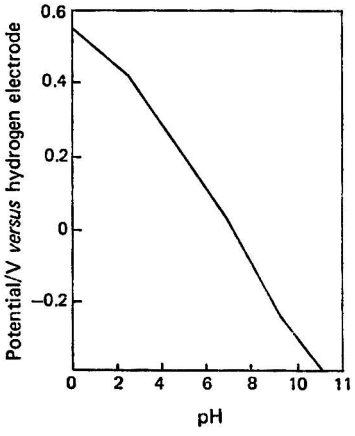

Fig. 1. Redox potential of arsenic(V) - arsenic(III) couple as a function of pH.

Experimental and Results

Apparatus

Atomic-absorption measurements were made with a Unicam SP 90A atomic-absorption spectrophotometer fitted with an EMI 9663B photomultiplier tube using an air-entrained hydrogen - argon flame. The flow-rates were hydrogen 1.5 l min^{-1} and argon 7.5 l min^{-1}.

The arsenic resonance line at 197.2 nm was used in preference to the more sensitive 193.7-nm line because at the latter wavelength the noise level was greater by a factor of about 2.5. The slit width was 0.1 mm for all measurements.

Signals were recorded on a digital integrator with a count rate of 100 digits mV^{-1} s^{-1}. This integrator was constructed by the Electronics Division of the Chemistry Department, University of Auckland. It was adapted to integrate absorbance with respect to time by connecting a logarithmic converter between the integrator and spectrophotometer.

The arsine generator is illustrated in Fig. 2. It consisted of a 250-ml separating funnel

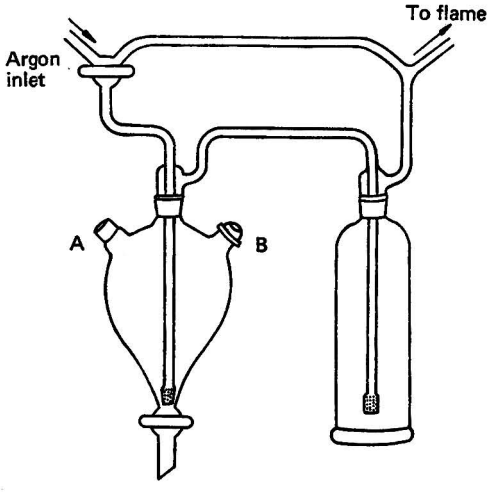

Fig. 2. Arsine generator.

fitted with two extra inlets, one (A) fitted with a glass stopper for introduction of samples, the other (B) fitted with a rubber septum through which the sodium borohydride solution was injected. The gas-bubbling tube was fitted with a sintered-glass frit, which was ground to enable it to be placed as close to the tap as possible in order to maximise the efficiency of removal of arsine from the generating solution. The 250-ml gas bottle was used as a ballast jar, which made it possible to operate the system with the aid of the single two-way tap.

Measurements were made in the following manner. The sample to be analysed and the appropriate acid solution were pipetted through inlet A, the stopper was replaced and argon passed through the reaction vessel to remove air. The two-way tap was then turned back to its original position so that the argon passed directly to the flame. Sodium borohydride solution (2 ml) was then injected through the septum (B) and the argon immediately diverted to pass through the generating vessel and transfer the liberated arsine to the flame. The absorption signal was integrated over a period of 25 s.

Chemicals

With the exception of sodium borohydride, all chemicals were of analytical-reagent grade and the preparation and dilution of standard solutions were carried out with water that had been deionised and double-distilled.

The sodium borohydride (BDH Chemicals, laboratory-reagent grade) contained relatively large amounts of arsenic, which were removed by dissolving the sodium borohydride in aqueous sodium hydroxide and then recrystallising it by the addition of purified dioxan. The purified crystals were cooled in ice and washed with methanol (yield 80–85%). For arsine generation, sodium borohydride was used as a 50 g l^{-1} solution in 0.1 M sodium hydroxide. This solution was sufficiently stable to be used for several days.

Determination of Arsenic(III)

The performance of the apparatus was evaluated by preparing calibration graphs using arsenic(III) standards and arsine generation from 5 M hydrochloric acid solution. These calibration graphs were linear for arsenic levels below 0.3 μg ml^{-1} and gave a detection limit of about 25 ng of arsenic. At the 0.5-μg level (5 ml of 0.1 μg ml^{-1} solution), the relative standard deviation was 2.5–3.0%. While this detection limit is not as impressive as those reported by Schmidt and Royer[2] and Knudson and Christian,[3] it should be borne in mind that the purpose of this work was to examine the chemistry of the system. The use of a more sophisticated spectrophotometer will undoubtedly lead to some improvement in the detection limit.

Preliminary experiments also included investigations of the variation in absorption signal with the concentration of sodium borohydride (Table I) and with sample volume (Table II). The fact that it was physically easier to inject a smaller volume more rapidly formed the basis for the selection of 2 ml of 5% sodium borohydride in the method. The results in Table II indicate that an eight-fold increase in sample volume results in only about a 5% loss in atomic-absorption signal for the same amount of arsenic. This is no doubt attributable to the careful design of the flushing system.

TABLE I

ATOMIC-ABSORPTION SIGNAL AS A FUNCTION OF SODIUM BOROHYDRIDE CONCENTRATION

Sodium borohydride		Absorption
Concentration/g l^{-1}	Volume/ml	signal*/counts
10	10	1 604
20	5	1 823
50	2	1 830
100	1	1 777
150	0.7	1 786

* 5-ml samples of 0.1 μg ml^{-1} arsenic(III).

The effect of pH on the generation of arsine from both arsenic(V) and arsenic(III) was determined by measuring the atomic-absorption signals obtained from 0.5-μg samples (5 m l

TABLE II

ATOMIC-ABSORPTION SIGNAL AS A FUNCTION OF SAMPLE VOLUME

Arsenic(III)		
Concentration/μg ml^{-1}	Volume/ml	Absorption signal/counts
0.2	5	3 526
0.1	10	3 578
0.05	20	3 581
0.025	40	3 418

of 0.10 μg ml^{-1} solution) in a series of buffers. It can be seen (Fig. 3) that while generation of arsine from arsenic(III) is essentially independent of pH in the region studied, generation from arsenic(V) is never quantitative and falls continuously with increasing pH until above pH 3.5 it becomes negligible with respect to that generated from arsenic(III).

The explanation for this behaviour appears to be that as the pH is raised the rate of reaction (1) decreases until it eventually becomes much slower than the rate of hydrolysis of sodium borohyride under the same conditions.

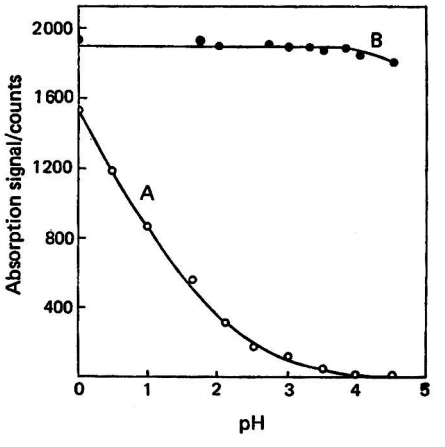

Fig. 3. Absorption signals from (A) arsenic(V) and (B) arsenic(III) as a function of pH.

Addition of alkaline sodium borohydride and its subsequent hydrolysis also affects the system by consuming protons. In order to ensure quantitative conversion of arsenic(III) into arsine, it is necessary to maintain the pH at a value no greater than 5.5. Citrate and acetate buffers (0.5 M) were found to have sufficient buffering capacity and maintained the pH to within 0.25 pH unit of their initial value during arsine evolution. However, when buffer solutions of lower concentration were used the solutions became alkaline during the reaction with sodium borohydride and lower atomic-absorption signals were recorded.

The application of the method to mixtures of arsenic(V) and arsenic(III) was examined by measuring the atomic-absorption signals obtained (a) for a series of samples containing different concentrations of arsenic(III) in the presence of a constant concentration of arsenic(V), and (b) for a series of samples containing a constant concentration of arsenic(III) in the presence of increasing concentrations of arsenic(V). In the first set of experiments, it was found that the presence of 1 μg ml^{-1} of arsenic(V) made no difference to the atomic-absorption signals obtained from arsenic(III) solutions (concentration less than 0.3 μg ml^{-1}) when arsine was generated from an acetic acid buffer at pH 5. When the arsine was generated from a buffer at pH 3.5, an increase of 3–5% was observed in the atomic-absorption signals. The results for the second set of experiments (Fig. 4) show that at pH 5 it is possible to measure arsenic(III) in the presence of arsenic(V) at ratios approaching 50:1; at pH 3.5, the critical ratio is about 40:1.

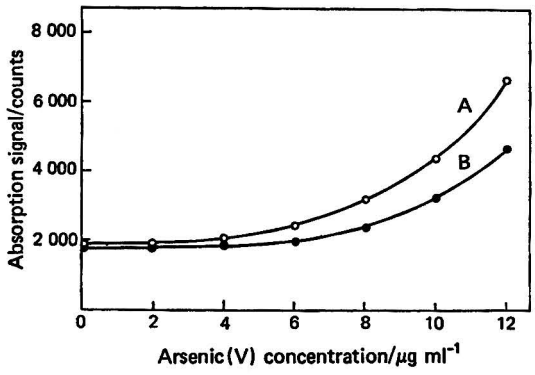

Fig. 4. Absorption signal from arsenic(III) (0.1 μg ml^{-1}) as a function of arsenic(V) concentration: A, citrate buffer, pH 3.5; and B, acetate buffer, pH 5.0.

Interference by a number of inorganic ions was examined in three different generating media, *viz.*, 5 M hydrochloric acid, 0.5 M acetate buffer (pH 4) and 1 M citrate buffer (pH 4).

Results for the first two systems are summarised in Tables III and IV. Of the species examined, only antimony(III) interfered in the citrate buffer and then only when its concentration was above 100 μg ml^{-1}. This interference by antimony has been shown to be caused by non-atomic absorption, although the species has not been positively identified.

At the time these studies were undertaken, very little information on inteferences in arsine generation methods was available. Vogel[4] had mentioned that copper, nickel and cobalt slow the evolution of arsine by zinc - hydrochloric acid. Braman *et al.*,[5] who used sodium borohydride generation from neutral solution coupled with emission detection, reported that both silver(I) and copper(II) interfered when present in 20-fold excess. However, while this manuscript was in preparation, Smith[6] published more detailed data on interferences in the sodium borohydride - hydrochloric acid method for arsine generation.

TABLE III

INTERFERENCES IN ARSINE GENERATION FROM 5 M HYDROCHLORIC ACID*

Results are percentages of original signal.

	Concentration/μg ml^{-1}		
Interferent†	10	100	1 000
Co(II)	100	100	73
Fe(III)	100	100	110
Mn(VII)	89	78	45
Ni(II)	108	51	2
Sb(III)	130	145	405

* Arsenic(III) concentration 0.1 μg ml^{-1}.
† The following species did not interfere at the 1 000 μg ml^{-1} level: Na(I), K(I), Ca(II), Mg(II), Cr(VI), Cu(II) and Zn(II).

The most significant discrepancy between the two sets of results appears to be the difference between the observed behaviour of copper(II). Smith reported that copper(II) suppressed the atomic-absorption signal by 10–50% when present at 1 000-fold excess, whereas we found no interference at this level. This discrepancy may be related to the difference in hydrochloric acid concentrations in the reaction solutions. Smith generated arsine from solutions that were approximately 1 M in hydrochloric acid, while in the present study, the concentration was almost 5 M. At the latter concentration, copper(II) is known to form chloro complexes, which would result in free copper(II) concentrations well below the formal concentration.

The ability of the acid anions to complex with inorganic cations also appears to explain the observed differences between interferences in the acetate and citrate buffers. In the

TABLE IV

INTERFERENCES IN ARSINE GENERATION FROM ACETATE BUFFER (pH 4)*

Results are percentages of original signal.

	Concentration/μg ml^{-1}		
Interferent†	10	100	1 000
Co(II)	45	2	<1
Cu(II)	29	20	<1
Fe(III)	44	37	34
Ni(II)	100	100	28
Sb(III)	110	122	410
Zn(II)	100	81	28

* Arsenic(III) concentration 0.1 μg ml^{-1}.
† The following species did not interfere at the 1 000 μg ml^{-1} level: Na(I), K(I), Ca(II), Mg(II), Cr(VI) and Mn(II).

latter buffer, which is more strongly co-ordinating, the degree of interference is minimal even with a 10 000-fold excess of interferent.

As a preliminary to the determination of arsenic(III) and arsenic(V) in solid biological samples, it is necessary to dissolve the solid without loss of arsenic or oxidation of arsenic(III). It was found that when the wet digestion procedure with concentrated nitric and sulphuric acids (Official Method 1A[7]) was used, arsenic(III) was oxidised to arsenic(V). However, when the acids were diluted 1 + 2 with water there appeared to be no significant loss of arsenic or oxidation of arsenic(III) (Fig. 5).

Belcher *et al.*[8] have recently reported that the addition of EDTA is effective in reducing interferences in the determination of arsenic by arsine generation. The action of EDTA is probably similar to that of citric acid. Both of these acids were investigated as potential generating media in the present study but the former was discarded because its lower solubility made it ineffective as a buffer on addition of alkaline sodium borohydride.

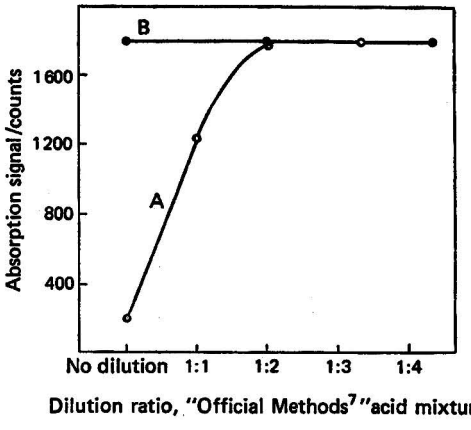

Fig. 5. Influence of digestion procedure on oxidation and recovery of arsenic: A, arsenic(III) with arsine generation at pH 3.5; and B, arsenic(V) with arsine generation from hydrochloric acid.

Suggested Procedure for Plant Materials

Transfer 2 g of the dried and powdered sample into a 100-ml flask and add 10 ml of 1 + 2 nitric acid. Place a watch-glass on the mouth of the flask and heat the mixture at 70 °C for 5 min. Cool, add gradually 10 ml of 1 + 2 sulphuric acid, then heat at 70 °C for 15 min. Allow the solution to cool, add a further 5 ml of water and boil the mixture gently for 5 min. Some flocculence remains if the sample is not thoroughly wetted with nitric acid

in the first stage. This flocculence can be removed by filtration or centrifuging without affecting the recovery of arsenic. The acidity of the sample is then adjusted to permit the determination of total arsenic from 5 M hydrochloric acid, or arsenic(III) from citrate buffer at pH 4.5–5.0, and the volume is made up to 50 ml. Aliquots of 5 ml are normally used for analysis, although larger volumes can be used to obtain greater precision at low concentrations.

These procedures have been used to determine the total arsenic and arsenic(III) contents of orchard leaves and apples in an orchard adjacent to a timber treatment plant and to analyse standard orchard leaves (NBS Standard Reference Material 1571 containing 14 μg ml^{-1} of arsenic). The arsine generation method has been used to determine the total arsenic and arsenic(III) content of water from geothermal areas. Typical results are given in Table V. The results for water were obtained by analysing 5-ml aliquots directly with sodium borohydride reagent. The results of these applications will be presented in detail when the studies are complete.

TABLE V

RESULTS OF ANALYSIS OF WATER AND LEAF SAMPLES

Sample	Arsenic (total)/μg g^{-1}	Arsenic(III)/μg g^{-1}
Water: Waikato River	0.164	0.035
Water: Geyser	6.75*	3.85
Orchard leaves (local)	19.0	2.30
Orchard leaves (NBS 1571) ..	13.5	4.90

* Analysis of this sample by the silver diethyldithiocarbamate method gave a total arsenic content of 6.62 μg ml^{-1}.

References

1. Shroeder, H. A., and Balassa, J. S., *J. Chron. Dis.*, 1966, **19**, 85.
2. Schmidt, F. J., and Royer, I. L., *Analyt. Lett.*, 1973, **6**, 17.
3. Knudson, E. J., and Christian, G. D., *Analyt. Lett.*, 1973, **6**, 1039.
4. Vogel, A. I., "A Text Book of Quantitative Inorganic Analysis," Third Edition, Longmans, Green and Co., London, 1961, p. 797.
5. Braman, R. S., Justen, L. L., and Foreback, C. C., *Analyt. Chem.*, 1972, **44**, 2195.
6. Smith, A. E., *Analyst*, 1975, **100**, 300.
7. Jolly, S. C., *Editor*, "Official, Standardised and Recommended Methods of Analysis," W. Heffer and Sons, Cambridge, 1963, p. 6.
8. Belcher, R., Bogdanski, S. L., Hendon, E., and Townshend, A., *Analyst*, 1975, **100**, 522.

Received *October 13th*, 1975
Accepted *December 31st*, 1975

Analyst, May, 1976, Vol. 101, pp. 348–355

The Determination of the Precious Metals by Flameless Atomic-absorption Spectrophotometry

G. L. Everett

Johnson Matthey Chemicals Ltd., Royston, Hertfordshire

The optimum conditions for the determination of palladium, platinum, rhodium, ruthenium, iridium and osmium by carbon rod atomic-absorption spectrophotometry are described and data for sensitivity, detection limit and reproducibility are given. With the exception of osmium the sensitivity and detection limits are such that the working range of the atomic-absorption technique can be extended downwards by an order of magnitude or more when compared with that with an air - acetylene flame.

The effect of mutual interference is investigated and methods of reducing it are proposed.

The atomic-absorption spectrophotometric determination of the precious metals was first performed in air - propane flames[1,2] but severe interference from other precious metals was generally found to occur. The use of the air - acetylene flame[3–6] did not remove these interferences but the introduction of various releasing agents such as uranium[7] and lanthanum[8] reduced them to acceptable levels such that concentrations of the metals down to 1 μg ml^{-1} could be measured. The nitrous oxide - acetylene flame was found to give relatively interference-free results for the determination of rhodium[9] and platinum[10] but in both instances the sensitivity was below that obtained with the air - acetylene flame.

Although the precious metals are relatively involatile, the use of non-flame methods of atomisation, with their high atomisation efficiencies and absolute sensitivities, could improve this limit of determination to well below the 1 μg ml^{-1} level. There is, however, only a limited number of publications on this subject and the results reported differ to some extent.

The first report was by L'vov,[11] who obtained 1% absorption sensitivities for palladium, platinum and rhodium of 4, 10 and 6 pg, respectively. Guerin[12] investigated the use of the NIM rod, a carbon filament containing a slot 20 × 1.9 mm and 1.5 mm deep, the light beam being collimated parallel to the filament and just above the slot. By using 50-μl samples this technique gave aqueous detection limits that were about ten times better than those obtained in an air - acetylene flame. Adriaenssens and Knoop[13] used a graphite tube furnace to investigate the determination of platinum, rhodium and iridium but the sensitivities achieved were only of the order of 1 ng and, owing to the high atomisation temperatures used, trouble was encountered from carbon particle scatter and emission signals from the glowing tube.

Pearton and Mallett[14] compared the NIM rod with a Mini-Massmann, the latter being a carbon rod with a transverse hole acting as an atom reservoir, and an atomiser consisting of a small graphite tube held between two support electrodes. They found the tube atomiser to be the most satisfactory in all respects, but it was subject to interferences from the other precious metals, although only a limited study of this aspect was undertaken. Donega and Burgess[15] have described a low-pressure non-flame cell that utilised atomisation from a tantalum ribbon but this cell was insensitive to platinum, a figure of 300 ng for 1% sensitivity being found.

This paper describes the optimum conditions for the atomic-absorption spectrophotometric determination of palladium, platinum, rhodium, ruthenium, iridium and osmium, using a carbon rod atomiser, and discusses some inter-element effects and their removal and also the scope of the technique for determining the precious metals.

Experimental

Apparatus

A standard Varian Techtron AA-5 atomic-absorption spectrophotometer was operated on minimum damping in conjunction with a Model 63 carbon rod atomiser. Except where mentioned, the carbon rod atomiser was used in the "tube" mode with pyrolytically coated graphite tubes (Ringsdorff-Werke GmbH, Bonn-Bad Godesburg, West Germany). The

spectral sources used were Varian Techtron hollow-cathode lamps and read-out was in the absorbance mode, peak height being measured via a Model 3000 fast-response chart recorder (Oxford Instruments, Oxford).

An Eppendorf microlitre pipette (5-μl capacity) with disposable tips was used for sample injections. Oxygen-free nitrogen and, when appropriate, argon containing 10% of methane were used as sheathing gases. The flow-rate of the cooling water was 0.8 l min^{-1}.

Reagents and Standard Solutions

All reagents used were of AnalaR grade and standard solutions were prepared from Johnson Matthey Specpure materials, namely gold, palladium and platinum sponges, ammonium chloroiridate, ruthenium(III) chloride and ammonium chlororhodite, dissolved in the minimum volume of a 20% solution of aqua regia. These solutions were then made up so as to give a concentration of 10 g l^{-1} of the appropriate metal in a 5% solution of aqua regia. Osmium standard solution was prepared by dissolving 2.01 g of osmium tetroxide in 100 ml of de-ionised water and was then diluted so as to give 1 l of a solution that was 2 M in sodium hydroxide. This solution contained 1 500 μg ml^{-1} of osmium. Working solutions were prepared by diluting these stock standards with de-ionised water, those at concentrations below 100 μg ml^{-1} being prepared daily and those below 1 μg ml^{-1} immediately prior to use.

Optimisation of Experimental Parameters

The optimisation of experimental parameters was undertaken with a view to maximising both the sensitivity of the method and the signal to noise ratios. However, owing to the low volatility of the analyte metals high temperatures of about 2 500 °C are required for atomisation. Sensitivity is at a maximum at the highest temperatures attainable (about 2 700 °C), but at this level a considerable signal due to carbon particle scatter is observed and some sensitivity must therefore be sacrificed by reduction of the atomisation temperature in order to produce a signal that is free from this non-atomic absorption. However, this reduction in atomisation temperature leads to a greater reproducibility and an increased lifetime of the carbon tubes.

Sensitivity is at a maximum at a low monochromator band pass but for a given lamp current the loss in sensitivity with increasing band pass is balanced by a decrease in noise that results from an increase in radiation reaching the detector and this causes the signal to noise ratio to pass through a maximum. In a similar manner the fall in sensitivity caused by an increase in lamp current at a given band pass is also balanced by a decrease in noise, which gives a maximum signal to noise ratio at a certain lamp current. The lamp currents and monochromator band passes were optimised so as to give these maximum values. In each instance the sensitivity at the optimum values chosen (Table I) was greater than 80% of the maximum attainable.

The nitrogen flow-rates gave a maximum sensitivity at the values tabulated and fluctuations of $\pm$1 l min^{-1} had little effect on the signal, changing it by less than 5%.

The ashing parameters given in Table I are the maximum settings that afford no loss of analyte from an aqueous sample and give an absorbance of about 0.4.

TABLE I

OPTIMUM EXPERIMENTAL CONDITIONS

Samples dried at 120 °C for 15 s.

	Pd	Pt	Rh	Ru	Ir	Os
Wavelength/nm	244.8	266.0	343.5	349.9	208.9	290.9
Lamp current/mA	7	8	10	20	15	15
Monochromator band pass/nm	0.33	0.66	0.33	0.17	0.17	0.33
Nitrogen flow-rate/l min^{-1}	4.4	6.0	4.4	4.4	4.4	4.0
Argon - methane flow-rate/l min^{-1}	—	—	—	0.9	0.9	0.9
Maximum ashing temperature tolerable/°C	1 450	1 730	1 450	1 730	1 730	1 730
Ashing time/s	30	60	60	60	60	60
Atomisation temperature/°C	2 200	2 200	2 200	2 400	2 400	2 400
Atomisation time/s	5	5	5	5	5	7

Results

Calibration Graphs, Sensitivities and the Origin of the Signal

The sensitivity values obtained under the optimum conditions chosen are listed in Table II and they compare very well with those obtained by other workers. Adriaenssens and Knoop[13] obtained 1% sensitivities of 1.0, 0.18 and 2.0 ng for platinum, rhodium and iridium, respectively, while Pearton and Mallett[14] compared three non-flame cells and found that the Model 63 CRA in the "tube" mode gave the best results, with a 1% sensitivity for platinum of 0.22 ng. Calibration graphs are reproduced in Fig. 1 and, as can be seen, the working ranges are fairly extensive except for osmium, because of incomplete atomisation of osmium at the higher levels. This effect was monitored by subjecting the carbon tubes to X-ray fluorescence analysis prior to and after atomisation, when it was found that approximately 60% of the osmium remains unatomised. However, with amounts from 10 to 100 ng the calibration graph can be used on a semi-quantitative basis.

TABLE II

CALIBRATION DATA

Metal	1% sensitivity*/ ng	Detection limit/ ng	Reproducibility/ ng	Relative standard deviation, %	Upper working limit	
					ng	μg ml^{-1}
Palladium	0.01	0.002 5	0.5	2.1	1.5	0.3
Platinum	0.18	0.05	2.5	2.7	25.0	5.0
Rhodium	0.01	0.007 5	1.0	1.6	1.25	0.25
Ruthenium	0.04	0.2	2.5	1.7	5.0	1.0
Iridium	0.11	0.5	10.0	3.5	25.0	5.0
Osmium	3.4	10.0	—	—	100.0	20.0

* The mass corresponding to 1% absorption.

An attempt to obtain better detection limits for ruthenium and iridium was made by investigating alternative spectral lines (Table III), but in each instance a considerable scatter signal was observed, especially at the intense iridium 264.0-nm line. Of these alternative lines those for ruthenium 343.67 nm and iridium 209.26 nm are in fact non-resonance lines but their lower energy levels, 0.15 eV and 0.35 eV, respectively, are sufficiently close to the ground state for atomic absorption to occur. The lower level of the non-resonance iridium 208.57-nm line (0.88 eV) is considerably higher than the ground state and therefore no atomic absorption is observed. This line is useful in correcting for non-atomic absorption at 208.9 nm.

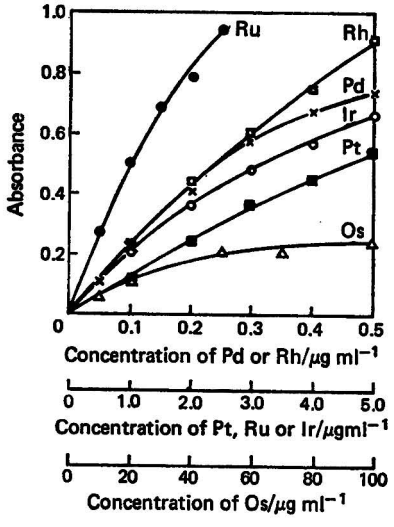

Fig. 1. Calibration graphs for various elements (5-μl aliquots).

The sensitivity of the method is critically affected by the condition of the graphite tubes. These tubes are pyrolytically coated in order to reduce surface porosity and to enable high atomisation temperatures to be used without the tube becoming pitted and porous. However, the quality of the tube declines with use and this effect can be monitored by the change in sensitivity with successive atomisations. There is an initial sharp fall in sensitivity before a plateau region is reached where the sensitivity and reproducibility remain fairly constant. Thereafter, the sensitivity and reproducibility fall sharply owing to rapid deterioration of the tube. For the more volatile metals (palladium, platinum and rhodium) this plateau persists for about 120 atomisations but, as the volatility of the analyte decreases, this number falls and is about 30 for iridium.

This decrease in sensitivity can be partially rectified by introducing into the sheathing gas flow argon containing 10% of methane at the rate of 0.9 l min^{-1}.[16] When the tube is heated to a very high temperature the methane is cracked and a deposit of carbon is left on the tube surface, which has the effect of regenerating the tube and, with iridium, considerably lengthening the plateau region to about 75 atomisations.

The origin of the signals was investigated for palladium, platinum and osmium by use of a hydrogen continuum lamp operated at 20 mA; for rhodium the 349.9-nm ruthenium line was used and for ruthenium, the rhodium 343.5-nm line. Non-atomic absorption at the iridium 208.9-nm line was measured by using either the hydrogen lamp or the iridium 208.57-nm line. In no instance was any absorption observed and it was therefore concluded that all analytical signals were those of atomic absorption.

TABLE III

1% SENSITIVITY FOR ALTERNATIVE LINES

Ruthenium		Iridium	
Wavelength/nm	1% sensitivity/ng	Wavelength/nm	1% sensitivity/ng
287.5	0.9	208.57	0.0
343.67	0.72	209.26	0.6
372.8	0.43	264.0	0.35

Reproducibilities and Detection Limits

The values for reproducibility obtained were very good but the precision was critically dependent on the condition of the graphite tube. Time must be allowed for the tube to cool after each sample cycle otherwise low precision results, because the sample soaks to a small but significant extent into the graphite surface and, as the porosity of the surface affects the analytical signal, this degree of absorption must be constant. Consequently, a steady supply of cooling water is required and a lapse of at least 30 s must occur between atomisation and the injection of the following sample. With no ashing step a sample cycle time of 1 min was found to be adequate, but if various ashing stages were included the sample cycle time had to be appropriately increased.

The detection limit obtained was defined as that amount of analyte which gives a mean signal equal to twice the standard deviation of a set of at least ten sample - blank pairs close to the detection limit. Those obtained for palladium, platinum and rhodium were very good but those for ruthenium, iridium and osmium were equivalent to about 5% absorption owing to a scatter signal of about 0.01 absorbance unit, which could not be reduced as it was caused by the high atomisation temperatures that were necessary for complete atomisation of the analytes. With the use of simultaneous background correction it should be possible to improve on the figures for ruthenium, iridium and osmium.

Interference Studies

The interference of the precious metals on each other was determined at ratios from 1- to 100-fold mass excess of interferent over analyte. Results for the 100-fold excess level at two levels of each analyte are given in Table IV.

In general, as the concentration of interferent increases for a given concentration of analyte, the tendency is towards a decrease in analytical signal, the exception being for 0.2 ng of palladium as analyte, with which the signal is constant from 5- to 100-fold excess. This

TABLE IV

PERCENTAGE CHANGE IN ABSORBANCE WITH 100-FOLD MASS EXCESS OF INTERFERENT

Analyte	Amount/ ng	Interferent					
		Pd	Au	Rh	Pt	Ru	Ir
Palladium	1.0		0	+20	0	0	+5
	0.2		+35	+40	+25	+10	+30
Rhodium	1.0	−5	−10		−35	−30	−40
	0.2	0	0		+10	+5	0
Platinum	25.0	−20	−35	−65		−70	−80
	5.0	0	0	0		−30	0
Ruthenium	5.0	−30	−60	−20	−30		−55
	1.0	0	−15	+5	+20		0
Iridium	25.0	−50	−45	−20	−25	−75	
	5.0	−10	−30	0	−10	−10	

effect is illustrated in Fig. 2, where data are given for interference at the 10-, 50- and 100-fold excess levels.

It seems that at higher levels ruthenium and iridium give greater signal depressions than the more volatile precious metals, this effect being due mainly to accumulation of interferent in the tube. This accumulation is so intense that when osmium is involved it is extremely difficult to obtain quantitative results and therefore none are included here. The accumulation of the less volatile elements and the resulting interferences can be reduced by using a higher atomisation temperature at the expense of precision and tube lifetime. In the determination of platinum, by increasing the atomisation temperature, the interference due to rhodium and iridium can be reduced by between 25 and 50% (Table V).

A mechanism for the cause of interference has been postulated[12] to be the formation of alloys between the metals involved, which thus changes the atomisation characteristics of the analyte. It is therefore to be expected that as the interferent concentration increases or the

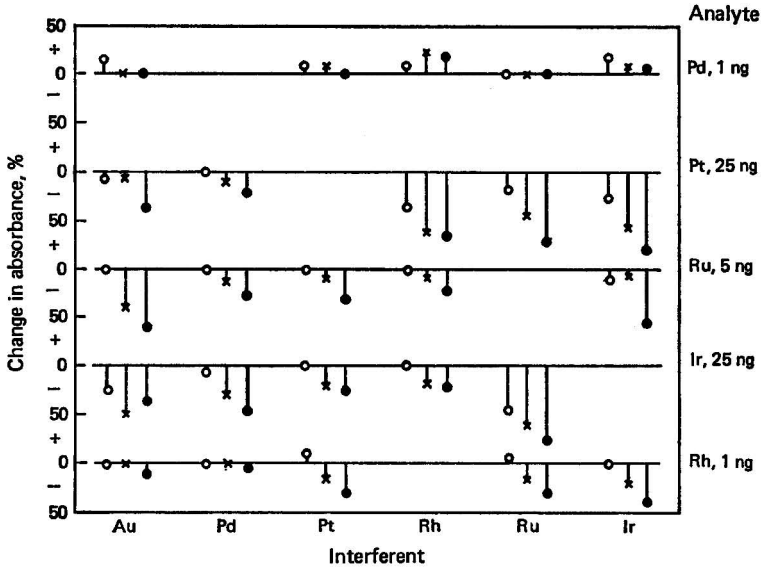

Fig. 2. Percentage change in absorbance with different interferents. ○, 10-fold; ×, 50-fold; and ●, 100-fold mass excess of interferent.

TABLE V

EFFECT OF ATOMISATION TEMPERATURE ON THE INTERFERENCE OF IRIDIUM AND RHODIUM ON 25 ng OF PLATINUM

Results are expressed as a percentage change in absorbance.

Interferent	Atomisation temperature/°C	Excess of interferent		
		50-fold	70-fold	100-fold
Iridium	2 200	−55	−55	−80
	2 400	−45	−50	−55
	2 700	−30	−30	−40
Rhodium	2 200	−50	−60	−60
	2 400	−35	−45	−45

tube ages, becoming more porous, the analytical peaks will broaden owing to slowing of the rate of atomisation; results that support this conclusion are given in Table VI. By visual comparison of peak shapes for other analyte - interferent pairs it can be seen that a similar broadening effect occurs.

TABLE VI

EFFECT OF INTERFERENT CONCENTRATION ON SIGNAL DURATION

Peak width at half-height measured in seconds.

Interferent	Excess of interferent over 25 ng of platinum							
	0	×1	×5	×10	×20	×50	×70	×100
Rhodium	1.06	1.14	1.17	1.35	1.57	2.14	2.23	2.60
Iridium	1.06	1.24	1.26	1.33	1.68	1.74	2.23	3.08

Various methods for the reduction and removal of these interferences were investigated. Simple dry ashing was found to be of no real use, the interferences being reduced in many instances but not removed. Pearton and Mallett[14] investigated the effect of releasing agents on the determination of palladium and found that whereas uranium, vanadium and lanthanum depress the palladium signal, copper and cadmium do show some releasing action. Therefore, the use of these two reagents was investigated for the other precious metals. The two solutions used were a 1 000 μg ml^{-1} solution of cadmium and a solution containing 0.5% each of copper sulphate and cadmium sulphate, the ashing setting being 1 290 °C for 30 s. By using the analyte and interferent levels described in Table IV it was found that both reagents were successful in removing interferences but the results obtained were not acceptable owing to the high irreproducibility entailed, relative standard deviations being about 20% for five replicates. The copper - cadmium mixture also caused a decrease in sensitivity of 30–50% for all elements.

The use of oxidising agents to remove ruthenium and osmium as their tetroxides was investigated. Perchloric acid (3–20%) had no effect but acidified sodium bromate solution (0.5% *m*/*V*) was found to be successful in removing these two elements, the only exception being the removal of ruthenium in the presence of iridium. However, again there was a problem of a 50% reduction in sensitivity and a low reproducibility (relative standard deviation of about 10%) from five replicates.

Ebdon[17] proposed that with the carbon filament technique the degree of interference was governed, not by the ratio of analyte to interferent, but by the absolute amount of interferent present. Preliminary experiments seemed to support this view and therefore simple dilution of the sample was investigated as a means of reducing interferences. By examining the results given in Table IV it can be seen that this step was most successful and, with the exception of palladium as analyte, the remaining small interferences could be removed by a further 5–10-fold dilution.

It can therefore be concluded that dilution of the sample is the best method of removing precious metal interferences, although there will be a limit to this practice as regards precision and the concentration of the analyte. When high levels of ruthenium and osmium are present

it would be practicable to use acidic bromate solution to remove them, although some degree of sensitivity and precision would need to be sacrificed in order to achieve an interference-free determination.

Applications

The carbon rod technique has been used in this laboratory to determine precious metals in a number of samples, some of the results obtained being presented in Tables VII, VIII and IX.

TABLE VII

DETERMINATION OF PRECIOUS METALS IN COPPER AND NICKEL STANDARDS

Results are expressed in p.p.m.

Sample	Pd	Pt	Rh	Ir
Euratom copper standards:				
1.19 p.p.m. of palladium and platinum	1.6 ± 0.3	1.2 ± 0.4	—	—
11.9 p.p.m. of palladium and platinum	11.9 ± 2.1	10.3 ± 1.0	—	—
Copper sample A	3.2, 3.2	15.5, 16.2	0.3, 0.3	—
B	—	12.3 ± 2.0	—	—
C	0.21, 0.25	1.2, 1.3	—	<1.3, <1.3
Nickel sample A	2.9, 2.3	2.3, 2.0	—	<1.3, <1.3

TABLE VIII

COMPARISON OF RESULTS FOR SOLUBLE PLATINUM IN AIR FILTERS

Results are expressed in micrograms.

Method	Sample A	B	C	D	E	Precision
This work	3	4	3	4	6	±0.1
X-ray fluorescence	10	2	4	<2	<2	±2.0

TABLE IX

DETERMINATION OF PLATINUM IN WHOLE BLOOD

Sample	Platinum/μg per 100 ml		
A	10	12	11
B	8	8	10
C	21	14	14
D	6	7	<5
E	21	36	21

Platinum in air

Platinum was leached from air filters with 1 M hydrochloric acid or 50% aqua regia in order to determine soluble or total platinum. The detection limit was improved by a factor of ten when compared with that obtained by the equivalent flame method. The volume of leaching solution used was dependent on the size of the air filter.

Platinum in human blood

Blood was diluted 1+1 with de-ionised water and 1-μl aliquots were added to the carbon rod. The detection limit achieved was 4 μg per 100 ml of whole blood as compared with 100 μg per 100 ml for a flame method in which no pre-concentration was used.

Palladium and platinum in organic substances

The improvement in sensitivity and detection limits by the non-flame method when compared with those obtained with a flame was 10-fold for platinum and 40-fold for palladium.

In the methods used the sample was simply dissolved in an appropriate solvent and applied directly to the carbon rod.

Precious metals in copper and nickel

Following dissolution in aqua regia the precious metals were pre-concentrated by ion exchange and the resulting solution was applied to the carbon rod. The detection limits compared with those by the equivalent flame procedure were improved by a factor of four for iridium and rhodium, of ten for platinum and of 40 for palladium. Ruthenium was not determined.

For most of these methods non-flame atomic absorption was used as the instrumental finish and therefore enhancement factors were due only to the inherent sensitivity of the technique resulting from higher atomisation efficiencies. However, for samples such as human blood, a flame can accept only dilute samples and therefore the enhancement afforded by non-flame atomisation is further increased.

With air filters, blood and organic substances the determinations were free of interference from the matrix, but for the copper and nickel samples, although there was no interference from these metals in the final solution, it was necessary to run standards in order to eliminate errors caused by incomplete ion exchange.

Conclusion

The carbon rod method provides a sensitive and precise method of determining the precious metals down to below parts per million levels. The sensitivity is such that the upper working limit of the method is approximately the same as the lower limit of the corresponding flame procedure. It thus provides a most useful extension of the atomic-absorption technique. Osmium, however, owing to its very low volatility, is the exception and in all but a few isolated instances the flame procedure will be preferred.

Although mutual interference between the precious metals is, in some instances, high, they can mostly be removed by simple dilution of the sample and if this device is not possible the standard additions procedure can be used without too great a loss in sensitivity. Also, integration of the analytical signal may prove a better measurement technique as it should compensate for the variation in the rate of evaporation of the analyte.

The author thanks the Board of Directors of Johnson Matthey Chemicals Limited for permission to publish this paper, and his colleagues at Royston for their help in analysing some of the copper, nickel and air samples.

References

1. Strasheim, A., and Wessels, G. J., *Appl. Spectrosc.*, 1963, **17**, 65.
2. Erinc, G., and Magee, R. J., *Analytica Chim. Acta*, 1964, **31**, 197.
3. Schnepfe, M. M., and Grimaldi, F. S., *Talanta*, 1969, **16**, 591.
4. Pitts, A. E., van Loon, J. C., and Beamish, F. E., *Analytica Chim. Acta*, 1970, **50**, 181.
5. Mallett, R. C., Pearton, D. C. G., Ring, E. J., and Steele, T. W., *Talanta*, 1972, **19**, 181.
6. Sen Gupta, J. C., *Mineral Sci. Engng*, 1973, **5**, 207.
7. Scarborough, J. M., *Analyt. Chem.*, 1969, **41**, 250.
8. van Loon, J. C., *Z. Analyt. Chem.*, 1969, **246**, 122.
9. Atwell, M. G., and Hebert, J. Y., *Appl. Spectrosc.*, 1969, **23**, 480.
10. Pitts, A. E., van Loon, J. C., and Beamish, F. E., *Analytica Chim. Acta*, 1970, **50**, 195.
11. L'vov, B. V., *Spectrochim. Acta*, 1969, **24B**, 53.
12. Guerin, B. D., *Jl S. Afr. Chem. Inst.*, 1972, **25**, 230.
13. Adriaenssens, E., and Knoop, P., *Analytica Chim. Acta*, 1974, **68**, 37.
14. Pearton, D. C. G., and Mallett, R. C., National Institute of Metallurgy, Johannesburg, 1974, Report No. 1598.
15. Donega, H. E., and Burgess, T. E., *Analyt. Chem.*, 1970, **42**, 1521.
16. Morrow, R. W., and McElhaney, R. J., *Atom. Absorption Newsl.*, 1974, **13**, 45.
17. Ebdon, L. C., Ph.D. Thesis, University of London, 1971.

Received *September 19th*, 1975
Accepted *November 28th*, 1975

Analyst, May, 1976, Vol. 101, pp. 356–366

The Development of Remote Spectrographic Heads for Metallurgical Analysis and their Application to Product Inspection Analysis

A. D. Ambrose

Research Centre, British Steel Corporation, Tubes Division, Corby, Northamptonshire, NN17 1UA

and J. D. Hobson

Dunford Hadfields Ltd, East Hecla Works, Sheffield, S9 1TZ

Direct-reading optical-emission spectroscopy has been widely adopted in the iron and steel industry as a rapid analytical technique for process control and for other laboratory analyses. The conventional spark stand, however, severely restricts the size and shape of the sample that can be accommodated on the instrument and thus limits the more general use of the technique for on-site analysis outside the laboratory. This disadvantage has now been overcome by using a fibre-optic light-guide to link a mobile excitation head to a conventional spectrometer.

Parallel but independent work has been carried out in the authors' laboratories, first on photographic and later on direct-reading spectrometers. At the Corby laboratories, further work has resulted in the manufacture and application of an inspection analyser that incorporates a 5-m guide. Typical calibration graphs, reproducibilities and examples of material identification are given, and possible applications are discussed.

Steel is produced to many different compositional specifications according to its intended usage. During recent years there have been outstanding advances in the analytical procedures used for process control, thus making possible the more economic production of ever higher tonnages of steel of assured composition. In the interval between primary production and the finished product and again before the latter reaches the customer, however, there may be many intermediate stages of manipulation, *e.g.*, forming, sub-division and transportation, at any of which normal identification marks may be obliterated. Although great precautions are taken, loss of identity, with the attendant risk of mix-ups, is a very serious matter and has been of concern to quality control engineers for many years. This problem has led to a continuing quest for rapid and reliable methods for the identification of material on-site.[1]

Generally, purely chemical methods of identification are not well suited to large-scale production, although local spot-testing, for example with dimethylglyoxime for nickel, is sometimes useful. Physical methods, based, for example, on the magnetic or electrical properties of the material, have been more widely applied but, unfortunately, they indicate chemical composition only indirectly and are too easily affected by other metallurgical factors. Other miscellaneous methods include spark testing, and the use of portable isotope analysers and direct-vision spectroscopes. Although each may be helpful in specific circumstances, none meets entirely the requirements of quality control inspection. Equipment that does have the potential for this kind of work is that based on measurement of spectral radiation, either optical or X-ray.

Traditionally, however, such equipment, while of excellent use in the laboratory and highly developed in this context, has been unsuitable for on-site quality control inspection, as it is too bulky, fragile and easily upset by vibration or temperature variations. Recent developments by the manufacturers have now enabled single-console, robust optical spectrometers to be provided, which are suitable for use in the plant with the minimum of protection.

Clearly, the outstanding problem is one of sample presentation. The need to cut and prepare a suitable size of sample limits the scope of the technique. On the other hand, the handling difficulties in locating massive components quickly and accurately on the spectrometer are obviously formidable. The novel solution to this problem that was conceived independently at the Research Centre of the British Steel Corporation, Tubes Division, Corby, and in the laboratories of Dunford Hadfields Ltd, Sheffield, embodies separation of the electrical discharge into a portable test-probe, which is independent of the main spectrometer but coupled to it by a light-guide several metres long if necessary.

Another spectroscopic approach to inspection analysis involves atomisation of the sample in an arc and transportation of the vapour in a stream of argon through a flexible pipe to a plasma arc coupled to a spectrometer.[2] Conveyance of light, however, appeared to have certain attractions and governed the lines of the present investigation.

The present paper describes the laboratory work carried out on this concept and then proceeds to describe a production version of the apparatus in operation at Corby since the beginning of 1974. Possibilities for further development are also indicated.

Experimental Work at Corby Research Centre

Preliminary Trials Using a Photographic Spectrograph

In order to determine the feasibility of the remote sampling concept and generally to gain experience in the use of fibre optics, preliminary experiments were conducted by using a Hilger large-dispersion Littrow spectrograph and a glass light-guide that was approximately 2 m long and 3 mm in diameter. The only optical modification was the removal of the condenser lens that normally stands before the spectrograph slit, and the insertion, immediately before the slit, of a short-focus lens (microscope eye-piece), which was adjusted until the spectra were in focus. In the first experiments the original 15 000-V uncontrolled a.c. condensed-spark source supplied with the instrument was employed and many spectra were successfully recorded.

In the expectation of possible plant usage, however, it was envisaged that, initially, the remote sampling head would be hand-held, and in view of the elaborate safety precautions that would be required for high-voltage operation, it was decided to replace this source by the power unit from a Metascop direct-vision portable spectroscope. This is a compact unit that is well suited to being built into a portable test head and, when operating at a relatively low potential, presents only a slight safety problem. In operation, a locating pin is in contact with the sample and the body of the unit is tilted until the discharge electrode just makes contact.

The current flow energises a coil, which lifts the electrode from the sample against the action of a spring, thus simultaneously drawing the arc and breaking the circuit. With the coil now de-energised, the electrode returns to the sample and the cycle is repeated.

Typical calibration graphs for manganese and chromium are shown in Fig. 1, in which arbitrarily selected lines in the 400.0-nm region were used. At this early stage in the feasibility trial, time was not spent in determining exact wavelengths. Alternatively, if these graphs are assumed to represent correctly located calibrations and the points are read from them as for analytical samples, results typified by those shown in Table I are obtained.

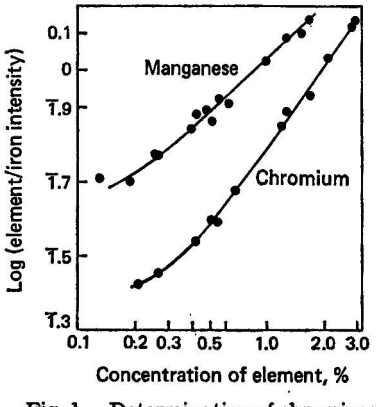

Fig. 1. Determination of chromium and manganese. Preliminary experiments with photographic spectrograph.

TABLE I

COMPARISON OF ANALYSES AND CHEMICAL RESULTS

Photographic spectrograph; Metascop source; 60-s exposure.

	Chromium, %		Manganese, %	
Sample	Analyser method	Chemical method	Analyser method	Chemical method
S.S. 401	0.18	0.18	0.97	1.0
402	0.51	0.55	0.16	0.19
403	0.42	0.42	1.70	1.69
404	0.68	0.68	0.46	0.52
405	0.21	0.21	1.31	1.28
406	2.21	2.12	0.53	0.53

These results were regarded as being highly encouraging, although it must be emphasised that they were obtained under controlled laboratory conditions with clean prepared samples and by using a relatively long exposure time of 60 s.

Other information to emerge from these photographic experiments was as follows:

(*a*) The useful wavelength range transmitted by the glass light-guide does not extend below about 380.0 nm, thus precluding the determination of carbon, sulphur and phosphorus. Above this wavelength range, however, spectral lines are listed for most of the alloying elements.

(*b*) From the manufacturer's data, a loss of light intensity to approximately 30% of the original value (70% loss) might be expected along the 2-m glass guide. In practice, inserting the guide between the source and the slit gave a slight gain in intensity, presumably because of the greater solid angle subtended.

(*c*) Comparisons were made with the guide straight, looped and coiled on itself several times. It was found that provided the coil diameter was not less than about 25 cm, no deleterious effect on the spectral lines could be detected.

Trials with a Direct-reading Spectrometer

The next important stage in the laboratory experiments was the transference of the system to a direct-reading spectrometer, for which purpose Applied Research Laboratories, Luton, kindly lent us a sequential single-channel instrument (the Quantoscan) so as to enable trials to be continued with electronic recording and in regions of the spectrum that were beyond the range of the photographic spectrograph. As before, a short-focus lens was fitted between the end of the light-guide and the spectrometer slit, and adjusted for maximum intensity.

The first trials with the Metascop source, which had given good results on the photographic spectrograph, however, proved to be unsatisfactory. With a smooth-running discharge, the light intensity was too small to record in a reasonable length of time. Only by increasing the gap almost to cut-off point was there sufficient sensitivity and then the arc was so erratic that multiple readings were necessary. Accordingly, attention had to be given to an alternative form of discharge.

In emission spectroscopy, two extreme forms of discharge are recognised. One is a high-voltage, low-current spark that has a relatively low light output but gives good quantitative results. At the other end of the scale is the d.c. arc (commonly 25–50 V on closed circuit and a current up to 10 A), which gives a high light output but is less accurate and subject to wander. Between these extremes, various interrupted arc sources have been designed so as to give optimum performance in any given situation.

With suitable engineering, no doubt any of these sources could be used in a remote sampling head but, bearing in mind the safety aspects mentioned in the previous section and in order to avoid the need for lengthy development, attention was concentrated on the possibilities of the d.c. arc, and it was found that with a simple source operating at 35 V and 2–2.5 A closed circuit (110 V open circuit), there was sufficient light intensity for a guide several metres long to be used if necessary. A circuit diagram is shown in Fig. 2.

Spectroscopically speaking, the d.c. arc is a crude source and it was realised that the accuracy achieved might not be high but it was considered that provided results were reasonable, refinements could be introduced. For the portable test head, a copper electrode

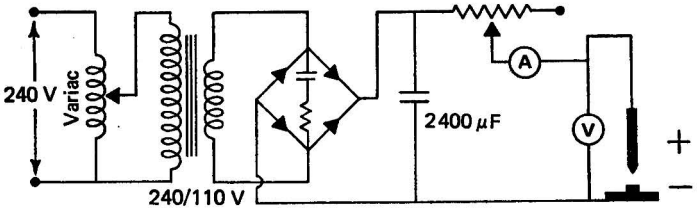

Fig. 2. The d.c. arc source.

was fastened into a simple press-down holder as illustrated in Fig. 3. In practice, the test head is applied to the sample. The electrode, which is gripped in an inner sliding tube, is then pressed down against the action of the spring so as to touch the sample and strike the electric arc. When the pressure is released, the electrode springs back a controlled distance and the arc continues to burn. This device is the subject of a patent application.

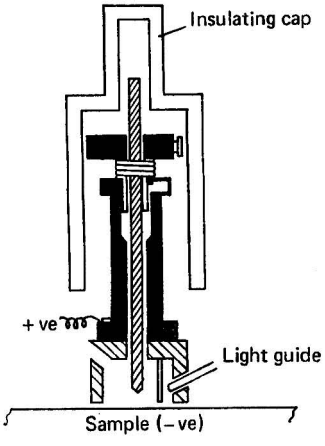

Fig. 3. First experimental test head in laboratory trials.

By using this relatively crude source, spectral lines were located in the visible region for the following elements of interest: manganese, 476.24; chromium, 520.61 (425.44); molybdenum, 386.41; nickel, 471.44; niobium, 405.89; and vanadium, 437.92 nm. Aluminium (396.15 nm) also falls within the transmission range of glass and could be added to the programme if required. The nickel line given above is rather insensitive and is not recommended for alloying concentrations below about 1%. The chromium line at 520.61 nm is less strongly self-reversing than that at 425.44 nm and is preferred, but it may be outside the range of some spectrometers.

As expected, analytical results using this relatively crude source were not as good as had previously been obtained on the photographic instrument, partly because of the inherent variability of the arc and partly because of the much shorter integration times that are now being used. Nevertheless, they were suitable for the identification of a number of important steel specifications, without risk of confusion between them. It should perhaps be emphasised that analytical accuracy, although always desirable, is not necessarily the major consideration in the identification of material on site. Speed of testing assumes a high priority, even at the expense of accuracy, provided, of course, that there is no risk of confusion between the individual specifications. Process control of the primary steelmaking ensures that the concentrations within a given specification are correct.

With this aspect in mind, and in order to relate the work still more closely to plant practice, a number of tube samples were then analysed directly on the surface without surface preparation of any kind. With chromium as a test case, it proved to be possible to separate the

principal steel grades of interest (residual, 0.5, 1, 2, 4%, etc. of chromium) unambiguously in testing times of under 10 s, of which 5 s were required for pre-burn. More details of the analytical results are given under the inspection instrument at the Corby works, where the production version of the instrument is described.

Experimental Work at Dunford Hadfields

Preliminary Investigations

In early 1969, the analytical laboratories were receiving fairly frequent requests for analyses to be carried out on rather large pieces of steel in the course of works investigations, and were using a number of the techniques discussed earlier, but at considerable expense and inconvenience. The purchase of a demonstration fibre-optic light-guide kit for general evaluation in the physics department led almost at once to the idea of bringing the light from a discharge, rather than an unwieldy specimen itself, to the spectrometer.

Preliminary tests with a 1-m glass light-guide of only 1.5 mm diameter coupled to a 3-m quartz photographic spectrograph at once showed that typical emission spectra of steel could be recorded on Kodak IIL panchromatic plates. It was clear that the idea was sound and patent applications[3,4] were filed. The patents envisaged that a widened scope could be achieved for spectrometry outside the laboratory by using an excitation head capable of being used under workshop conditions, for example, as part of a routine inspection on a production line, with the head coupled via a flexible light-guide to an automatic spectrometer housed nearby in a small enclosed space capable of being maintained under controlled conditions akin to those of a normal spectrographic laboratory. In subsequent experiments carried out in order to evaluate the idea, the gradual introduction of more sophisticated equipment has shown the validity of the original idea, and exploitation of the patent is now the subject of a licence agreement with Applied Research Laboratories.

The first step was to produce some actual analyses by use of the Hilger and Watts 3-m Quartz Littrow spectrograph that had previously been calibrated for steel analysis. However, all of the spectrographic lines used in normal photographic or direct-reading procedures for steel were in the ultraviolet region. Examination of the spectrum of light from a mercury lamp transmitted through the fibre optic gave its transmission range and showed that the use of the conventional Kodak B10 plate was satisfactory. However, constant exposure times gave spectra of decreasing density, although the intensity recovered after a period of inactivity.

The fading effect was found to be due to the concentration of heat on the end of the fibre optic by the lens used to gather light from the source discharge, and it was eliminated by introducing a pair of heat filters (Evans Electroselenium, Type HA1). These also removed longer wavelengths and the transmission band of the optical path was reduced to approximately 380.0 to 440.0 nm. Provided that allowance was made for variations in the gamma characteristics of the plate with wavelength, as determined by step exposures on the spectrum of a mercury-discharge lamp, satisfactory calibration graphs could be produced for the line pairs manganese 403.1 - iron 400.7 nm, chromium 425.4 - iron 426.0 nm and molybdenum 386.4 nm with an unidentified iron line of suitable sensitivity. However, the vanadium line at 437.9 nm was unsatisfactory because of an inter-element interference from nickel, and it was shown that 1% of nickel was recorded as being equivalent to 0.1% of vanadium.

Experimental Work with Silica Optics

Use of a silica optic guide was desirable in order that transmission in the ultraviolet region should match that of the lenses and prisms of the spectrograph, allowing the use of normal working techniques. In the early stages, silica light-guides were not commercially available, so experimental light guides were made, first from home-made hand-drawn fibres, and later from Thermal Syndicate Spectrosil fibres of 40 μm diameter. The home-made optic guides showed satisfactory transmissions in the ultraviolet region but they were internally fragile and gradually lost their power of transmission. Eventually, a commercially manufactured guide that was 1 m long and 3 mm in diameter became available (from Jena Glaswerk Schott, Mainz) and this was used in conjunction with a water-filled fused silica cell of 10-mm path length, substituted for the glass filters because the latter did not transmit in the ultraviolet region, for heat absorption.

Adequate light for photographic recording was available; the silica guide had a quoted transmission of 50% at wavelengths above 250.0 nm, but falling to about 20% at 200.0 nm. Fortunately, this loss was more than compensated for by the improved aperture of the guide and the quartz lenses used for light collection. Satisfactory calibrations for steel analysis were obtained for silicon, manganese, chromium, nickel and molybdenum. As an example, the calibration for chromium using the two-line pair method is shown in Fig. 4.

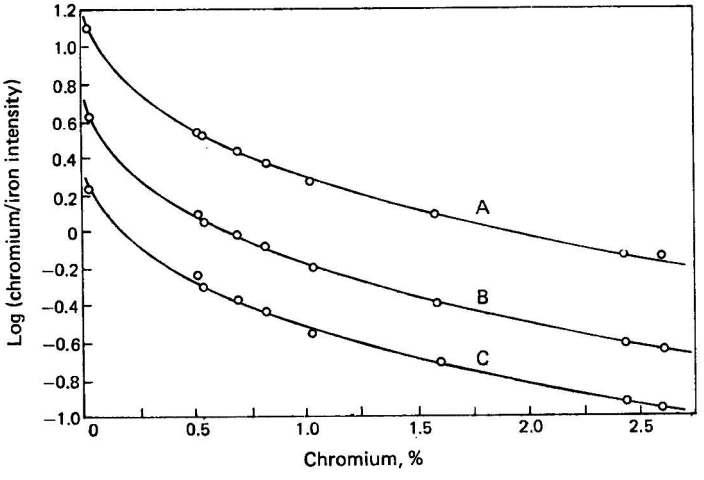

Fig. 4. Calibration of 3-m quartz photographic spectrograph using 1-m silica fibre-optic light-guide. Chromium 282.2371-nm line. Iron lines: A, 282.8634 nm; B, 282.7434 nm; C, 281.9294 nm.

An early success for the technique was gained with the large forging shown in Fig. 5 (see Plate). Analysis showed that steel of incorrect composition had been used, thus explaining metallurgical problems; a parallel analysis on a piece of steel of correct type gave results that were in close agreement with its composition determined conventionally (Table II), which confirmed the reliability of the results for the unidentified material.

TABLE II

ANALYSIS OF A LARGE FORGING USING THE FIBRE-OPTIC LIGHT-GUIDE

Standard test piece N.5659		Si	Mn	Cr	Ni	Mo
Known composition, %	..	0.20	0.29	1.94	2.02	0.61
Result by fibre optic, %	..	0.18	0.27	1.99	1.98	0.64
Stem forging						
Result by fibre optic, %	..	0.28	0.47	1.33	4.70	0.23

Experiments with a Direct-reading Instrument

The next development was to couple the silica guide to an air-path Applied Research Laboratories direct-reading spectrometer, which was built in 1954 but was still operational. At first, in order to avoid major modifications, a pair of glass prisms was also interposed into the optical path. This arrangement limited the choice to lines in the visible region, but a good calibration graph was produced for the vanadium line 437.923 8 nm in low-alloy steels (Fig. 6). The standard deviation for ten consecutive results at 0.50% of vanadium was 0.011%.

This finding justified further work and the spectrometer was moved to another laboratory where it could be linked with a Hilger and Watts FS 139 source unit. A manually operated digital measurement unit replaced the original chart-recorder system. The external optics were rearranged so as to eliminate the use of glass, the silica fibre and quartz lenses being used to bring the light to the entrance slit. Experiments were conducted in the use of reflecting

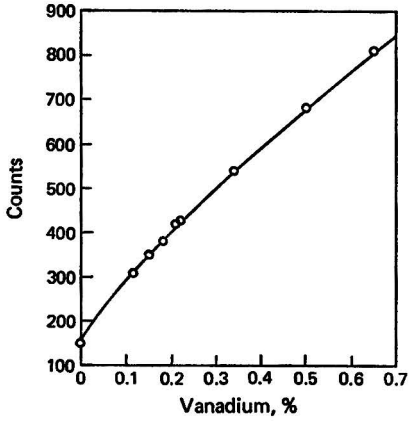

Fig. 6. Calibration graph for vanadium with air-path direct-reading ultraviolet spectrometer incorporating silica fibre-optic and glass components. Vanadium 437.9238-nm line.

optics,[5] which have advantages over lenses in providing wide aperture and improved coupling to fibre-optic guides, and in eliminating problems of absorption. Fig. 7 shows typical ways in which reflecting systems can replace lenses.

Excitation in air gave unsatisfactory reproducibility, but the use of a conventional Petrey table and a triggered discharge in argon (for which the source unit had been designed) gave results that were much more reproducible. By using this hybrid instrument, calibrations were obtained for many of the elements for which lines had been selected when the spectrometer was built as an air-path instrument, including silicon 251.612 3, manganese 293.306 3, chromium 267.715 9 (Fig. 8), nickel 231.603 7, molybdenum 281.615 4, copper 327.296 2 and the previously mentioned vanadium 437.923 8 nm, with the iron line at 271.441 2 nm as internal standard. Aluminium at 396.15 nm gave results that were too scattered to be of use.

Subsequent work has been directed at developing a mobile head; experiments with a rough prototype have been used to aid in the design of a correctly engineered version. The prototype showed the necessity for a good seal in order to exclude air from the argon atmosphere. It also showed the importance of keeping the end of the optic guide free from sputtered steel, which caused gradual loss of light transmission. It is hoped that these problems will have been overcome in the unit now being built.

Applications of the Hybrid Instrument

Considerable use is made of spark-testing at Dunford Hadfields as a routine test, but this method of identification of a steel type requires very considerable skill and practice. More-

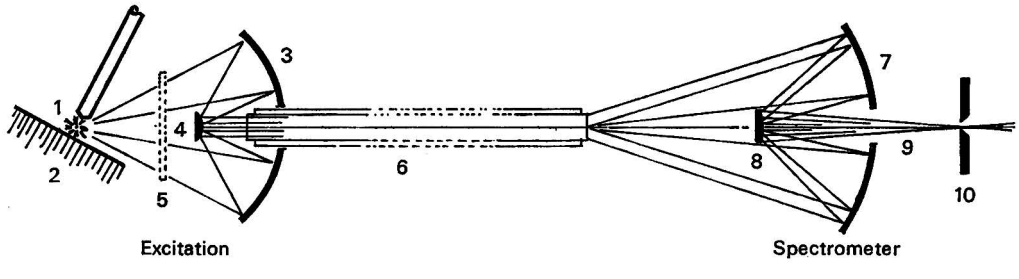

Fig. 7. Use of mirror optical systems with light guide in ultraviolet region. 1 = Radiation source; 2 = metal; 3 = primary mirror; 4 = secondary mirror; 5 = transparent screen; 6 = fibre-optic light-guide; 7 = primary mirror; 8 = secondary mirror; 9 = beam; and 10 = spectrometer slit.

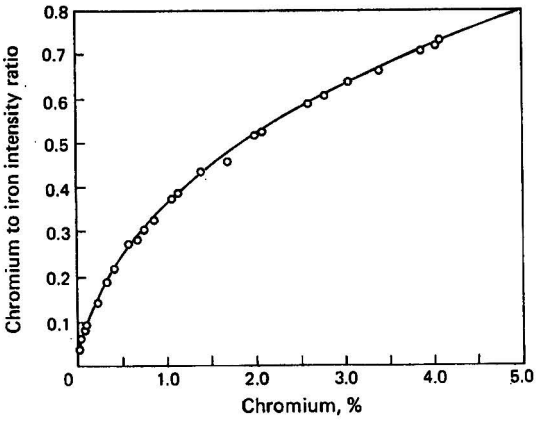

Fig. 8. Calibration graph for air-path direct-reading ultraviolet spectrometer with excitation in argon and 1-m silica fibre-optic light-guide. Chromium 267.7159-nm line.

over, when two similar steels or two heats of similar nominal composition are involved it cannot be applied. When such an instance arose, involving 101 bars, it was decided to test the capabilities of the fibre-optic experimental apparatus at its present stage of development. The steels had the nominal compositions shown in Table III, and vanadium was the obvious choice of element for rapid sorting.

TABLE III

COMPOSITION OF STEELS (%) IN MIXED BATCH OF BARS, INSEPARABLE BY CONVENTIONAL SPARK-TESTING

Steel	C	Si	S	P	Mn	Cr	Ni	Mo	Al	V
A ..	0.39	0.28	0.042	0.017	0.65	1.00	0.26	0.11	0.005	0.02
B ..	0.45	0.28	0.022	0.022	0.60	1.02	0.20	0.11	0.006	0.15

Linishing the specimens took 16.5 min, the 101 unknown bars were analysed for vanadium in only 59 min, and a further 14 analyses on standard steels and checking of results took 16 min. Thus 115 analyses required 91.5 min. The samples were given a 7.5-s pre-spark and a 7.5-s exposure in accordance with the calibration graphs mentioned previously, and the separation into two groups was so obvious as to render calculation unnecessary. Subsequent examination of the integrated counts from vanadium and iron, using the usual ratio method, produced the statistics given in Table IV.

TABLE IV

REPRODUCIBILITY OF VANADIUM ANALYSES OBTAINED DURING SORTING OF A BATCH OF MIXED BARS, USING FIBRE-OPTIC SPECTROMETRY

Identification of bars from mixed batch	Vanadium, % Mean value	Vanadium, % Standard deviation
Steel A, 36 bars	0.01_5	0.00_2
Steel B, 65 bars	0.15_3	0.01_0

Other tests have established that a wide variety of low-alloy steels can be successfully analysed. As an example, Table V gives the results obtained from 15 successive analyses of one piece of a bar of EN 353 quality, and for one analysis from each of ten bars from a single cast of EN 8 steel.

TABLE V

REPRODUCIBILITY OF SPECTROMETRIC ANALYSES FROM EXPERIMENTAL EXCITATION HEAD LINKED BY SILICA FIBRE OPTIC

	Si	Mn	Cr	Ni	Mo	Cu
EN 353 *steel*						
Mean (1 bar, 15 exposures), % ..	0.23	0.95	1.02	1.34	0.11	0.22
Standard deviation, %	0.01_4	0.01_2	0.01_2	0.00_9	0.00_5	0.00_6
EN 8 *steel*						
Mean (10 bars from one cast), % ..	0.23	0.85	0.15	0.16	0.03	0.16
Standard deviation, %	0.04_6	0.02_5	0.00_7	0.00_5	0.00_5	0.01_1

The Inspection Instrument at the Corby Works

The success of the experiments described in the Corby trials with a direct-reading instrument gave convincing proof of the feasibility of inspection analysis within the plant, using optical spectroscopy and a remote sampling head, and led the Corby Research Centre to have discussions with Applied Research Laboratories about the construction of a production version of the instrument for tube inspection at Corby.

This was based on a modified version of the Quantometer 28, a compact, single-console instrument with internal temperature control that is sufficiently robust to operate under plant conditions with the minimum of protection. The conventional spark stand is replaced by a flexible metal sheath about 5 m long, carrying the light guide, the electrical cables and water-cooling pipes and terminating at the portable test head. This head, a robust version developed from the experimental model, is illustrated in Fig. 9 (see Plate). Below the main block, two adjustable rails serve to accommodate tubes of different diameters. The vertical cylinder, which is of insulating material, contains the electrode-locking mechanism. A quarter turn of this cylinder enables the electrode to be gripped or released at will. After setting the initial gap, it is simply necessary, on transferring the head from one tube to another, to depress the cylinder until the electrode makes contact with the sample and then to release it again so as to draw the arc. This test head is also subject to a patent application.

The apparatus can be operated in two modes: manual and semi-automatic.

Manual Operation: Graphical Evaluation

In the manual mode, a meter reading is obtained for each element in turn and plotted against concentrations in the usual way. Typical calibration graphs are shown in Fig. 10 (*a*, *b* and *c*). Niobium suffers interference from manganese but this effect is automatically allowed for by signal subtraction within the instrument. Rather surprisingly, it was found that manganese formed two graphs, the low-alloy steels forming a separate line from the plain carbon steels. This is probably a function of the spectral line used and the excitation, and is obviously undesirable. Nevertheless, it does not invalidate the use of the instrument in practice, given familiarity with the specifications being sought.

Typical results for the instrument used in the manual mode are shown in Table VI, and results of reproducibility tests on tube samples with unprepared surfaces in Table VII. The coefficients of variation can be seen to be of the order of 10%.

TABLE VI

ANALYSIS OF TUBE SAMPLES (UNPREPARED SURFACES)

Manganese, %		Chromium, %		Molybdenum, %		Vanadium, %	
A	L	A	L	A	L	A	L
0.73	0.72	0.35	0.33	0.55	0.60	0.29	0.25
0.52	0.53	2.5	2.33	0.88	0.93	—	—
1.6	1.50	< 0.1	0.04	0.26	0.26	—	—
0.62	0.64	0.43	0.40	0.52	0.52	0.22	0.22
0.57	0.58	2.4	2.23	1.0	0.94	—	—
0.44	0.43	1.0	0.96	0.55	0.50	—	—

A, analyser; d.c. arc, 5-s pre-burn, 8-s integration.
L, laboratory analysis of parent metal.

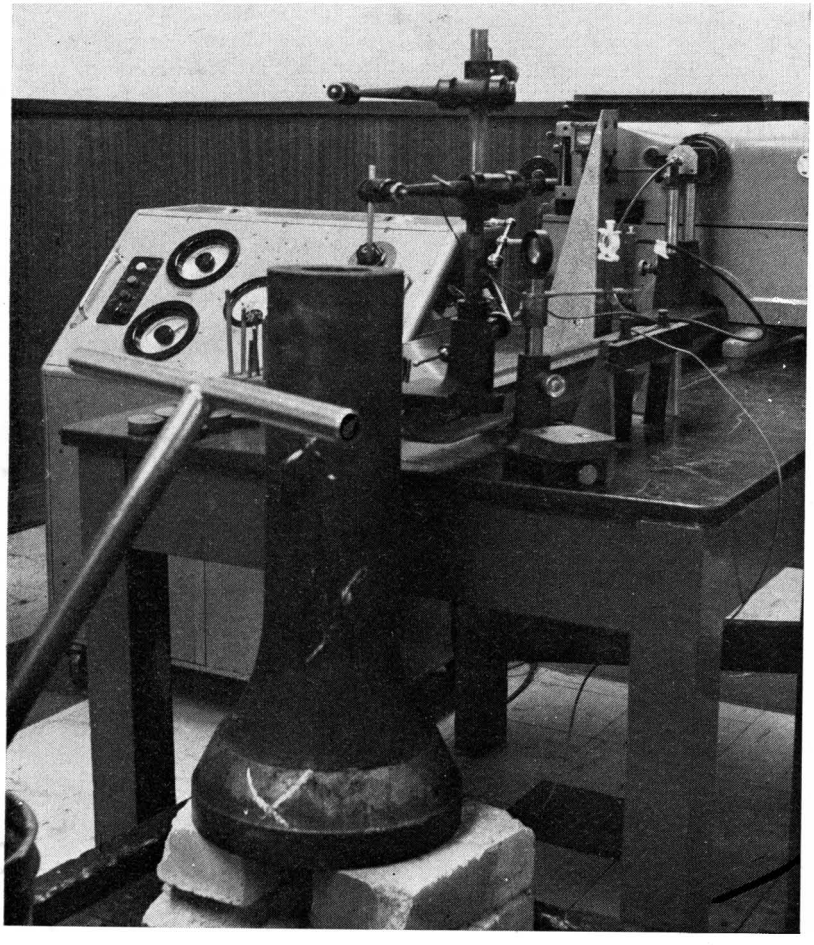

Fig. 5. Analysis of a large forging using a silica fibre-optic coupling between the excitation source and a photographic spectrograph.

[*To face p. 364*

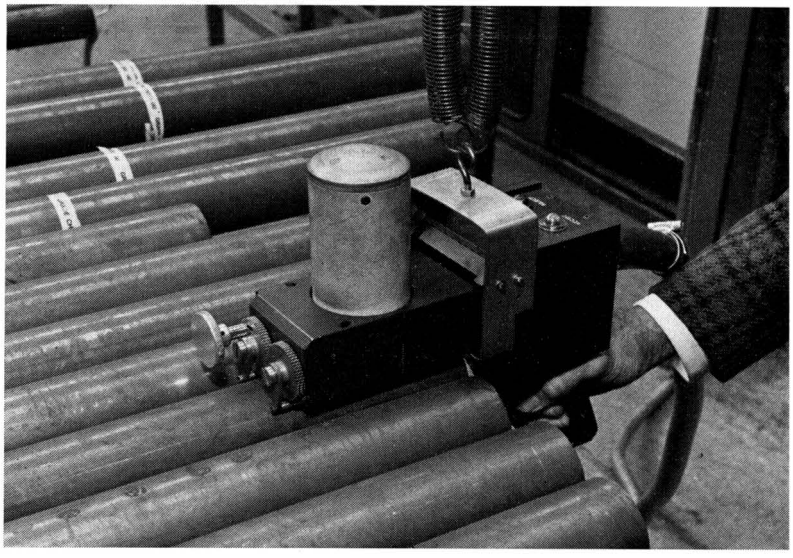

Fig. 9. Production version of portable test head.

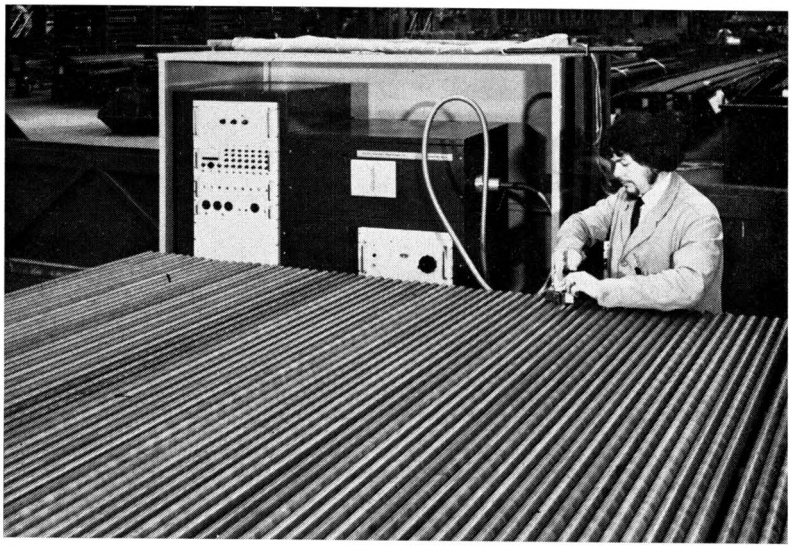

Fig. 11. General view of the inspection analyser in operation.

[*To face p. 365*

TABLE VII

REPRODUCIBILITY OF RESULTS (TUBE SAMPLES, UNPREPARED SURFACES)

Element	Mean, %	Standard deviation, %	Coefficient of variation, %
Manganese	0.69	0.052	7.5
Chromium	0.46	0.053	11.5
Molybdenum	0.60	0.059	9.5
Vanadium	0.23	0.017	7.4

Automatic Operation: Alloy Selector

In practical inspection analysis, the instrument is seldom used in the manual mode. More usually, it is used in a semi-automatic mode in which the meter readings for each element are automatically compared with pre-set values that correspond to the steel specifications being sought. If these values agree, a green light shows on the console and is repeated in the test head. If the readings do not agree, red lights are illuminated and an audible signal sounds. If desired, the readings for the individual elements can then be examined manually in order to decide on which element the discrepancy has occurred.

While it is not proposed to give a detailed description of the instrument design, it may be said briefly that, in principle, the instrument makes provision for the selection of 60 different specifications by means of alloy selector switches on the front of the console. On turning the switch from one position to another, permanently wired circuits come into operation so as to provide an acceptance "gate" for each element corresponding to a segment of the calibration graph, appropriate to the specification being sought. Since its installation at the beginning of 1974, many thousands of tubes have been successfully tested.

With added experience, the measurement time normally employed for automatic inspection comprises a pre-burn period of 3–4 s and an integration time of approximately 4 s. Allowing for occasional changing of electrodes, wiping the glass window that protects the end of the light-guide and transference of the test head from one tube to another, testing time averages 12 s per tube over a large batch of determinations. If other ancillary operations, such as untying the roped bundles, laying out the tubes on the inspection table and affixing identification tapes on the samples after test, are also taken into account, throughputs of over 200 tubes per hour can be achieved. Fig. 11 (see Plate) shows a general view of the equipment in a tube-making mill at Corby. Such equipment is now being manufactured for retail sale by Applied Research Laboratories Ltd. under agreement with the British Steel Corporation.

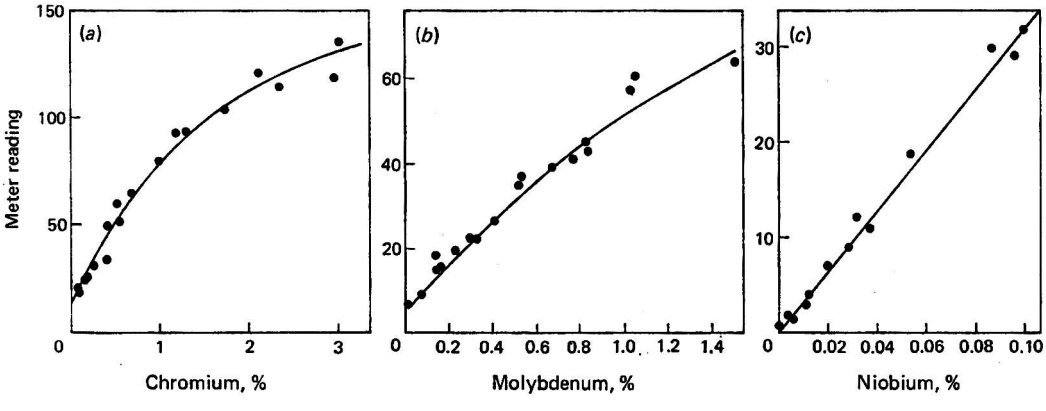

Fig. 10. Typical calibration graphs for (*a*) chromium (425.44 nm), (*b*) molybdenum (386.41 nm) and (*c*) niobium (405.89 nm) (corrected for manganese). D.c. arc.

Discussion and Further Developments

The success of these various experiments offers considerable promise for applications in metallurgy and engineering. This optimism is borne out by the successful application of the

production analyser at Corby. This particular apparatus was designed to meet a specific set of circumstances, which it appears to be doing very well. Taking the work of the two laboratories together, it is obvious that variations and further developments are possible. Alternative source units, for example, can be used to improve analytical accuracy, especially in a fully automated system in which the probe is not handled manually. When necessary, a form of specimen preparation could be introduced in order to deal with more heavily scaled materials, and instruments can be designed with a conventional spark stand and a remote sampling head leading to the same spectrometer.

Light-guides made of silica can be used to extend the wavelength range into the ultraviolet region, thus allowing the determination of most of the elements commonly required in steel analysis. The conventional manganese line at 293.3 nm used in the Dunford Hadfields instrument does not suffer from the need for separate calibrations for plain carbon and low-alloy steels mentioned earlier. So far the determination of carbon has not proved possible with the guide materials available, but is not necessarily permanently ruled out.

The use of a mini-computer for processing the data into individual specifications should provide more flexibility than the gating system described.

Clearly there are two distinct roles for very rapid analysis obtained with this type of equipment. One application is for routine inspection for quality assurance, with the instrument as part of a production line, for example in billet or bar-finishing departments. With dedicated instrumentation there may well be a need for several such units in various departments of the works. The other role is in the operation of a mobile metallurgical analytical service made available to several works within a group. In this instance the instrument would be housed in a suitably sprung and insulated vehicle, which could be taken to the site of any metallurgical problem and plugged into a local power supply, or supplied from its own generator. This arrangement would enable large masses of steel to be analysed non-destructively and on site, for example when required by a customer's quality check. It could be used in the stockyards on ingots or blooms of uncertain identity, for example during stocktaking, or for the analysis of scrap, or of materials returned for re-processing, for example, large rolls returned for re-hardening after use.

There is little doubt that this development in optical spectroscopy, embodying remote sampling and flexible coupling between source and spectrometer, frees the technique from traditional restrictions in sample presentation, should open up many new analytical possibilities and seems certain to prove an invaluable weapon in the armoury of makers and users of many kinds of metals and alloys.

The authors are grateful to Mr. J. W. Shaw, Technical Director, British Steel Corporation, Tubes Division, and Mr. G. F. Smith, Technical Director, Dunford Hadfields Ltd, for permission to publish this paper. They also thank their colleagues who have collaborated in this work, in particular acknowledging the contributions of Mr. D. W. Swingler (BSC Corby) and Mr. T. W. Lomas (DH).

References

1. Ambrose, A. D., Croall, G., Henrys, F., and McGavin, R. F., *in* "Proceedings of the Conference on the Determination of Chemical Composition," Brighton, September, 1970, Iron and Steel Institute, London, pp. 153–163.
2. Hoyt, R. E., Dryer, H. T., and Runkler, L. D., *Pittsb. Conf. Analyt. Chem. Appl. Spectrosc.*, Cleveland, Ohio, 1975.
3. British Patent No. 1 335 657, 1973.
4. U.S. Patent No. 3 909 133, 1975.
5. *Res. Disclosure*, August, 1974, No. 124, p. 12, 12419.

Received *October 15th*, 1975
Accepted *November 11th*, 1975

Analyst, May, 1976, Vol. 101, pp. 367–378

Detection and Determination of Polynuclear Aromatic Hydrocarbons by Luminescence Spectrometry Utilising the Shpol'skii Effect at 77 K

Part II.* An Evaluation of Excitation Sources, Sample Cells and Detection Systems

B. S. Causey, G. F. Kirkbright and C. G. de Lima†

Department of Chemistry, Imperial College of Science and Technology, London, SW7 2AY

A comparison has been made of the use of a xenon arc continuum source, a mercury-vapour lamp and a fixed-wavelength helium - cadmium laser source for excitation in the production of quasi-linear luminescence emission spectra of some polynuclear aromatic hydrocarbons in n-alkane solvents at 77 K. The performance of a new design of cryostat sample cell for work at 77 K has been evaluated and compared with that of a commercially available Dewar-flask sample cell. The use of d.c. integration, photon counting and of repetitive optical scanning in conjunction with a signal averager has been investigated for registration of the luminescence obtained from PAH compounds by use of the Shpol'skii effect.

Although numerous papers have described the application of quasi-linear luminescence spectrometry at 77 K to the determination of polynuclear aromatic hydrocarbons (PAH), in most instances the apparatus employed has incorporated a mercury-vapour discharge lamp[1–4] as source and a glass or quartz Dewar[5–9] tube as the sample cell. In continuation of our previous work,[10] which was concerned with the application of low-temperature luminescence spectrometry utilising the Shpol'skii effect at 77 K, we have undertaken an evaluation of the use of different sources of excitation, two different types of sample cell and a number of techniques of signal registration in order to develop the optimum instrument assembly and to exploit the inherent high sensitivity and selectivity of which the technique is capable. This paper therefore describes a comparison of the use of a xenon arc, a mercury-vapour discharge lamp and a helium - cadmium laser as excitation sources. The use of a commercially available Dewar-flask sample cell and the performance of a new design of cryostat sample cell for work at 77 K is described. The application of d.c. integration techniques, photon counting and repetitive optical scanning with signal averaging using a multi-channel time averager for registration of low-temperature luminescence signals is reported.

Experimental

The basic assembly of the spectrometer was similar to that described previously[10]; a 1-m grating monochromator (Rank Hilger Ltd., Monospek 1000) with an aperture of *f*/8 and a reciprocal linear dispersion of 0.8 nm mm^{-1} at the exit slit was employed in conjunction with an EMI 62565 or 9601B photomultiplier for most of the work. In the study of d.c. integration for registration of luminescence, a modified Quantoscan monochromator (Applied Research Laboratories Ltd., Luton, Bedfordshire) was used. The electronics associated with this monochromator made it possible to use both constant-time and constant-charge modes.

For the constant-charge mode a second (reference) photomultiplier was used; the radiation originating from the sample was partially reflected in a quartz fibre and the reflected beam was used as a reference beam. The reference and measuring photomultipliers were side-window Hamamatsu R-106 (response type S-19) instruments. The reciprocal linear dispersion of this monochromator was also 0.8 nm mm^{-1}.

* For details of Part I of this series, see reference list, p. 378.

† Present address: Departamento de Quimica, Universidade de Brasilia, Brasilia, Brasil.

Sources

A 150-W xenon arc lamp (Osram XBO W/1) continuum source powered by a suitable supply unit (Perkin-Elmer, Model 150), which provided the start voltage and an operating voltage of 20 V d.c., was employed. The medium-pressure mercury-vapour discharge lamp (Philips, Type MBW/U) was operated at 125 W after removal of the outer envelope of Wood's glass.

A 3-mW helium - cadmium laser (Electro-Photonics/Liconix, Model 401/301) was used in the laser experiments. This laser has its principal emission at a wavelength of either 325 or 441 nm, which can be selected by changing the appropriate mirrors.

Sample-handling Systems

A commercially available Dewar-flask sampling system (American Instrument Co., Maryland) similar to that described previously was employed. This system included a sample cell (length 200 mm, i.d. 3 mm and wall thickness 0.6–1 mm) constructed from silica tubing and which required sample volumes of between 0.3 and 0.5 ml.

A new design of liquid-nitrogen cryostat sample cell for use with small liquid samples was assembled. The construction of this sample-handling system is shown in Fig. 1. The system is similar to those proposed by Parker[11] and Svishchyov,[12] without the inconvenience of the latter, with which it is necessary to warm the whole system in order to change the sample. Also, for compounds with biological activity, for example, some of the carcinogenic polycyclic aromatic hydrocarbons and aflatoxins, the system must be of practical use without the possibility of spillage or contamination.

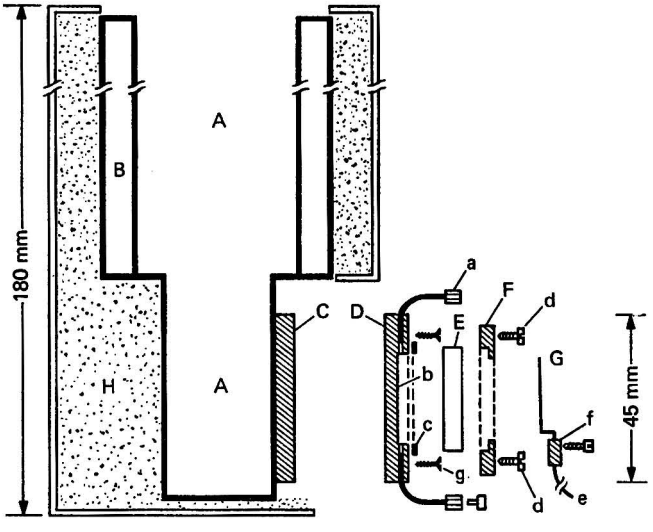

Fig. 1. Copper cryostat sample cell assembly (for key, see text).

The copper reservoir, A, into which the liquid nitrogen is introduced was surrounded with expanded polystyrene, H, as insulator. In the upper end of the reservoir a vacuum space, B, was provided in order to decrease heat transmission. At the lower end of the reservoir is a plane, vertical surface, C, to which the sample cell (D, E, F and G, when assembled) is attached by means of four copper screws, g. The copper cell, D, forms a sample compartment with a capacity of about 0.5 ml. Two small-diameter steel tubes (i.d. 1.58 mm), which end in syringe-type connections, a, provide for filling and flushing the cell. The sample compartment extends slightly outside the excitation area in order to allow for solvent contraction on cooling.

The sample compartment was isolated from the ambient temperature by a quartz microcell, E, which was evacuated several times, filled to a low pressure (10 torr) with dry nitrogen and sealed in order to create a vacuum chamber. This chamber was used as a window and a thin rubber washer, c, was used to seal it against the wall of the copper cell. The chamber was tightened in position by using an aluminium front plate, F, and nylon screws, d. These

screws are slightly flexible at room temperature and have the advantage of being poor conductors. Care must be exercised not to over-tighten the nylon screws as this would cause damage to the vacuum chamber. The front window of the chamber was defrosted by using a single turn of Nichrome wire (2.18 Ω m^{-1}), G, which was heated by using the mains supply applied at 2–3 V a.c. across a rheostat.

Temperature measurements

In order to compare the cooling rate of the conventional Dewar-flask cell and the new cryostat assembly two copper - constantan thermocouples were used; the e.m.f. developed at these was measured by using a Servoscribe recorder (Model Re 511.20). The thermocouples were tested and the measurements were calibrated by using liquid nitrogen, ice and solid carbon dioxide; the e.m.f. was related to temperature by reference to the appropriate published calibration values.[13] The measuring thermocouple was introduced into the solvent either inside the silica tube in the Dewar flask or in the sample compartment of the cryostat cell. The reference thermocouple was introduced into a Dewar flask containing melting ice.

Detection Systems

D.c. integration

For the experiments with constant-time and constant-charge integration the Quantoscan spectrometer was used with a sample cell and a mercury-vapour lamp excitation source as described above. The measurement electronics employed consisted of simple integrator circuitry, which permitted display of the luminescence signal integration to constant charge or for a constant time.

Photon counting

A simple single-channel system (Model 300, EDT Research, London) having a capacity of 10^7 counts was used for the photon-counting experiments and the output from the photomultiplier was developed across a 50-Ω load resistor. A pulse-pair resolution of 10 ns was obtained with this system. The EMI 62565 photomultiplier tube used for the photon-counting experiments had low dark current (at ambient temperature about 40 counts s^{-1} and at dry-ice temperature about 4 counts s^{-1}). A specially built cooling chamber based on the design used by Sharp[14] was used in order to cool the photomultiplier during the experiments. A constant flow of dry nitrogen was circulated around the photomultiplier so as to avoid condensation either during the cooling or the warming cycle.

In most of the photon-counting experiments the copper cryostat cell was used.

Signal averaging

Experiments on the use of signal averaging were made with a 1 000-channel signal-processing system (Unimac 4 000, Data Laboratories Ltd., Mitcham, Surrey). This apparatus has an on-line digital processing system for the storage and analysis of waveforms in both the time and frequency domains. The instrument is modular and has four basic units. The first unit is a memory with 1 024 words and the second is a sweep timer, which generates timing pulses so as to control the rate at which the data is sampled during analysis. Delay times after each sweep (from 1 μs to 999 ms) and before each sweep (from 1 ms to 999 μs) can be selected. A programme unit is provided in which not only single-channel averaging is possible but also multi-channel averaging, as four single-channel inputs, which can be averaged simultaneously, are available. A display control module provides for the selection of the number of sweeps in intervals of 2^x from 1 (2^0) to 16 384 (2^{14}).

In order to produce rapid repetitive scanning of small wavelength ranges in the luminescence emission spectra an oscillating, transparent refractor-plate mechanism, the operation of which is similar, in principle, to that of those proposed by McWilliam,[15] Roldan[16] and Snelleman *et al.*,[17] was constructed. This assembly was located within the monochromator, behind the entrance slit. A frequency of oscillation of about 5 Hz was used in all the experiments. As the refractor plate, a poly(methyl methacrylate) (Perspex) block of path length 25 mm, which was transparent from 345 nm throughout the visible region, was employed. The plate was driven by an oscillator circuit, similar to that described by Snelleman *et al.*,[17] by which the

amplitude of oscillation could be controlled. The wavelength range scanned with this system was typically between 2 and 3 nm. A simple pre-amplifier (of gain 50) was constructed and used between the photomultiplier and the signal averager. The signal being averaged was continuously observed at a display oscilloscope (Hewlett-Packard, Model 175A) and after averaging was either photographed or point plotted by using a potentiometric chart recorder.

Reagents

The n-alkanes used in this work were of laboratory reagent grade and were purified by percolation through silica gel (60–120 mesh), which had been activated overnight at 110–130 °C.

Materials

Samples of pure polynuclear aromatic hydrocarbons were kindly donated by Tobacco Research Council Laboratories, Harrogate, British American Tobacco Co., Southampton, and Shell Research Ltd., Chester.

Results and Discussion

Application of Different Excitation Sources

A comparison was made of the use of the 150-W xenon arc source, the medium-pressure mercury-vapour discharge lamp and the helium - cadmium laser for excitation in the production of the quasi-linear luminescence spectra, at 77 K, of selected PAH compounds. Radiation from the xenon and mercury sources was selected by using interference filters of peak-transmission wavelength either 250 or 300 nm (with corresponding spectral half-band widths of 14 and 30 nm and about 30% transmission at the central wavelength). The laser excitation wavelength was either 325 or 441 nm without the use of an interference filter in the irradiation optics. Table I shows the detection limits (expressed as that concentration of the compound studied which produced a luminescence signal to noise ratio of 2 at the detector electronics) for the compounds selected for study. As shown for coronene and 3,4-benzopyrene, the detection limits obtained were typically lower by a factor of about two when the mercury-vapour lamp rather than the xenon arc source was used. The use of the mercury-vapour lamp rather than the xenon arc lamp also gives rise to less scattered radiation from the sample at wavelengths near to the wavelength of measurement of the luminescence emission. Fig. 2 shows the luminescence spectra obtained with the xenon and mercury sources in the region of 400 nm for a solution of 3,4-benzopyrene in octane at 77 K; the effect of the scattered background radiation and noise from the continuum source is clearly visible. Although the mercury 404.7-nm line also contributes scattered radiation to the 3,4-benzopyrene spectrum its presence is readily identified and does not distort the shape of the observed emission

TABLE I

DETECTION LIMITS, p.p.m., OBTAINED AT 77 K WITH DIFFERENT EXCITATION SOURCES FOR PAH COMPOUNDS

		Excitation source			
				He - Cd laser	
Compound	Luminescence emission wavelength/nm	150-W xenon arc	Mercury-vapour discharge lamp	325 nm	441 nm
Coronene*	445.05	2×10^{-3}†	1×10^{-3}†	5×10^{-4}	—
3,4-Benzopyrene‡	403.00	2×10^{-4}†	1×10^{-4}†	1×10^{-4}	—
1,2-Benzanthracene‡	383.75	—	2×10^{-3}†	5×10^{-4}	—
1,2,5,6-Dibenzanthracene‡	394.25	—	5×10^{-3}†	3×10^{-3}	—
Perylene*	443.95	—	1×10^{-2}§	—	2×10^{-3}
3,4,8,9-Dibenzopyrene	449.15	—	1×10^{-4}‖	—	7×10^{-4}

* In hexane.
† 300 nm interference filter.
‡ In octane.
§ 250 nm interference filter.
‖ 300 or 325 nm interference filter.

spectrum. The use of the xenon arc source has an advantage, however, when the compound to be determined exhibits one of its principal, narrow, Shpol'skii luminescence emission bands at a wavelength at which it would be overlaid by scattered radiation from mercury line radiation from the mercury-vapour discharge-lamp source. An example of this effect was observed for the weak quasi-linear emission of dibenzofuran in tetrahydrofuran at 77 K, which occurs at 434.10 nm; this emission is obscured by scatter of the strong broad mercury line from the mercury source at 435.8 nm, whereas with xenon arc lamp excitation the dibenzofuran luminescence at 434.10 nm is readily observed. For compounds whose luminescence excitation maxima lie at short wavelengths near to the mercury 253.7-nm line, however, the use of the mercury-vapour lamp produces greater sensitivities, the output intensity of the xenon source at these wavelengths being relatively low. For perylene, for example, whose wavelengths of maximum excitation occur at 246 and 266 nm, xenon arc source excitation resulted in a luminescence signal of intensity only one tenth of that obtained with the mercury-vapour lamp source. We are of the opinion that in instrument assemblies for low-temperature luminescence spectrometry it is useful to have both mercury and xenon-discharge lamp excitation sources available as for particular analyses the best performance willl be attainable with only one of these sources.

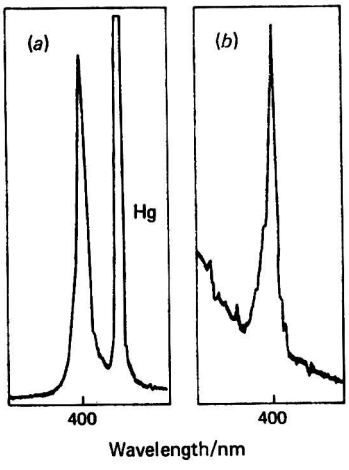

Fig. 2. Quasi-linear luminescence emission at 403 nm for 3,4-benzopyrene in octane at 77 K. (*a*), Mercury-vapour discharge-lamp source; (*b*), xenon arc lamp source.

Investigations in our laboratories, which are to be described in a later paper,[18] have demonstrated that in n-alkane matrices at 77 K the luminescence excitation spectra of PAH compounds that exhibit the Shpol'skii effect in their emission spectra also exhibit characteristic narrow-band absorption maxima. In order to achieve efficient excitation at these wavelengths a source which has high energy at the specific excitation wavelengths required for each compound is therefore desirable. Ideally, a tunable dye laser source would be employed. The only laser excitation source available to us, however, was a CW helium - cadmium laser with power output at 325 nm of 3 mW or at 441 nm of 7 mW. Obviously the use of this source can only be fully effective if the principal excitation maximum of the luminescence of the compound investigated overlaps the narrow-line output from the source at 325 or 441 nm. With excitation at 325 nm no gain in signal intensity was observed, compared with that obtained by use of the mercury-vapour lamp, for most of the compounds studied. As shown in Table I, however, some gain in detection limit for coronene, 1,2-benzanthracene and 1,2,5,6-dibenzanthracene was observed with excitation at 325 nm with the laser source. For excitation at 441 nm an enhancement in both signal intensity and signal to noise ratio (detection limit) was obtained only for perylene. This results from the fact that the 441.6-nm laser line almost

exactly overlaps the longest wavelength (0,0) band in the absorption spectrum of perylene.[19] This finding confirms our opinion that the use of a tunable dye laser source would be beneficial for low-temperature luminescence spectrometry of PAH compounds utilising the Shpol'skii effect.

Sample-handling Systems

The Dewar-flask sampling system is in widespread use and is commercially available. The system consists of a silica sample tube, which is immersed in liquid nitrogen contained in a Dewar flask with a transparent silica bore so that incident radiation can enter in order to excite luminescence in the sample, which can then be viewed by an analysing spectrometer. While this system has a number of advantages, including a fast cooling rate and a stable final temperature of 77 K, the system suffers from a number of disadvantages. Poor reproducibility can arise from difficulty experienced in the positioning of the sample tube in the Dewar flask; this disadvantage has already been noted by Hollifield and Winefordner[20] and Kirkbright *et al.*,[21] who have recommended alternative arrangements for use with a Dewar-flask or tube assembly. Other problems result from the condensation of atmospheric water vapour on the external surface of the Dewar flask and within the liquid-nitrogen coolant. Signal noise is also increased owing to the constant boiling of the liquid nitrogen in the optical path surrounding the sample cell. In order to eliminate these effects, and to improve the sensitivity and precision of low-temperature luminescence spectrometry, a new sample cell and cryostat system was designed and constructed (Fig. 1). The performance of this cell, with particular reference to sample cooling rate, final temperature achieved and precision and detection limits obtainable for PAH compounds, was compared with that of the conventional Dewar-flask sample cell.

Cooling rate and final temperature

The rate of cooling of sample solutions plays an important role in determining the width and intensity of the quasi-linear luminescence emission observed from PAH compounds in n-alkane solvents at 77 K.[22] Temperature measurements for both the Dewar-flask and copper cryostat assembly were made by using a copper - constantan thermocouple inserted directly into sample solutions of coronene in hexane in each cell. As shown in Table II, the mean cooling rate for the copper cryostat assembly was found to be considerably slower than that for the silica tube and Dewar-flask system, although the cooling rate for the latter system is a function of the wall thickness of the sample tubes employed. As expected, the final temperature attained by the sample in the silica-tube sample cell immersed in liquid nitrogen within the Dewar flask was 77 K, while for the copper cryostat assembly the equilibrium temperature attained by the frozen sample solution was higher, *i.e.*, 80 K.

TABLE II

COMPARISON OF MEAN COOLING RATES FOR THE TWO SAMPLE CELLS EMPLOYED

Sample cell	Mean cooling rate/K min^{-1}	Ratio of intensities of components of coronene doublet ($I_{445.15\ nm}/I_{443.44\ nm}$)
Silica tube:		
0.6 mm wall thickness	720	3.3 ± 0.3
1.0 mm wall thickness	540	3.7 ± 0.3
Copper cryostat cell	75	4.05 ± 0.1

Personov[2] has observed an increase in the ratio of the intensity of the coronene 445.15-nm emission to that of its emission at 443.44 nm in hexane at 77 K when slow rather than rapid cooling is employed. As shown in Table II we have confirmed this observation, although the change in the intensity ratio observed is relatively small in relation to the large difference in cooling rate that occurs in the two sampling systems.

Precision attained with Dewar-flask and copper cryostat sample cells

A comparison was made of the reproducibility of quasi-linear luminescence signals obtained at 77 K for coronene and 3,4-benzopyrene, each at two different concentrations in both sample

cells. The emission intensity obtained at the principal luminescence wavelengths was measured repetitively for sample solutions introduced into each cell. The relative standard deviations obtained for these sample solutions are shown in Table III. It is evident that a substantial improvement in precision is possible with the copper cryostat assembly. This results principally from the reproducible alignment of the sample in the optical path of the spectrometer and the elimination of noise, which is caused in the silica-tube system by the boiling of the coolant liquid nitrogen in the path of the incident and luminescence radiation.

TABLE III

REPRODUCIBILITY OF RESULTS OBTAINED WITH SILICA-TUBE AND COPPER CRYOSTAT CELLS

Compound	Concentration/ M	Solvent	Luminescence emission wavelength/nm	Relative standard deviation, %	
				Silica-tube cell	Copper cryostat cell
Coronene	2.5×10^{-5}	Hexane	445.05	9.7	1.5
Coronene	2.5×10^{-7}	Hexane	445.05	12	2.5
3,4-Benzopyrene	1×10^{-7}	Octane	403.00	4	1.3
3,4-Benzopyrene	1×10^{-9}	Octane	403.00	18	11

Techniques Employed for Registration of Luminescence Signals

The use of three techniques for recording the luminescence signal received by the photomultiplier at the exit slit of the monochromator was evaluated. These techniques were d.c. integration, photon counting and repetitive optical scanning used in conjunction with a 1 000-channel signal-averaging system.

D.c. integration

An investigation was made of the use of simple signal-integration techniques as an alternative to direct presentation of the voltage generated across a load resistor on the output of the photomultiplier. Experiments with integration for a constant time and also for the time required to reach a constant charge were undertaken with sample solutions of 3,4-benzopyrene in octane at 77 K. In the constant-time mode of operation an integration time of 60 s was used with an applied voltage of 700 V at the side-window photomultiplier fitted in the Quantoscan spectrometer. As the background luminescence intensity was high in these experiments the integrated background signal at 400 nm was measured and backed-off electrically before integration of the luminescence at the 403-nm emission line of 3,4-benzopyrene. Table IV shows that, as expected, a considerable improvement in the relative standard deviation of the luminescence signals is obtained when constant-time integration is employed rather than direct display of the instantaneous luminescence signal recorded from the photomultiplier across a load resistor. The detection limit for 3,4-benzopyrene is improved from about 5×10^{-7} M to

TABLE IV

RELATIVE STANDARD DEVIATIONS OBTAINED IN MEASUREMENT OF LUMINESCENCE OF 3,4-BENZOPYRENE IN OCTANE AT 77 K WITH INSTANTANEOUS AND D.C. INTEGRATION TECHNIQUES OF SIGNAL REGISTRATION

Concentration/M	Relative standard deviation, %		
	Direct read-out	Integration for fixed time period	Integration to constant charge
1×10^{-4}	2.5	3.0	—
1×10^{-5}	3.0	3.0	—
5×10^{-6}	9.0	4.6	0.9
5×10^{-7}	25.0	3.0	1.4
1×10^{-7}	—	9.4	0.7
5×10^{-8}	—	5.5	1.6
2.5×10^{-8}	—	6.0	4.4
2.5×10^{-9}	—	4.4	6.0

about 1×10^{-9} M. Table IV also shows that further improvement in the relative standard deviation of the luminescence signals was obtained when the constant-charge mode of integration was adopted. This further improvement in precision is explained by the availability of the signal from the reference photomultiplier, which is employed to correct the measured signal integration period for variation in the over-all light input due to fluctuation in the intensity of the excitation source during this time.

It should be noted that, while the use of signal integration can result in substantially improved detection limits for PAH compounds by measurement at a known wavelength corresponding to a peak in the luminescence emission spectrum, this improvement obviously cannot be achieved in qualitative identification studies at low concentrations, for which the luminescence emission spectrum must be scanned over a wide wavelength range.

Photon counting

In the technique of photon counting the current pulses that result at the anode of the photomultiplier due to photon impact at the photocathode are counted directly. When sufficient resolution is available electronically this technique provides a direct digital measurement of the intensity of the radiation incident upon the photomultiplier. Advantages established for the technique include the ability to provide detection at low light levels, improvement in signal to noise ratios, the decrease of effective dark current, accurate signal integration, improved precision of analytical results and direct digital output of data.[23] A number of workers have described the advantages of the application of the photon-counting technique to spectrofluorimetry and have demonstrated that enhanced detection limits can be obtained for many compounds.[24–26] In our work we have examined the use of a simple single-channel photon-counting detection system for registration of the quasi-linear luminescence of 3,4-benzopyrene and 3,4,9,10-dibenzopyrene at 77 K.

Fig. 3(*a*) shows the variation with the voltage applied to the photomultiplier of the quasi-linear emission count and background count (over 5 s) for a 1×10^{-9} M solution of 3,4-benzopyrene in octane. The signal was recorded at the 403.0-nm peak for 3,4-benzopyrene and the background signal was recorded at 400.0 nm. The influence, at this low concentration, of the relatively high level of background radiation due to scattered source radiation with the "frontal" illumination of the polycrystalline octane matrix is clearly visible. Fig. 3(*b*) shows the variation of the observed signal to noise ratio with the voltage applied to the photomultiplier, the signal to noise ratio having been calculated from the expression proposed by Franklin, Horlick and Malmstadt[23] for situations in which the background count rate is high.

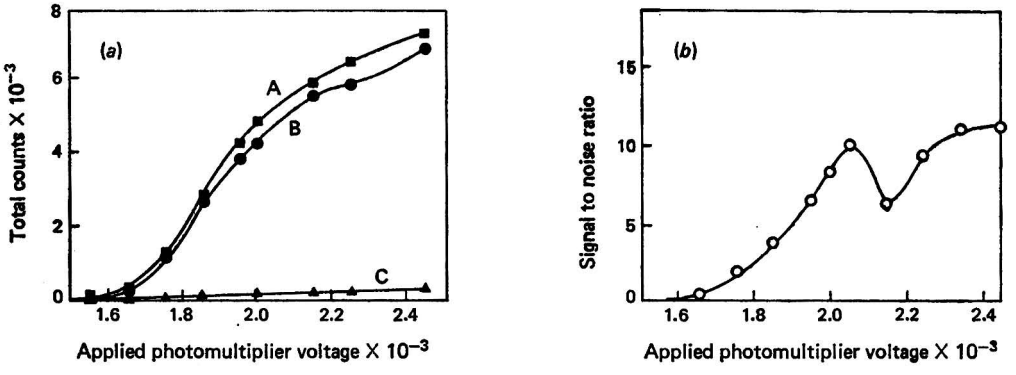

Fig. 3. (*a*), Variation of signal count with photomultiplier voltage for 3,4-benzopyrene: A, total signal count at 403.00 nm in 5 s; B, background count at 400.00 nm in 5 s; and C, dark-current count in 5 s. (*b*), Variation of signal to noise ratio with applied voltage at photomultiplier using photon counting for 3,4-benzopyrene luminescence at 403.00 nm.

Thus, if the signal to noise ratio is given by Z, then

$$Z = \frac{(R_S T)^{\frac{1}{2}}}{(2R_B/R_S)^{\frac{1}{2}}} = \frac{R_S T^{\frac{1}{2}}}{(2R_B)^{\frac{1}{2}}} \quad \ldots \quad \ldots \quad \ldots \quad \ldots \quad (1)$$

where R_S is the signal count rate, R_B is the background count rate and T is the total counting time. The anomolous decrease in signal to noise ratio observed at 2 200 V is an anomalous characteristic of our particular experimental assembly and the photomultiplier tube employed; no explanation for this effect can be offered. Further experiments were conducted with an applied voltage to the photomultiplier of 2 000 V.

Fig. 4 shows the luminescence growth curves obtained for 3,4-benzopyrene, utilising both photon-counting and analogue signal registration: the counting time was 5 s. Both photon-counting and analogue readings correspond to the net difference between the signals obtained at the luminescence maximum (403.0 nm) and for the background at 400.0 nm. A similar range of linearity is observed with each system, *i.e.*, in the concentration range 10^{-7}–10^{-9} M. The deviation from linearity at concentrations above 10^{-7} M can be attributed to an "inner-filter" effect caused by self-absorption of luminescence radiation. Similar experiments were carried out with 3,4,9,10-dibenzopyrene and it was again observed that the ranges of linearity in the luminescence growth curves obtained for this compound at 77 K by photon-counting and analogue measurement techniques were similar.

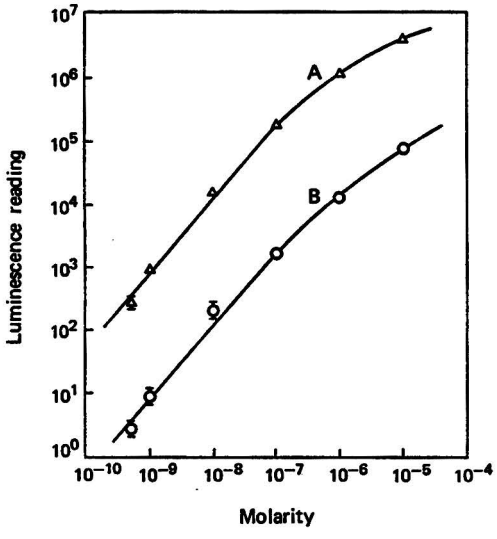

Fig. 4. Comparison of analytical growth curves for 3,4-benzopyrene obtained with photon-counting and analogue signal registration: A, photon counting (count obtained for 5 s count time); and B, analogue read-out (mV).

Although the growth curves of Fig. 4 might suggest that an improvement in detection limit is obtainable by using photon counting, the determination of the relative standard deviation for replicate measurements of low concentrations indicates that the limit of detection for the compounds investigated is similar for photon-counting and analogue techniques. Table V shows the relative standard deviations obtained for solutions of 3,4-benzopyrene and 3,4,9,10-dibenzopyrene at different concentrations. It is evident that, whereas the detection limits for the compounds are similar, at determinable concentrations above the detection limit the over-all precision attainable with the photon-counting technique is superior to that of simple analogue measurements.

The effect of variation in the counting time on the signal to noise ratio obtained for 3,4-benzopyrene was investigated for a 10^{-9} M solution of the compound in octane (Table VI). As shown in Table V, the signal to noise ratio obtained is approximately proportional to $T^{\frac{1}{2}}$, where T is the counting time, as would be expected for situations in which the background count rate is high (equation 1). The counting times required in order to obtain a substantial improvement in detectability, however, are unacceptable for routine practical application. From the results obtained in this study of the use of photon counting for detection of quasi-

TABLE V

PRECISION OF PHOTON COUNTING AND ANALOGUE SIGNAL REGISTRATION FOR LUMINESCENCE OF PAH COMPOUNDS AT 77 K

Compound	Concentration/M	Relative standard deviation, %	
		Photon counting	Analogue
3,4-Benzopyrene	5×10^{-10}	46	32
	1×10^{-9}	2.0	25
	1×10^{-8}	1.3	5.3
	1×10^{-7}	0.5	3.9
	1×10^{-6}	0.2	1.6
	1×10^{-5}	0.7	1.4
3,4,9,10-Dibenzopyrene	1×10^{-9}	44	33
	1×10^{-8}	5.0	6.2
	1×10^{-7}	0.6	4.4
	1×10^{-6}	0.3	4.0
	1×10^{-5}	0.6	2.2
	1×10^{-4}	0.9	1.1

linear luminescence from PAH compounds we must conclude that the fundamental advantages are difficult to exploit. This difficulty undoubtedly results from the presence of high background light levels due to scattered source radiation with the copper cryostat sample cell and optical arrangement employed.

Signal averaging

In order to permit rapid spectral-data acquisition over chosen small wavelength ranges in the region of the principal quasi-linear luminescence emission maxima for PAH compounds, and to obtain enhanced sensitivity of detection, a simple repetitive optical scanning device was constructed for use in conjunction with a multi-channel analyser. The repetitive optical scanning was achieved in a manner similar to that described by Snelleman *et al.*,[17] using an oscillating refractor plate mounted within the monochromator behind the entrance slit. This device then causes repetitive displacement of the monochromator and allows rapid scanning of small wavelength ranges. The lateral displacement of the light beam, which governs the wavelength range that can be scanned, is given, approximately, by the relationship

$$\Delta_1 = t\,\alpha(n-1)/n \qquad (2)$$

where t is the thickness of the plate, α is the angle of incidence (radians, in air) and n is the refractive index of the refractor-plate material. In addition, the light beam is displaced longitudinally owing to change in the optical path length so that the image is defocused at the exit slit.[17] This displacement approximates to

$$\Delta_2 = t\,(n-1)/n \qquad (3)$$

The longitudinal displacement can be corrected for either by using a refractor plate whose surface has been ground so as to produce a convex curvature of long focal length or by corresponding displacement of the exit slit. In order to scan a relatively large wavelength range a Perspex refractor plate ($n_D^{20} = 1.49$) of 25 mm thickness was used. With the oscillator drive circuit used the maximum amplitude of oscillation obtainable for this plate was $\pm 13°$, which produced a limit of 3 nm on the wavelength range scanned.

The output from the photomultiplier was led to the pre-amplifier and then to the 1 000-

TABLE VI

VARIATION IN SIGNAL TO NOISE RATIO WITH COUNTING TIME FOR 3,4-BENZOPYRENE

Counting time/s	Signal to noise ratio
5	10
10	15
15	19
30	26
60	36

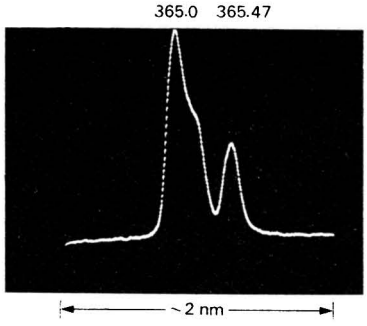

Fig. 5. Calibration spectrum from mercury-vapour lamp obtained for repetitive optical scanning with signal averaging.

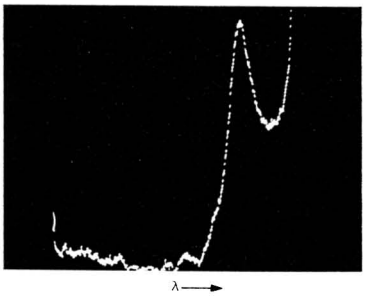

Fig. 6. Quasi-linear luminescence emission at 403.0 nm of 3,4-benzopyrene (5×10^{-9} M) in octane obtained by repetitive optical scanning with time averaging.

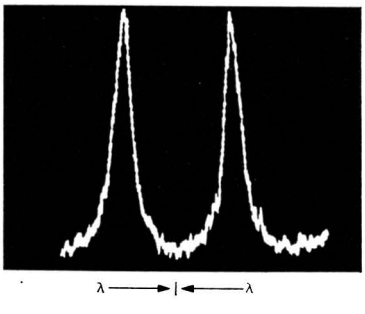

Fig. 7. Quasi-linear luminescence emission of coronene at 445.05 nm obtained by repetitive optical scanning with time averaging (forward and return scans shown).

[*To face p.* 377

channel signal-averaging system. The output from the signal averager was displayed on an oscilloscope or printed out on to a chart recorder. The wavelength calibration and scan range were determined by using the emission of the mercury triplet from a low-pressure mercury-vapour discharge lamp; as shown in Fig. 5 the attainable scan range is between 2 and 3 nm, depending upon the power applied to the oscillator. With repetitive presentation of spectral data to the signal averager the signal increases linearly in the register, depending on the number of sweeps, N, whereas random fluctuations (noise) will accumulate only as the square root of the number of sweeps, $N^{\frac{1}{2}}$. Consequently the signal to noise ratio obtained increases as $N^{\frac{1}{2}}$.

An evaluation has been made of the application of repetitive scanning with signal accumulation by time averaging to the detection of the quasi-linear luminescence of low concentrations of 3,4-benzopyrene in octane and coronene in hexane at 77 K. Typical spectra obtained for their luminescence emission at 403.00 and 445.05 nm are shown in Figs. 6 and 7. Early experiments with this technique indicate that it is likely to prove most valuable in low-temperature luminescence spectroscopy. The technique allows the high resolution of the spectrometer to be utilised rapidly to study the effect of such parameters as cooling rate and solvent type on the half-width and intensity of quasi-linear emission bands and also enables study of the effect of solute concentration and the presence of other PAH compounds on these features of the luminescence emission. In addition, and most importantly from the viewpoint of practical application to the determination of PAH compounds, the technique of repetitive optical scanning used in conjunction with signal averaging permits improved analytical sensitivity to be obtained.

Conclusions

The instrument system assembled permits the quasi-linear luminescence of PAH compounds at 77 K to be used for their detection and determination with high sensitivity. For practical analytical work the use of a xenon arc or mercury-vapour lamp source with the copper cryostat sample cell and d.c. integration of the luminescence signals provides a simple and reliable system. The use of the photon-counting technique for registration of quasi-linear luminescence signals with our instrument assembly does not result in a substantial gain in sensitivity, probably because of the high background light levels encountered with front-surface illumination using our sample cell.

Repetitive optical scanning shows considerable promise for both qualitative and quantitative analytical work using quasi-linear luminescence spectrometry. The use of this technique with signal averaging permits operation of the spectrometer at the low light levels obtained when the maximum spectral resolution is required at narrow slit-widths for fundamental studies or when only very low concentrations of the analyte are present. Pilot studies with a simple helium - cadmium laser indicate that a significant gain in sensitivity and spectral selectivity would be obtained by the use of a tunable laser source for excitation of quasi-linear luminescence of PAH compounds.

We thank the Science Research Council for an equipment grant for construction of the luminescence spectrometer. One of us (C.G. de L.) thanks the University of Brasilia for study leave and UNESCO for the grant of a Fellowship.

References

1. Dikun, P. P., *Vop. Onkol.*, 1961, **7**, 42.
2. Personov, R. I., *J. Analyt. Chem. USSR*, 1962, **17**, 503.
3. Varshavskii, I. L., Shabad, L. M., Khesina, A. Ya., Khitrovo, S. S., Chalabov, V. G., and Pakhol'nik, A. I., *J. Appl. Spectrosc. USSR*, 1965, **2**, 68.
4. Eichhoff, H. J., and Köhler, N., *Z. Analyt. Chem.*, 1963, **197**, 272.
5. Fedoseeva, G. E., and Khesina, A. Ya., *J. Appl. Spectrosc. USSR*, 1968, **9**, 838.
6. Personov, R. I., and Teplitskaya, T. A., *J. Analyt. Chem. USSR*, 1965, **20**, 1176.
7. Jäger, J., and Lugrová, O., *Chemické Zvesti*, 1965, **19**, 774.
8. Dikun, P. P., Krasnitskaya, N. D., Gorelova, N. D., and Kalinina, I. A., *J. Appl. Spectrosc. USSR*, 1968, **8**, 254.
9. Florovskaya, V. N., Teplitskaya, T. A., and Personov, R. I., *Geochem. Int.*, 1966, **3**, 419.
10. Kirkbright, G. F., and de Lima, C. G., *Analyst*, 1974, **99**, 338.
11. Parker, C. A., "Photoluminescence of Solutions," Elsevier, Amsterdam, 1968.
12. Svishchyov, G. V., *Optics Spectrosc., N.Y.*, 1965, **18**, 350.

13. Weast, R. C., *Editor*, "The Handbook of Chemistry and Physics," Forty-eighth Edition, The Chemical Rubber Publishing Co., Cleveland, Ohio, 1967–68.
14. Sharp, B. L., PhD Thesis, University of London, 1972.
15. McWilliam, I. G., *J. Scient. Instrum.*, 1959, **36**, 51.
16. Roldan, R., *Rev. Scient. Instrum.*, 1969, **40**, 1 388.
17. Snelleman, W., Rains, T., Yee, K., Cook, H., and Meins, O., *Analyt. Chem.*, 1970, **42**, 394.
18. Farooq, R., and Kirkbright, G. F., to be published.
19. Personov, R. I., Al'shit, E. I., Bikovskaya, L. A., *Optics Communs.*, 1972, **6**, 169.
20. Hollifield, H. C., and Winefordner, J. D., *Analyt. Chem.*, 1968, **40**, 1759.
21. Kirkbright, G. F., Mayne, P. J., and West, T. S., *Analytica Chim. Acta*, 1971, **54**, 353.
22. Dokunikhin, N. S., Kizel, V. A., Sapozhnikov, M. N., and Solodar, S. L., *Optics Spectrosc., N.Y.*, 1968, **25**, 42.
23. Franklin, M. L., Horlick, E., and Malmstadt, H. V., *Analyt. Chem.*, 1969, **41**, 2.
24. Aoshima, R., Iriyama, K., and Asai, H., *Appl. Opt.*, 1973, **12**, 2 748.
25. Curry, R. E., Pardue, H. L., Mieling, G. E., and Santini, R. E., *Clin. Chem.*, 1973, **19**, 1259.
26. Tuan, V. D., and Wild, U. P., *Appl. Opt.*, 1973, **12**, 1286.

NOTE—Reference 10 is to Part I of this series.

Received *November 28th*, 1975
Accepted *January 6th*, 1976

Determination of Dicumenyl Peroxide by Gas Chromatography

P. Hudec, B. Novotná and J. Petrůj

Research Institute of Macromolecular Chemistry, Tkalcovská 2, Brno, Czechoslovakia

A method is described for the determination of small amounts of dicumenyl peroxide in liquid samples or powdered materials. Dicumenyl peroxide is isolated from the latter by extraction.

Dicumenyl peroxide is one of the additives that are widely used in the industrial processing of polymers. Continuous checking of its concentration during processing is desirable because its residues could considerably affect the product.

For the determination of dicumenyl peroxide the method of reduction in a mixture of acetic acid and alkali-metal iodide, followed by quantitative determination of the liberated iodine,[1] is frequently used. This method, combined with thin-layer chromatography, has also been successfully applied to the determination of dicumenyl peroxide in mixtures with other organic peroxides.[2–4]

The determination of low relative molecular mass peroxides and hydroperoxides by use of gas chromatography has been described in the literature.[5,6] The usefulness of this method is restricted by the volatility, as well as the stability, of the substance under analysis, 2,5-dimethyl-2,5-bis(*tert*-butylperoxy)hexane being the limiting case reported.

The thermal stability of dicumenyl peroxide is relatively high,[7] and we therefore attempted to find suitable conditions for its gas-chromatographic determination.[8]

Experimental

Reagents and Materials

Dicumenyl peroxide, more than 98% pure. Obtained from Noury van der Lande.
Octadecane, more than 99% pure.
Cumen-7-ol, boiling point 102 °C *at* 2.6 kPa. Prepared in this Institute.
Phenol, acetone, toluene, acetophenone and methanol. Analytical-reagent grade.
Chromosorb W DMCS (60–80 *mesh*).
Fluorosilicone oil QF 1.

The material to be analysed for determination of the dicumenyl peroxide content was in the form of clays, modified by the reaction with butyl 1,3-diacrylate in the presence of dicumenyl peroxide as initiator. The determination of unreacted dicumenyl peroxide served as the test for the efficiency of modification.

Apparatus and Conditions

A Perkin-Elmer F11 gas chromatograph, fitted with a flame-ionisation detector, was used. A 3 ft × $\frac{1}{4}$ in o.d. glass column was packed with 3% of QF 1 on 60–80-mesh Chromosorb W DMCS and argon was used as the carrier gas at a flow-rate of 40 ml min^{-1}. The optimum column temperature was found to be 110 °C, whereas the injection port was not heated. The sample (2 μl) was introduced by use of a 10-μl syringe that was fitted with a 4-in needle. A Hitachi Perkin-Elmer 2.5-mV recorder, Model 159, was attached to the instrument.

Preparation of Samples

A sample of modified clay (1 g) was weighed into a 25-ml beaker and mixed with toluene (10 ml). After stirring it for 5 min the slurry was filtered through a sintered-glass filter stick and the filtrate collected; another portion of toluene was then added to the clay and the procedure was repeated. The extracts were combined, 5 ml of octadecane solution in toluene (internal standard) were added and, by making the total volume up to 50 ml, the solution was prepared for gas-chromatographic analysis.

Results and Discussion

Optimum Gas-chromatographic Conditions

Fluorosilicone oil QF 1 was selected for its low polarity and short retention time. Initially, when the analysis was carried out at column temperatures above 140 °C, the decomposition of peroxide was observed even when the injection port was not heated. In view of the fact that the kinetics and mechanism of dicumenyl peroxide decomposition have been described elsewhere,[9] we employed the published rate constants in order to find the optimum column conditions for the gas-chromatographic determination. The chosen column temperature of 110 °C was a compromise value at which the decomposition could still be neglected (it was less than 1%) although the retention time was acceptable.

Under the column conditions described above the peaks due to the solvent, octadecane and dicumenyl peroxide are well resolved. The retention times are given in Table I.

TABLE I

RELATIVE RETENTION TIMES OF CHROMATOGRAPHED MATERIALS

Compound	Relative retention time
Toluene	0·04
Octadecane	1·00 (7·2 min)
Dicumenyl peroxide	2·13

Calibration

On plotting a graph of the mass ratio of the dicumenyl peroxide and octadecane peaks *versus* the ratio of their concentrations, a straight line passing through the origin was obtained. For equal concentrations of dicumenyl peroxide and octadecane the mass ratio of the peaks was found to be 0.788. The mean deviation of experimental points from the calibrated plot was $\pm 3.2\%$ over the entire concentration range.

The main products of the decomposition of dicumenyl peroxide are methane, acetone, acetophenone, phenol and cumen-7-ol; their relative concentrations are governed by the reaction mechanism.[9] With the column used in this work all of these compounds are eluted before octadecane so that the determination is not affected.

Quantitative Study of the Extraction of Dicumenyl Peroxide from Modified Clays

A sample (1 g) of surface-modified clay was repeatedly extracted with toluene as described above. The toluene extracts were analysed in order to determine the dicumenyl peroxide content. Three extractions were found to recover 99% of the peroxide from the clay.

Example

Modified clay with an initial dicumenyl peroxide content of 0.10% was used for the experiments. Following the modification process the samples were analysed to determine the residual dicumenyl peroxide concentration by using the method described. The mean value of the results of four analyses was $0.089 \pm 0.006\%$ *m/m*. Thus, in the course of modification, only about 10% of the dicumenyl peroxide that was present initially participated in the reaction.

The authors are grateful to Ing. K. Otto of this Institute for fruitful discussions.

References

1. Mair, R. D., and Graupner, A. J., *Analyt. Chem.*, 1964, **36**, 194.
2. Brammer, J. A., Frost, S., and Reid, V. W., *Analyst*, 1967, **92**, 91.
3. Buzlanova, M. M., Stepanovskaja, V. P., and Antonovskij, V. L., *J. Analyt. Chem. USSR*, 1966, **21**, 1324.
4. Hayano, S., Ota, T., and Fukushima, Y., *Bunseki Kagaku*, 1966, **15**, 365; *Chem. Abstr.*, 1967, **67**, 39941r.
5. Bukata, S. W., Zabrocki, L. L., and McLaughlin, M. F., *Analyt. Chem.*, 1963, **35**, 885.
6. Abraham, M. H., Davies, A. G., Llewellyn, D. R., and Thain, E. M., *Analytica Chim. Acta*, 1957, **17**, 499.
7. Swern, D., "Organic Peroxides," Wiley-Interscience, New York, 1971.
8. Hudec, P., and Petrůj, J., *Chemický Prům.*, 1975, **25/50**, 95.
9. Markert, H., and Wiedermann, R., *Siemens Forsch. -u. Entwicklungs-ber.*, 1973, **2**, 85.

Received *October 21st*, 1974
Amended *October 31st*, 1975
Accepted *November 24th*, 1975

Analyst, May, 1976, Vol. 101, pp. 381-385

The Determination of Uric Acid in Animal Feeding Stuffs Using High-performance Liquid Chromatography

G. B. Cox, C. R. Loscombe and J. A. Upfield

Department of Industry, Laboratory of the Government Chemist, Cornwall House, Stamford Street, London, SE1 9NQ

A method is given for the extraction of uric acid from animal feeding stuffs that contain dried poultry waste. The extract obtained is analysed by high-performance liquid chromatography using a strong anion-exchange bonded silica column and an ultraviolet method of detection. Results of a series of recovery checks together with those for the analysis of actual samples are given. The application of the method to ammonium urate additions is investigated.

Uric acid is the principal end product of nitrogen metabolism in birds. It can be utilised by rumen bacteria and hence by ruminants.[1] Accordingly, dried poultry waste may be added to cattle feeds in order to provide this additional source of non-protein nitrogen.[2,3] Legislation is now under consideration in the United Kingdom for the control of such additions of dried poultry waste. One method of monitoring the dried poultry waste is to determine the amount of uric acid present in the feed. In consequence, the Government Chemist was requested, by the Ministry of Agriculture, Fisheries and Foods, to produce a suitable method for determining uric acid in animal feeding stuffs at levels from 0.2 to 1.0%. In connection with chicken nutrition studies, Terpstra and de Hart[4] have recently described a method for determining uric acid in poultry waste, and a procedure for monitoring dried poultry waste and feeding stuffs containing it, based on modifications to this method, has been developed by the Fertilisers and Feeding Stuffs sub-division of this laboratory.

Several papers have been published on the determination of uric acid by column chromatography,[5–9] which suggest that a method based on high-performance liquid chromatography could be developed that would provide an alternative to the modified Terpstra and de Hart procedure. The determination of uric acid in serum by high-performance liquid chromatography with electrochemical detection has already been described.[10–12] An alternative chromatographic procedure for its determination in animal feeding stuffs is presented here.

Experimental

Apparatus

Liquid chromatograph. A liquid chromatograph based on a piston-diaphragm pump (Whitey, Model LP10) with variable air leak was used.[13] The column was a 150 mm × 4.6 mm i.d. stainless-steel tube packed with the anion exchanger[14] and was operated at a temperature of 45 °C. Injection valve and end fittings were as previously described.[14,15] Samples (2 μl) were introduced with a 10-μl syringe using the stop - flow technique. The mobile phase used was a solution of sodium acetate (0.1 M) plus acetic acid (0.04 M) in de-gassed de-ionised water with a flow-rate of 1 ml min^{-1}. A variable wavelength ultraviolet monitor (Cecil Instruments, Model CE 212) set at 290 nm was used as a detetector.

Reagents

All reagents are of analytical-reagent grade.
Acetic acid, glacial.
Boric acid.
Lithium carbonate.
Sodium acetate, trihydrate.
Uric acid.
Buffer solution. Dissolve 2.5 g of lithium carbonate together with 5.0 g of boric acid in 1000 ml of water.

High-performance liquid chromatography mobile phase. Dissolve 13.6 g of sodium acetate together with 2.3 ml of acetic acid in 1000 ml of de-gassed water.

Uric acid standard solution. Dissolve 1 mg of uric acid in 50 ml of cold buffer solution. Prepare a fresh standard for each analysis.

In addition, the following reagents were used for the recovery checks with ammonium urate.

Ammonium urate. This material was obtained from Pfaltz and Bauer and on analysis was found to consist of the acid salt, $NH_4(C_5H_3N_4O_3)$. The purity was not specified.

Hydrochloric acid, 3.0 g l^{-1}.

Ammonium urate standard solution. Dissolve 2.5 mg of ammonium urate in 50 ml of buffer solution plus 2 ml of hydrochloric acid solution by shaking the mixture for 10 min, and then dilute to 250 ml with buffer solution.

Procedure

Weigh accurately approximately 1 g of feeding stuff, transfer it into a 250-ml calibrated flask and add approximately 50 ml of buffer solution. Heat the mixture on a steam-bath for 15 min, with occasional shaking. Cool it rapidly and make it up to volume with buffer solution. Shake the mixture well and allow it to stand for a few minutes, then decant the solution under vacuum through a pre-filter and a 1.2-μm pore size cellulose filter (*e.g.*, the Millipore system). Determine the uric acid in the filtrate, using a suitable liquid-chromatographic system (see above), by comparison of the average peak height of a minimum of three injections with the average peak height of the standard.

Results and Discussion

The chemical group most commonly employed in strong anion-exchange column packings is the quaternary ammonium ion. A synthetic route towards such a group bonded to silica gel has been developed in this laboratory,[14] the resulting silica containing the functional group $C_3H_6N(CH_2C_6H_5)(CH_3)_2Cl$. This group has been bonded to irregular-shaped porous silica particles (LiChrosorb SI 60, 5-μm average particle size; obtainable from BDH Chemicals Ltd.) and spherical porous silica particles (Spherisorb S5W SAX, 5-μm average particle size; Phase Separations Ltd., Clwyd); both packings were used successfully in this analysis. A chromatogram of a uric acid standard on the bonded spherical silica is shown in Fig. 1.

Uric acid is readily detected by conventional high-performance liquid chromatographic ultraviolet monitors (log $E = 4.04$ at 290 nm). A calibration graph of peak height against concentration of uric acid in the range 10–140 mg l^{-1} (2-μl injections) is rectilinear and passes through the origin. The use of electrochemical detection methods for the chromatographic determination of uric acid in serum has been advocated by several workers because of their high degree of selectivity.[10–12] In the analysis of feeding stuffs, however, this very high selectivity is not required as the extraction process and subsequent chromatography, which are both of a selective nature, give rise to negligible blanks (see Table I and Fig. 2). An examination of three other purines (xanthine, hypoxanthine and purine) showed that only xanthine was eluted under the conditions used for uric acid. Xanthine has a shorter retention time (1.75 min compared with 4 min for uric acid) and its ultraviolet light absorption is considerably lower.

The advantage of the proposed method over other chromatographic procedures is the short time required for analysis. The two gas-chromatographic methods reported in the literature require an additional step involving the formation of either the trimethylsilyl[16] or the tetraethyl[17] derivative. Unfortunately, neither publication presents the results that are required for a quantitative comparison with the high-performance liquid chromatographic procedure. Extraction with cold lithium carbonate solutions (5.0 g l^{-1}), one of the methods recommended by Tinsley and Nowakowski,[18] gave low recoveries. The alternative procedure involving extraction for 15 min with hot buffered lithium carbonate solution (2.5 g of lithium carbonate plus 5.0 g of boric acid in 1 000 ml of water) gave a significant improvement. The duration of this hot extraction is important; extraction for 30 min gave low results, typically 83–90% recoveries, which are thought to be due to decomposition of the uric acid during heating, a mechanism that was suggested by Tinsley and Nowakowski in order to explain why decomposition of the standards occurred. Filtration of the extract through a conventional type 3 porosity glass sinter gave cloudy solutions, the uric acid contents of which were considerably

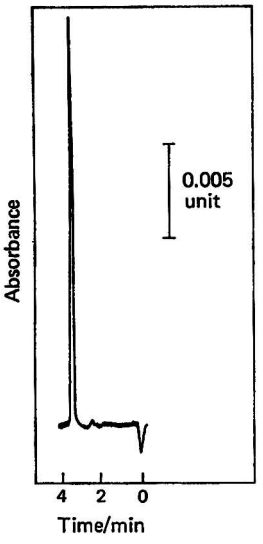

Fig. 1. Uric acid standard. Column, Spherisorb S5W SAX. Sample, 2-μl injection of 16.8 mg l^{-1} solution.

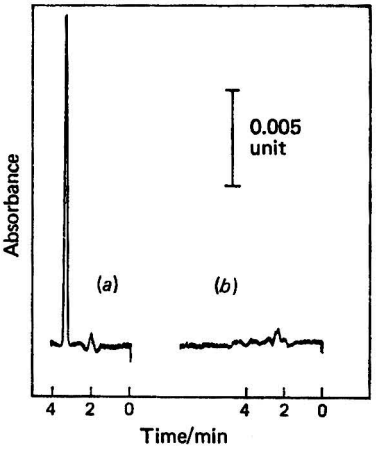

Fig. 2. Uric acid in samples. Column, Spherisorb S5W SAX. (*a*) Feed + dried poultry waste; and (*b*) feed.

TABLE I

URIC ACID ADDITIONS TO FEEDING STUFFS

Feed	Uric acid added/ mg g^{-1}	Uric acid recovered: Total/ mg	Uric acid recovered: Corrected for blank/mg	Recovery, %
Dairy pellets (29% of protein)	—	0.13	—	—
	5.88	5.68	5.55	94.4
	5.68	5.81	5.68	100.0
	4.26	4.30	4.17	97.9
	4.08	4.24	4.11	100.7
	2.26	2.28	2.15	95.4
	2.12	2.20	2.07	97.6
			Mean	97.7
Milk nuts (16% of protein)	—	0.07	—	—
	5.78	5.75	5.68	98.3
	5.38	5.28	5.21	96.8
	4.01	3.89	3.82	95.3
	4.06	3.97	3.90	96.1
	2.25	2.26	2.19	97.3
	2.07	2.14	2.07	100.0
			Mean	97.3
Dairy standard meal (14% of protein)	—	<0.005	—	—
	6.09	5.82	—	95.6
	5.95	5.81	—	97.6
	4.42	4.45	—	100.7
	3.96	3.91	—	98.7
	2.18	2.21	—	101.4
	1.87	1.89	—	101.1
			Mean	99.2
			Over-all mean	98.1
			Coefficient of variation, %	2.2

reduced on standing. However, this problem was overcome by using a 1.2-μm cellulose filter, which gave clear solutions.

The extraction procedure used by Terpstra and de Hart is based on the use of an aqueous ethanolic formaldehyde solution,[4] dissolution of the uric acid being achieved via the formation of an adduct with formaldehyde. This extraction solvent was found to be unsuitable for use with the high-performance liquid chromatographic method because of the incomplete formation of the adduct. Thus each sample injection gave rise to two distinct peaks on the chromatogram, corresponding to the free acid and the adduct.

Uric acid was added to three different types of feed (2–6 mg of uric acid with approximately 1 g of feed), which were then treated as described above. Percentage recoveries (shown in Table I) range from 94.4 to 101.4% with a mean of 98.1% and a coefficient of variation of 2.2%.

Several authors[18–21] report that a proportion of the uric acid in dried poultry waste is present in the form of ammonium urate. Uric acid and ammonium urate have the same biochemical effect[22] as both are broken down to allantoin[23] by uricase. A series of recovery checks was performed on a feed to which ammonium urate had been added. The results obtained are given in Table II and are comparable with those obtained for uric acid. Initially, difficulty was experienced in preparing stable ammonium urate standards. The earlier work of Tinsley and Nowakowski on uric acid suggested that this difficulty could have been caused by a pH effect, and it was found that a slight lowering of the pH to a value comparable with that of the feed extract, by the addition of dilute hydrochloric acid, overcame the problem.

TABLE II

AMMONIUM URATE ADDITIONS TO FEEDING STUFFS

Ammonium urate added/mg	Recovery, %
5.37	99.3
4.23	100.3
3.64	95.0
3.24	96.2
2.89	98.8
2.78	100.1
2.75	95.0
2.12	101.1
Mean	98.2
Coefficient of variation, %	2.5

Three samples were analysed for uric acid content: a feed, a feed plus dried poultry waste and the dried poultry waste itself, and the results of these analyses are given in Table III; Fig. 2 shows the typical chromatograms obtained; the negligible amount of uric acid found in the feed alone is well illustrated.

TABLE III

DETERMINATION OF URIC ACID IN ACTUAL SAMPLES

Sample	Uric acid, %	Average, %
Feed	<0.005*	<0.005
Dried poultry waste	2.75, 2.58, 2.68	2.67
Feed + dried poultry waste ..	0.32, 0.31, 0.28, 0.27, 0.31,† 0.29†	0.30

* Three determinations.

† Determination using Partisil-10 SAX column. Other figures using Spherisorb S5W SAX.

As an alternative to the strong anion exchanger bonded to LiChrosorb and Spherisorb silicas, as prepared in this laboratory, a commercial column was used, which was a 250 mm × 4.6 mm i.d. Partisil-10 SAX column (Reeve Angel Scientific Ltd.) based on silica with an average particle size of 10 μm. The sample of feed plus dried poultry waste was analysed in duplicate on this column and was found to give results that agree with those obtained by using the other columns (Table III). A comparison of the efficiencies of the three columns is given in

Table IV. One reason for the improved efficiency of the LiChrosorb and Spherisorb columns is that their particle size is smaller. Although the use of the Partisil column results in a decrease in separating ability it illustrates the feasibility of carrying out this analysis with a commercial column.

TABLE IV

COMPARISON OF EFFICIENCIES OF THREE COLUMNS

Column packing	Length/mm	Number of theoretical plates	HETP/mm
LiChrosorb SI 60 SAX (5 μm)	150	5 500	2.73×10^{-2}
Spherisorb S5W SAX (5 μm)	150	4 100	3.66×10^{-2}
Partisil-10 SAX (10 μm)	250	2 300	10.85×10^{-2}

Conclusion

Hot extraction with buffered lithium carbonate solution followed by high-performance liquid chromatography on a strong anion exchanger provides a simple and accurate method for the determination of uric acid in animal feeding stuffs containing dried poultry waste.

We thank Dr. K. Field and Mr. J. Harris of the Fertilisers and Feeding Stuffs subdivision of this laboratory for valuable discussion and for supplying samples of feed and dried poultry waste, originally obtained from the Trade and ADAS (Agricultural Development and Advisory Service). We also thank the Government Chemist for permission to publish this paper.

References

1. McDonald, P., Edwards, R. A., and Greenhalgh, J. F. D., "Animal Nutrition," Second Edition, Oliver and Boyd, Edinburgh, 1974, p. 145.
2. Tillman, A. D., "Proceedings of the Symposium on Effects of Processing on the Nutritional Value of Feeds 1972," U.S. Government Printing Office, Washington, 1973, p. 180.
3. Fontenot, J. P., and Webb, K. E., jun., *Fed. Proc. Fed. Am. Socs Exp. Biol.*, 1974, **33**, 1936.
4. Terpstra, K., and de Hart, N., *Z. Tierphysiol. Tierernähr. Futtermittelk.*, 1974, **32**, 306.
5. Wolford, J. C., Dean, J. A., and Goldstein, G., *J. Chromat.*, 1971, **62**, 148.
6. Farina, E., and Mule, A., *Boll. Soc. Ital. Biol. Sper.*, 1962, **38**, 1554.
7. Kelemen, S. P., and Degens, E. T., *Nature, Lond.*, 1966, **211**, 857.
8. Jagenburg, R., Rodjer, S., and Sansioe, G., *Scand. J. Clin. Lab. Invest.*, 1968, **22**, 54.
9. Simkin, P. A., *J. Chromat.*, 1970, **57**, 103.
10. Varadi, M., Feher, Z., and Pungor, E., *J. Chromat.*, 1974, **90**, 259.
11. Kissinger, P. T., Felice, L. J., Riggin, R. M., Pachla, L. A., and Wenke, D. C., *Clin. Chem.*, 1974, **20**, 992.
12. Pachla, L. A., and Kissinger, P. T., *Clinica Chim. Acta*, 1975, **59**, 309.
13. Cox, G. B., *J. Chromat.*, 1973, **83**, 471.
14. Cox, G. B., Loscombe, C. R., Slucutt, M. J., Sugden, K., and Upfield, J. A., *J. Chromat.*, 1976, **117**, 269.
15. Cox, G. B., *J. Chromat.*, 1976, **116**, 244.
16. Sasaki, Y., and Hashizume, T., *Analyt. Biochem.*, 1966, **16**, 1.
17. Ismail, A. A. A., and Dakir, T. A., *J. Chromat.*, 1975, **110**, 182.
18. Tinsley, J., and Nowakowski, T. Z., *Analyst*, 1957, **82**, 110.
19. Woodman, H. E., *J. Agr. Sci., Camb.*, 1924, **14**, 413.
20. Davidson, J., and Thomas, O. A., *Br. Poult. Sci.*, 1969, **10**, 53.
21. Stewart, D. J., Holmes, W. M., and Fletcher, G. L., *J. Exp. Biol.*, 1969, **50**, 527.
22. Belasco, I. J., *J. Anim. Sci.*, 1954, **13**, 601.
23. Poulson, T. L., and McNabb, F. M. A., *Science, N.Y.*, 1970, **170**, 98.

Received *October 24th*, 1975
Accepted *November 18th*, 1975

Analyst, May, 1976, Vol. 101, pp. 386–390

Determination of Residues of Inorganic Bromide in Grain

Report by The Panel on Fumigant Residues in Grain

OF THE COMMITTEE FOR ANALYTICAL METHODS FOR RESIDUES OF PESTICIDES AND VETERINARY PRODUCTS IN FOODSTUFFS OF THE MINISTRY OF AGRICULTURE, FISHERIES AND FOOD

The background and membership of the Panel were described in its first report.[1]

As the Panel had recommended[1] the use of the method of Heuser and Scudamore[2] for determining residues of volatile fumigants in grain, it was agreed that an extension of the method, again by these workers, to the determination of inorganic bromide[3] should be investigated. In this modification of the original method, bromide ion is allowed to react selectively with ethylene oxide to form 2-bromoethanol, which is separated and determined by gas chromatography using an electron-capture detector.

The Joint Meeting of the FAO Working Party of Experts on Pesticide Residues and the WHO Expert Committee on Pesticide Residues in 1971[4] recommended tolerances for bromide that relate exclusively to bromine as the negative ion and not to bromine present in intact, bromine-containing organic fumigants, for which separate figures were set. However, total-bromide methods that have hitherto been commonly used for determining compliance with inorganic bromide tolerances also include the bromine contained in any organobromine compounds present. Methods involving an initial hydrolysis step with sodium or potassium hydroxide or with ethanolamine, neutron-activation methods and X-ray fluorescence methods all measure the total bromine content of foodstuffs. The Panel therefore examined the method of Heuser and Scudamore[3] for its specificity in addition to assessing the correlation of results obtained in a number of laboratories.

Preparation of Samples

For the first of these collaborative studies, common samples of unfumigated wheat and maize were spiked with inorganic bromide (bromide ion). A measured volume of an aqueous solution of 99.9% pure potassium bromide of known concentration was slowly added to wheat or maize of moisture content approximately 15% contained in a large desiccator, with thorough mixing. The sample was then spread in thin layers on trays and kept at 10 °C under a controlled relative humidity of 70% for 12 d, thus reducing the moisture content of the grains to about the original 15%. At the end of this period, taking into account the final mass of the grain, the bromide-ion contents calculated on the wet masses were 49.5 ± 0.3 mg kg^{-1} for the wheat and 50.4 ± 0.3 mg kg^{-1} for the maize. (The standard deviation was derived from the accuracy of the individual weighings made.)

For the second collaborative study, wheat and maize were fumigated with bromomethane and 1,2-dibromoethane and the resulting residual fumigants allowed to desorb and/or partially decompose. A 4-kg amount of wheat and 4 kg of maize, spread in thin layers on trays, were fumigated in a 1700-l steel fumigation chamber at 25 °C for 24 h (concentration - time products were 500 mg h l^{-1} for bromomethane and 400 mg h l^{-1} for 1,2-dibromoethane). After fumigation, the grains were kept at 10 °C under a controlled relative humidity of 70% for 40 d before the sample was sub-divided and dispatched to the collaborating laboratories. The moisture content of both wheat and maize on dispatch was again about 15%.

First Collaborative Study

In the first collaborative study, wheat and maize were spiked with inorganic bromide using the technique outlined above and analysed by use of the method described in the Appendix. The results for bromide-ion content are given in Table I. In about half of the instances, the final solutions were examined in another laboratory as well as in the laboratory in which the

extraction was made; agreement between these pairs of laboratories was good, giving added confidence in the reproducibility of the gas-chromatographic step.

TABLE I

DETERMINATION OF BROMIDE ION IN WHEAT AND MAIZE SPIKED WITH INORGANIC BROMIDE

Results are given in milligrams of bromine per kilogram of sample.

Laboratory	Wheat	Maize
1	52, 51, 53, 50	50, 50, 50, 51
2	44, 46, 48, 49	44, 44, 46, 43
3	53, 50, 49, 51	51, 46, 50, 61
4	51, 47, 49	44, 47, 44
5	54, 55, 54, 52	55, 56, 53, 53
6	56, 54, 52	50, 50, 52
7	47, 46, 46, 44	45, 40, 44, 40
8	47, 48, 49, 48	48, 44, 45, 45
Mean	50	48
Standard deviation	±3.2	±4.8

Second Collaborative Study

The second collaborative study was carried out on wheat and maize in which ionic bromide had been produced by fumigation under conditions simulating commercial practice, and in which organobromine residues in the form of unchanged 1,2-dibromoethane were also present. (The period of aeration after fumigation would have ensured the removal of all of the bromomethane that had not already reacted with the cereal constituents but allowed part of the adsorbed 1,2-dibromoethane to remain intact.) In addition to the determination of ionic bromide by the method of analysis described in the Appendix, residual 1,2-dibromoethane was determined by the Panel's method for volatile fumigants.[1] Four laboratories also determined 1,2-dibromoethane concurrently with the ionic bromide analyses (see Discussion).

The results obtained for each form of bromine compound present are given in Table II. No values for actual content of bromine compounds can be given for these determinations because of the manner of their introduction.

TABLE II

DETERMINATION OF BROMIDE ION IN WHEAT AND MAIZE CONTAINING RESIDUES OF INORGANIC BROMIDE AND 1,2-DIBROMOETHANE

Results are given in milligrams of inorganic bromide (calculated as Br^-) per kilogram of sample.

	Wheat*			Maize*		
	Br^-	1,2-Dibromoethane		Br^-	1,2-Dibromoethane	
Laboratory						
1	52, 52, 51	22, 22, 22	—	69, 70, 71	13, 11, 11	—
2	39, 46, 44	14, 15, 15†	—	51, 57, 56	8, 9, 8†	—
3	47, 47, 47, 48	23, 24	19, 19, 19, 19	68, 67, 67, 62	12, 11	10, 10, 9, 9
4 (A)‡	62, 61, 58	26, 24, 23	12, 9, 11	71, 70, 73	13, 11, 11	7, 8, 8
(B)	58, 58, 58	—	—	64, 70, 68	—	—
5	52, 53, 49	21, 21, 18	—	70, 68, 68	11, 11, 11	—
6	44, 46, 47	25, 25, 23	7, 8, 8	64, 66, 68	11, 11, 11	4, 4, 4
7	42, 41, 43, 47	23, 22, 23	18, 16, 18, 19	54, 61, 55, 57	10, 11, 9	8, 9, 11, 10
Mean	50			65		
Standard deviation	±6.4			±6.2		

* Columns 2 and 5: determined on whole grains by the Panel method for volatile fumigants in grain[1]; columns 3 and 6: determined on ground grain by the method described in the Appendix.

† Samples held for 4 d before analysis.

‡ Two workers participated from Laboratory 4.

Some laboratories found a peak on gas chromatograms at about twice the retention time of 2-bromoethanol. In order to avoid interference on subsequent chromatograms, if such peaks are noted care should be taken to allow them to clear before making fresh injections.

Discussion

The results given in Table I show that known amounts of bromide added in ionic form to wheat and maize (first study) were determined satisfactorily by the gas-chromatographic method described in the Appendix, the collaborating laboratories obtaining mean rates of recovery ranging from 92 to 109% for wheat and from 84 to 107% for maize. In this study, the standard deviation of individual results from the mean was 10% or less of the mean and was regarded as acceptable. However, it can be seen in Tables I and II that laboratories 2 and 7 consistently obtained significantly lower results for ionic bromide than the other laboratories in both collaborative tests (at 95% confidence limits). Statistical tests on the individual analyses in the first study for skewness and kurtosis showed no significant deviation at 95% confidence limits. Determinations on spiked grain were therefore regarded as satisfactory.

In the second study, although the absolute value for the bromide-ion content of the cereal samples was not known, a comparable spread of results of determinations was obtained by the collaborating laboratories (Table II, columns 1 and 4 of results). Statistical tests on all of the individual analyses for skewness and kurtosis again showed no significant deviation at 95% confidence limits.

The results of determinations of the 1,2-dibromoethane content of cereals examined indicate clearly that the compound was present and show a measure of agreement in the amount reported. The lower and varying results for 1,2-dibromoethane obtained by the method given in the Appendix, as compared with those obtained when the method previously recommended by the Panel was used, can probably be ascribed to loss of the compound through its volatility on grinding. It is probable, therefore, that most of the remaining 1,2-dibromoethane stayed chemically intact during the analysis. In their paper, Heuser and Scudamore[3] provided evidence that bromomethane similarly remained unconverted during analysis for ionic bromide by the gas-chromatographic method.

Conclusion

The method of Heuser and Scudamore[3] has been established by collaborative study to be suitable for the specific determination of residues of bromide ion (inorganic bromide) in wheat and maize in the presence and absence of 1,2-dibromoethane. Evidence provided by these authors[3] encourages the Panel to accept the validity of the method for determining bromide ion in other similar commodities.

APPENDIX

Recommended Method for Determining Residues of Ionic Bromide in Grain

Apparatus

Conical flasks. Capacity 150 ml, with 24/29 ground-glass sockets and glass stoppers.
Graduated cylinders. Capacity 25 and 10 ml, stoppered.
Microlitre syringes. Capacity 1 and 5 μl.
Laboratory mill.
Gas chromatography column. A 2 m × 2.2 mm (recommended) i.d. stainless-steel column, packed with 15% *m/m* polypropylene glycol (LB 550 X, Ucon fluid, maximum recommended temperature 150 °C, obtainable from Perkin-Elmer Corporation) on 60–80-mesh Chromosorb W.
Gas chromatograph. An isothermal model, fitted with a tritium or nickel-63 source electron-capture detector and a glass-lined heated injector, or provision for on-column injection.
Recorder. A 1-mV potentiometric recorder with a 1-s maximum response and 0.5 cm min^{-1} chart speed is suitable.

Reagents

Acetonitrile (free from interfering peaks).
Diisopropyl ether. Laboratory-reagent grade, stabilised.
Ethylene oxide, 4% V/V in diisopropyl ether. Prepared fresh daily.

Caution

1. Pure diisopropyl ether readily forms explosive peroxides on standing. As supplied, it is normally stabilised by the addition of hydroquinone or another reducing agent and should be stored in a dark place. If the reagent is distilled before use, the distillate should not be stored.
2. Ethylene oxide boils at about 11 °C and the vapour is highly flammable, forming explosive mixtures with air. The liquid may polymerise on prolonged standing and should be stored in a spark-proof refrigerator at 0 °C for not longer than 3 months. The preparation of the solution of ethylene oxide in diisopropyl ether should be carried out by an experienced chemist. A minimum requisite volume of liquid ethylene oxide should be poured from the storage bottle into a previously cooled, ungreased burette, which is then stoppered with a rubber bung. After delivery of the required volume under the surface of the diisopropyl ether, any remainder should be discarded in the open air.

Sulphuric acid, 0.6 N.
Ammonium sulphate. Analytical-reagent grade.
Sodium sulphate, granular, anhydrous. Dried for 4 h at 180 °C.
Potassium bromide. Analytical-reagent grade, dried for 1 h at 130 °C.
2-Bromoethanol. Laboratory-reagent grade.

Method

To a 150-ml conical flask containing 10 g of the well mixed and finely ground cereal sample, quickly add a prepared mixture of acetonitrile (40 ml), a 4% *V/V* solution of ethylene oxide in diisopropyl ether (10 ml) and 0.6 N sulphuric acid (10 ml), stopper the flask and allow it to stand for at least 4 h at about 20 °C with occasional swirling. After this period, shake the contents, allow the supernatant liquid to clear and pour about 10 ml of this liquid into a 25-ml graduated cylinder. Add 2 g of ammonium sulphate, stopper the cylinder, shake the contents vigorously and allow the resulting two phases to separate. Pour approximately 5 ml of the upper layer into a 10-ml graduated cylinder, add 1 g of anhydrous sodium sulphate, stopper the cylinder, shake it briefly, then allow it to stand for 30 min with occasional further shaking.

Withdraw 0.5–5-μl aliquots from the supernatant liquor using a suitable microlitre syringe for gas chromatography and record relative peak heights for 2-bromoethanol. Run blank determinations using all of the reagents.

Conditions for Gas Chromatography

Using the column specified at an oven temperature of 120 °C, with nitrogen as carrier gas supplied at a pressure of 1.4 kg cm^{-2} (20 lb in^{-2}) and flow-rate approximately 30 ml min^{-1}, the retention time for 2-bromoethanol is 5–6 min, with 1,2-dibromoethane emerging at 3–4 min.* A column temperature of 120 °C should not be exceeded, and therefore heat transfer from injector or detector must be minimal. During the Panel's investigations, Ucon fluid from one supplier gave excessive bleed-off at 120 °C and it was found necessary to reduce the column temperature to 80 °C and also to increase the flow-rate of nitrogen.

Calibration

Dissolve suitable amounts of dried potassium bromide in solutions containing the same proportions of mixed reagents as were used in the extraction and blank experiments; allow the solutions to stand as before, then proceed through the dehydration procedure on 10-ml volumes of these solutions. Inject into the gas chromatograph aliquots of the final solutions

* This method is not recommended for measurement of more volatile fumigant residues owing to possible losses on grinding. The method recommended by the Panel[1] for volatile residues should be employed for this purpose.

containing 2-bromoethanol so produced, and record relative peak heights, taking the blank value, if any, into account. The injected volume represents an aliquot of a solution having a volume that is five sixths of the original, but containing all of the added bromine.

Plot peak heights for 2-bromoethanol against nanograms of potassium bromide [= nanograms of 2-bromoethanol × (119/125) for 100% conversion] represented in aliquots from a total volume of 50 ml of dried solution.

Solutions of 2-bromoethanol in acetonitrile can be used for secondary calibration after determining their apparent 2-bromoethanol content from the primary potassium bromide calibration. They are unsuitable for primary calibration as commercial 2-bromoethanol has been found to contain up to 2% of 1,2-dibromoethane.

Calculation

Taking 10 g of commodity and 60 ml of mixed reagents, yielding the equivalent of 50 ml of dehydrated solvent:

$$\mathrm{Br^-\ (mg\ kg^{-1})} = \frac{\text{ng KBr represented in volume injected}}{\text{volume injected } (\mu\text{l})} \times 5 \times \frac{79.9}{119.0}$$

or

$$= \frac{\text{apparent ng 2-bromoethanol in volume injected}}{\text{volume injected } (\mu\text{l})} \times 5 \times \frac{79.9}{125.0}$$

$$\text{1,2-Dibromoethane (mg kg}^{-1}) = \frac{\text{ng 1,2-dibromoethane in volume injected}}{\text{volume injected } (\mu\text{l})} \times 5$$

(but see footnote on p. 389).

References

1. Report by the Panel on Fumigant Residues in Grain, *Analyst*, 1974, **99**, 570.
2. Heuser, S. G., and Scudamore, K. A., *J. Sci. Fd Agric.*, 1969, **20**, 566.
3. Heuser, S. G., and Scudamore, K. A., *Pestic. Sci.*, 1970, **1**, 244.
4. "Pesticide Residues in Food. Report of the 1971 Joint Meeting of the FAO Working Party of Experts on Pesticide Residues and the WHO Expert Committee on Pesticide Residues," WHO Technical Report Series, No. 502, WHO, Geneva, 1972; FAO Agricultural Studies, No. 88, FAO, Rome, 1972.

Received *November 26th*, 1975
Accepted *December 12th*, 1975

Committee for Analytical Methods for Residues of Pesticides and Veterinary Products in Foodstuffs (Dr. N. A. Smart, Secretary),
Ministry of Agriculture, Fisheries and Food,
Plant Pathology Laboratory,
Hatching Green,
Harpenden,
Hertfordshire, AL5 2BD

Analyst, May, 1976, Vol. 101, pp. 391–395

Improved Extraction of Steroid Hormones from the Blood Plasma of the Domestic Fowl (*Gallus domesticus*) Using Light Petroleum at Elevated Temperatures

J. Culbert

The Agricultural Research Council's Poultry Research Centre, King's Buildings, West Mains Road, Edinburgh, EH9 3JS

A study has been made of the extraction of steroid hormones from hen blood plasma into light petroleum (boiling range 60–80 °C) at 50 °C. This technique results in a high yield of steroid hormones such as testosterone, androst-4-ene-3,17-dione, progesterone, corticosterone and oestrone, which partition into the epiphase with a low level of lipid contaminant. 17β-Oestradiol was poorly extracted, giving only a 52.5% yield. The relatively high temperature used did not cause steroid degradation. The low level of lipid contaminant in the light petroleum fraction contrasted favourably with that found with use of conventional methods when using solvents such as diethyl ether or dichloromethane.

One of the principal difficulties of isolating a steroid hormone fraction from plasma consists in the contamination of the extract with lipids. This is particularly true of the plasma of the laying hen, in which lipids are present at a relatively high concentration as they are being transported to the yolk as lipoprotein complexes.[1] The lipids include phospholipids, fatty acids, triglycerides, cholesterol and its esters and materials soluble in fat solvents, such as carotenoids and the fat-soluble vitamins A, D, E and K.

Several extraction methods for isolating hormones are currently in use, most of which involve a conventional organic solvent extraction followed by the removal of lipids by partitioning between organic solvents, by chromatography, or by precipitation from ethanol at low temperatures. The most widely used solvent-extraction method was introduced by Sommerville,[2] who extracted steroid hormones from alkaline plasma. Short[3] demonstrated that when sodium hydroxide was added to plasma before an extraction with diethyl ether, emulsions were avoided and most of the lipids remained in the aqueous phase while the steroid hormones went into the diethyl ether phase. This method of extraction proved to be very successful with human and mammalian plasma, but only partially successful with laying hen plasma. Although the lipid content in the steroid hormone fraction was considerably reduced, a substantial amount remained and made further purification necessary. The extraction of untreated plasma with diethyl ether[4] or dichloromethane[5] required the use of thin-layer chromatography in order to remove lipids and other interfering steroidal and non-steroidal material. However, with hen plasma, the lipid content of the extract, using any of these solvents, is such that its quantitative transfer to thin-layer chromatography plates or to paper for paper chromatography is difficult. Some form of preliminary clean-up is therefore necessary, such as solvent partitioning,[6] column chromatography[7] or gel filtration with Sephadex LH20.[8]

Although the removal of lipids by rendering the steroids soluble using hot saline with subsequent cooling in ice has been used to advantage,[9] the technique was unsuccessful when applied to laying hen plasma, probably because the amount of lipid material present is so large. However, a degree of success in obtaining steroid-rich extracts with a low lipid content from fowl plasma was achieved in this laboratory. The technique[10] involved the removal of steroid hormones from the plasma by adsorption on to Florisil (synthetic magnesium silicate) and washing the Florisil free of lipoprotein with saline before recovery of the steroids. Light petroleum has been used successfully to extract progesterone from plasma at room temperature and recoveries of 85–90% have been achieved.[11]

The manipulation of steroid hormones in aqueous solution at temperatures above 37 °C (approximate body temperature) has previously been considered unwise, most workers assum-

ing that thermal degradation of the steroid molecules would result. However, it has recently been shown[12] that these hormones can be extracted from plasma by adsorption on Amberlite XAD-2 at 64 °C and that there was no apparent degradation during 1 h at this temperature. In the present study steroids containing very little lipid contamination were recovered reproducibly and could be used directly in radioimmunoassay systems provided that the antiserum used had a high steroid specificity.

Materials and Methods

Reagents and Apparatus

Solvents, AnalaR grade. Light petroleum (boiling range 60–80 °C), diethyl ether, dichloromethane, hexane and methanol were redistilled before use.

Florisil (magnesium silicate) 100–200 *mesh.* This material had fine particles removed by repeated washing with distilled water; it was then dried and re-activated by heating at 300 °C for 16 h.

Radioactive steroids. [1,2,6,7(n)-^{3}H]Testosterone, 84 Ci mmol^{-1}, [1,2,6,7(n)-^{3}H]androst-4-ene-3,17-dione, 116 Ci mmol^{-1}, [1,2,6,7(n)-^{3}H]progesterone, 82 Ci mmol^{-1}, [1α,2α(n)-^{3}H]corticosterone, 40 Ci mmol^{-1}, [2,4,6,7(n)-^{3}H]oestrone, 97 Ci mmol^{-1} and [6,7-^{3}H]oestradiol, 56 Ci mmol^{-1} (supplied by The Radiochemical Centre, Amersham) were purified by thin-layer chromatography before use.

Polygram Sil G/UV$_{254}$ sheets (Camlab).

Radioactivity measurement. Radioactivity was detected using a Panax Thin Layer Scanner RTLS-1 and was measured with a Philips Scintillation Analyser PW45100/00 using a scintillator consisting of 2(4′-*tert*-butylphenyl)-5-(4″-biphenyl)-1,3,4-oxadiazole (Butyl PBD) dissolved in toluene, 0.4% *m/V*.

Blood Sampling

Blood from laying hens, derived from a commercial Shaver strain, was collected from a subclavian vein by use of heparinised syringes and was then transferred to centrifuge tubes in ice - water. The blood was centrifuged at 5 °C and 2 000 *g* for 15 min in order to obtain plasma, which was pooled and deep frozen before use.

Procedure for the Extraction of Steroids from Laying Hen Plasma at 50 °C

Incubate approximately 40 nCi of [1,2,6,7(n)-^{3}H]testosterone with 1 ml of plasma at 37 °C for 15 min, then allow the mixture to cool to room temperature. Add a similar aliquot of the labelled testosterone to each of two counting vials, which are to act as total activity standards. Next pipette 10 ml of light petroleum into the tubes containing the plasma, stopper them and shake them mechanically in a water-bath at 50 °C for 1 h. Draw off the light petroleum epiphase, add a further 10 ml of solvent to the plasma and again shake at 50 °C for 30 min. Remove the light petroleum epiphase and pool both extracts, then use a stream of nitrogen in order to evaporate the pooled light petroleum phase to dryness and re-dissolve the residue in 2–4 ml of methanol. Transfer half of the extract to a counting vial, again evaporate to dryness with a stream of gas, then re-dissolve the residue in 5 ml of scintillator solution for radioactive counting. In order to check whether or not thermal degradation of the steroids occurs at 50 °C, add 10 μg of testosterone to the remaining half of the extract and reduce to a small volume by blowing nitrogen over the mixture. Apply the residue as a small spot to a Polygram silica gel chromatographic strip and develop the chromatogram in a mixture of toluene and acetone, 2 + 1 or 4 + 1 as appropriate. The unlabelled testosterone is detected by ultraviolet light absorption and the labelled material detected by scanning the strip on a Panax Thin Layer Scanner. Repeat the procedure for the steroids androst-4-ene-3,17-dione, progesterone, corticosterone, oestrone and oestradiol.

Results

Recovery of Steroid Hormones from Plasma at 50 °C

The recovery of radioactively labelled steroid hormones from laying hen plasma (Table I) by using the present method indicates that this method facilitates the extraction of a high yield of steroids. Also, the problem of a large amount of lipid material being present in the

steroid extract has largely been overcome by using light petroleum at 50 °C. Scans of the chromatograms for radioactivity showed one peak, which coincided with that of the authentic steroid that had been added as a marker before chromatography.

TABLE I

PERCENTAGE RECOVERY OF LABELLED STEROID HORMONES FROM LAYING HEN PLASMA (1 ml) WITH LIGHT PETROLEUM (2 × 10 ml) AT 50 °C

Steroid	Recovery, %	Standard deviation, % (n = 12)
[^{3}H]Testosterone	90.9	2.5
[^{3}H]Androst-4-ene-3,17-dione	96.4	3.7
[^{3}H]Progesterone	99.5	2.6
[^{3}H]Corticosterone	78.5	2.0
[^{3}H]Oestrone	74.5	2.4
[^{3}H]Oestradiol	52.5	6.9

Comparison of the Amount of Lipid Material Extracted from Hen Plasma by Selected Organic Solvents

Aliquots (2 ml) from a hen plasma pool were extracted with diethyl ether (2 × 10 ml) and further aliquots (2 ml) were extracted with dichloromethane (2 × 10 ml). The Florisil adsorption technique was used to extract a third series of plasma aliquots (2 ml) and a final series (2 ml) was extracted with light petroleum (2 × 10 ml) at 50 °C. A comparison of the amount of lipid material extracted by each of these methods is presented in Table II and it shows clearly that the extraction with light petroleum results in a much lower level of lipid contaminant in the steroid extract.

TABLE II

LIPID MATERIAL EXTRACTED FROM 2 ml OF LAYING HEN PLASMA USING VARIOUS EXTRACTION TECHNIQUES

	Extractant			
	Diethyl ether	Dichloromethane	Florisil	Light petroleum at 50 °C
Mean/g	0.099 3	0.089 2	0.010 9	0.000 9
Standard deviation/g (n = 6)	0.009 2	0.003 5	0.001 8	0.000 25

Effect of Temperature on Steroid Extraction

The partitioning of testosterone between plasma and light petroleum was studied at various temperatures, *viz.*, 0, 10, 20, 30, 40, 50 and 60 °C. The labelled testosterone and cold testosterone carrier showed increasing solubility in the light petroleum as the temperature increased, reaching a maximum at about 60 °C, as is shown in Fig. 1. As there was no appreciable difference between the recovery at 50 and 60 °C, a temperature of 50 °C was adopted in order to minimise the possibility of thermal degradation.

Effect of Time on Steroid Hormone Extraction

The partitioning of radioactively labelled testosterone between light petroleum and plasma was carried out at 50 °C for 10, 20, 30, 40, 50 and 60 min and little change was apparent between 40 and 60 min (Fig. 2).

Solubility of Testosterone in Plasma Lipids

With the development of highly specific antibodies that are suitable for the radioimmunoassay of steroid hormones, rapid extraction methods have been introduced and assays performed directly on a buffered solution of the residue from a simple solvent extraction of plasma with an organic solvent. However, the plasma of the laying hen contains a large amount of lipid material, and when diethyl ether or dichloromethane is used to extract the steroids,

most of the lipid material is extracted simultaneously; in subsequent steps of a rapid procedure this co-extraction leads to difficulty in obtaining all of the available steroid in the final aqueous phase. In order to demonstrate this difficulty a study was made of the partitioning of [^{3}H]testosterone (100 nCi) and cold, carrier testosterone (10 μg) between phosphate-buffered saline (5 ml) and lipid material (250 mg) that had previously been extracted from plasma with diethyl ether. It was found that only 53% (mean of six results, standard deviation 3.26%) of the [^{3}H]testosterone partitioned into the aqueous buffer after 30 min equilibration by rotation.

The above low recovery prompted further investigation under conditions normally encountered in the extraction of steroids for radioimmunoassay. Thus, 1-ml plasma samples containing radioactively labelled testosterone were taken from a pool and extracted twice with diethyl ether. Further 1-ml plasma samples were extracted twice with light petroleum at 50 °C. The extracts were dried and the residues mixed thoroughly with phosphate-buffered saline (2 ml); they were then allowed to stand. The lipid present in the residue from the diethyl ether extracts settled out on the surface of the buffer solution and on the walls of the tube. In the first instance the lipid was excluded when aliquots of the buffered solution were taken for a radioactivity count. The mean recovery of [^{3}H]testosterone for these extracts was only 43.7%, with a standard deviation of 2.5% ($n = 7$). However, when the aqueous buffer solution and the lipid were shaken vigorously, aliquots of the resulting turbid suspension showed a [^{3}H]testosterone recovery of 99.3%, with a standard deviation of 4.7% ($n = 4$). By comparison, the light petroleum extract of plasma was virtually clear when dissolved in the aqueous buffer and on measurement for radioactivity showed a [^{3}H]testosterone recovery of 89.8%, standard deviation 1.38% ($n = 7$).

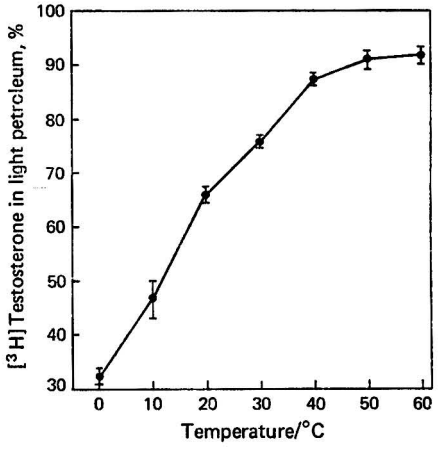

Fig. 1. Extraction of [^{3}H]testosterone from hen plasma (1 ml) by use of light petroleum (2 × 10 ml) at temperatures of 0, 10, 20, 30, 40, 50 and 60 °C. Values plotted are the means of three results; the bars indicate standard deviations.

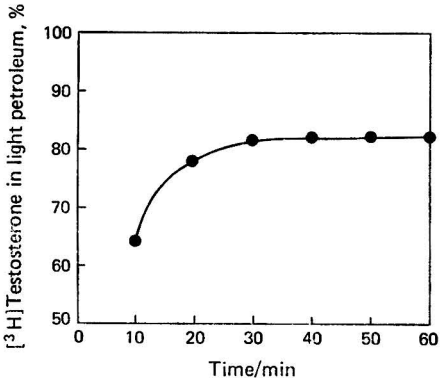

Fig. 2. Effect of time on the partitioning of [^{3}H]testosterone between plasma (1 ml) and light petroleum (10 ml) at 50 °C. Values are the means of duplicates, standard deviation = 2.44%, calculated by the difference between duplicates method.[18]

Discussion

An investigation of the factors affecting the partition coefficients of steroid hormones in a water - light petroleum system indicated that the partitioning was greatly influenced by temperature when it was greater than room temperature, in which circumstance the association constants of the steroid hormones and their binding proteins are greatly reduced.[14] From the results of the present study it appears that doubts about the stability of steroid hormones at temperatures in excess of 30 °C are unfounded. No degradation of the steroids investigated was apparent after 90 min at 50 °C when investigated by use of thin-layer chromatography.

Rupture of the plasma lipoprotein complexes was avoided when the reported extraction

method was used. In contrast, when diethyl ether or dichloromethane was used, lipid material from the ruptured lipoprotein was extracted into the steroid-rich solvent phase.

The present method results in a 10-fold reduction in the amount of lipid extracted from plasma when compared with the previous technique using Florisil and about a 100-fold reduction compared with the use of diethyl ether or dichloromethane. Although the extracts obtained with the Florisil technique were clean enough for direct radioimmunoassay, the recovery of steroids was not quantitative and a radioisotopic yield determination was necessary in order to correct for losses occurring during the extraction procedure.

For the steroids testosterone, androst-4-ene-3,17-dione and progesterone, which gave recoveries of 91.0, 96.4 and 99.5%, respectively, it should be possible, by using the present extraction method, to carry out radioimmunoassay without further purification. In this way a suitable compromise can be reached between the requirements of specificity, accuracy of measurement and rapidity of the assay system.

The recovery of corticosterone from plasma by use of the present method demonstrates the effect of temperature on the partition coefficient. Light petroleum extracted 78.5% of the corticosterone at 50 °C and only about 1% at 0–5 °C. The latter value is in agreement with that reported by Johansson.[11] The selective extraction of the non-polar progesterone at lower temperatures may find application in the determination of corticosterone.

The oestrogens were not extracted quantitatively by light petroleum, perhaps because of their phenolic nature; 72.4% of the oestrone and 52.5% of the more polar 17β-oestradiol were recovered from plasma. The oestrogen-binding proteins present in the plasma may also be responsible, in part, for the low level of the recoveries.

References

1. McIndoe, W. M., *Biochem. J.*, 1959, **72**, 153.
2. Sommerville, I. F., *J. Clin. Endocr. Metab.*, 1957, **17**, 317.
3. Short, R. V., *J. Endocr.*, 1958, **16**, 415.
4. Hotchkiss, J., Atkinson, L. E., and Knobil, E., *Endocrinology*, 1971, **89**, 177.
5. Stabenfelt, G. M., Ewing, L. L., Paton, P., and McDonald, L., *J. Endocr.*, 1969, **44**, 23.
6. Lindner, H. R., and Mann, T., *J. Endocr.*, 1960, **21**, 341.
7. Mayes, D. M., Nugent, C. A., and Furuyama, S., *Steroids*, 1970, **16**, 415.
8. Murphy, B. E. P., and Patter C. J., *J. Clin. Endocr. Metab.*, 1964, **24**, 919.
9. Love, D. N., and Ismail, A. A. A., *J. Endocr.*, 1969, **43***P*, liv.
10. Culbert, J., *Lab. Pract.*, 1973, **22**, 348.
11. Johansson, E. D. B., *in* Diczfalusy, E., *Editor*, "Steroid Assay by Protein Binding, Karolinska Symposia on Research Methods in Reproductive Endocrinology," Karolinska Institute, Stockholm, 1970, p. 188.
12. Sjovall, J., and Axelson, M., *J. Steroid Biochem.*, 1974, **5**, 733.
13. Snedecor, G. W., *Biometrics*, 1952, **8**, 85.
14. Chen, P. S., Mills, I. H., and Bartter, F. C., *J. Endocr.*, 1961, **23**, 129.

Received *November 18th*, 1975
Accepted *January 6th*, 1976

Analyst, May, 1976, Vol. 101, pp. 396–403

The Determination of Boron in Magnesites Using Carminic Acid*

N. F. C. Shelton

GR-Stein Refractories Ltd., Central Research Laboratories, Sandy Lane, Worksop, Nottinghamshire

and R. A. Reed

British Ceramic Research Association, Queens Road, Penkhull, Stoke on Trent, ST4 7LQ

The development of a method for the determination of boron in magnesite materials as used in the refractories industry is described. The method is based on the spectrophotometric measurement of the boron - carminic acid complex, and the most probable sources of error have been investigated and either eliminated or compensated for by appropriate experimental techniques.

The undesirable effects which the presence of small amounts of boron has upon the high-temperature strength of magnesia refractories have been the subject of much research work in the refractories industry in recent years, and fundamental studies by Taylor *et al.*[1,2] have shown that the loss of strength is due to the high rate of fluxing of the calcium silicate-type bonding between the periclase grains by relatively small amounts of boron oxide.

In order to study and develop a standard method for the chemical determination of boron in magnesites a Working Group was formed by the British Ceramic Research Association Analysis Committee. The concentration range of interest is from 0.005 to 0.3% of boron as B_2O_3, and two members of the Group, representing major manufacturers of refractories, were already using spectrophotometric carminic acid methods based upon modifications of the method of Hatcher and Wilcox.[3] After consideration of other methods involving the use of curcumin, methylene blue and dianthrimide, which had also been tried previously by various members of the Group, it was decided to consider the carminic acid method in detail and to study any effects that might affect the accuracy of the method. Modifications were introduced when necessary and the final method, described in this paper, is considered suitable for the determination of boron oxide in magnesia refractory products and raw materials. The method has subsequently been submitted to the British Standards Institution as an Addendum to BS 1902, Part 2E, 1970, "The Chemical Analysis of Magnesites and Dolomites."

Experimental

The carminic acid method already being used for the determination of boron in magnesites was essentially as follows. A suitable amount of sample was extracted with hot dilute sulphuric acid (1 + 3), diluted to a standard volume with water and the insoluble silica filtered off on a dry filter-paper. A 4-ml volume of the filtrate was diluted to 50 ml with concentrated sulphuric acid containing the carminic acid reagent and allowed to react for 2 h at room temperature; the absorbance of the boron - carminic acid complex was then measured at 585 nm.

In order to test its general suitability the method was used in its original form by all members of the Working Group to determine the boron oxide content of four magnesite samples within the desired range. The results from this initial trial are shown in Table I, and it was agreed that these showed sufficient precision for interest in the method to be maintained. The parameters which were then studied in detail and which resulted in modifications where necessary were: (1) the effect of the presence of magnesium upon the calibration, (2) possible pick-up of boron from glassware or loss of boron by volatilisation during the initial dissolution stage, (3) the determination of any boron that remained in the acid-insoluble residue, (4) interference by titanium and vanadium, (5) the time and temperature

* A report of work carried out by members of the Boron in Magnesites Working Group of the British Ceramic Research Association.

for development of the boron - carminic acid complex, (6) ageing of the carminic acid solution and (7) the effect of the presence of excess of water upon the absorbance.

TABLE I

SUMMARY OF RESULTS OF FIRST BORON OXIDE DETERMINATION EXPERIMENT

	Boron oxide, %			
Laboratory	BCS 319	AN 43	AN 44	AN 45
A	0.107	0.006	0.026	0.200
	0.106	0.006	0.026	0.208
	0.108	0.006	0.025	0.204
B	0.101	0.005	0.029	0.211
	0.101	0.006	0.029	0.211
	0.101	0.006	0.029	0.212
	0.101	0.006	0.030	0.213
	0.103	0.005	0.029	0.212
C	0.107	0.008	0.025	0.225
	0.108	0.007	0.027	0.222
	0.107	0.008	0.026	0.225
D	0.101	0.007	0.031	0.228
	0.104	0.007	0.031	0.235
	0.105	0.008	0.032	0.234
E	0.104	0.007	0.033	0.220
	0.108	0.006	0.031	0.226
	0.107	0.007	0.031	0.226
F	0.106	0.006	0.029	0.217
	0.101	0.006	0.030	0.216
	0.105	0.007	0.029	0.216
	0.107	0.007	0.029	0.213
	0.108	—	0.029	0.209
Mean, %	0.105	0.007	0.029	0.217
Standard deviation, %	0.003	0.001	0.002	0.009
Coefficient of variation, %	2.6	13.4	7.6	4.3

Effect of the Presence of Magnesium upon the Calibration

Although some positive results of very small magnitude were observed, these varied with the source of the magnesium and it was concluded that the effects were due to the presence of small amounts of boron in the materials and not due to a true interference by magnesium.

Gain or Loss of Boron during the Dissolution Stage

There was a possibility that boron could be either picked up from the borosilicate beaker or lost by volatilisation during the dissolution stage, which involves heating the sample with dilute sulphuric acid. Boron was therefore determined on two samples after incipient and vigorous boiling, each for 30 min, with dilute sulphuric acid (1 + 3) in Pyrex beakers and also after vigorous boiling in PTFE or platinum containers, the volumes being maintained reasonably constant by the addition of distilled water. From the results shown in Table II, it was concluded that the gain or loss of boron during the dissolution stage was insignificant.

TABLE II

GAIN OR LOSS OF BORON DURING DISSOLUTION

	Boron oxide, %		
Sample	Incipient boiling, 30 min in Pyrex	Vigorous boiling, 30 min in Pyrex	Vigorous boiling, 30 min in PTFE
AN 44	0.030	0.030	0.029
AN 45	0.228	0.229	0.230

Boron Content of the Acid-insoluble Residue

The sample was boiled with dilute sulphuric acid and the insoluble residue (mainly silica) filtered off and washed well with water. After gently burning off the paper the residue was fused with sodium carbonate, dissolved in sulphuric acid (1 + 3) and made up to volume.

Boron was then determined on an aliquot. Some difficulty was experienced, owing to the presence of sodium sulphate, but in the experiments that were successful the level of boron was of the order of 0·002%. The total boron oxide content of the sample was 0.10%.

Interference by Titanium and Vanadium

Preliminary tests indicated that the presence of titanium resulted in a spuriously high result for the boron oxide content of samples, and co-operative trials throughout the Group confirmed this result.

The magnitude of this interference was determined by making additions of pure titanium solution equivalent to 0, 0.01, 0.025, 0.05, 0.25 and 0.5% of titanium(IV) oxide to two samples of magnesite followed by the determination of the boron oxide contents. Typical results are shown in Table III and Fig. 1, which indicate that the positive interference by titanium is linearly proportional to the amount present over the range covered and that 0.5% of titanium(IV) oxide will increase the apparent boron oxide content by about 0.02%.

TABLE III

INTERFERENCE BY TITANIUM

		Titanium(IV) oxide added, %					
Sample	Value	0	0.01	0.025	0.05	0.25	0.50
BCS 319 (1 g per 100 ml)	Absorbance ..	0.143	0.145	0.145	0.148	0.157	0.168
	B_2O_3, % ..	0.105	0.106	0.106	0.109	0.115	0.123
AN 43 (2 g per 50 ml)	Absorbance ..	0.032	0.034	0.037	0.043	0.084	0.133
	B_2O_3, % ..	0.006	0.006	0.007	0.008	0.016	0.025
TiO_2 only (basis 2 g per 50 ml)	Absorbance ..	—	0.004	0.008	0.014	0.056	0.108
	B_2O_3, % (apparent)..	—	0.001	0.002	0.003	0.011	0.020

Although the titanium(IV) oxide content of the naturally occurring and synthetic magnesites used in the refractories industry does not usually exceed about 0.07%, the corresponding enhancement of about 0.003% in the apparent boron oxide content could still be significant in some of the materials with very low boron contents. Based upon modifications of the work of Calkins and Stenger,[4] a method was devised for correcting the interference due to titanium without the need to actually determine the amount of titanium present.

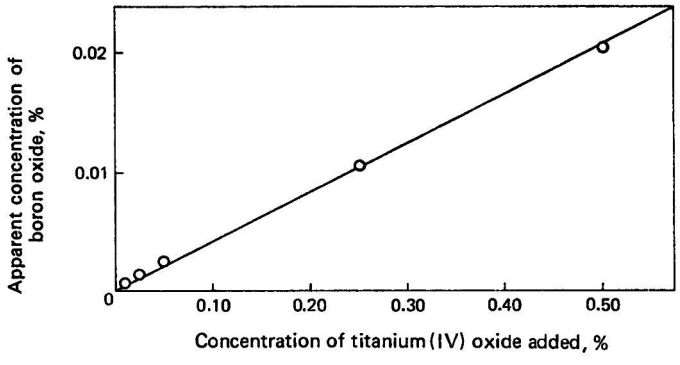

Fig. 1. Effect of titanium on boron content.

Fig. 2 shows the absorption graphs obtained for the carminic acid complexes of boron, titanium and vanadium. It is seen that the graph for boron has a flat top centred at about 610 nm, and it was decided to carry out future work at this wavelength instead of at 585 nm. This change had a two-fold benefit: (i) the reproducibility of measurement of the absorbance of the boron complex was improved as the wavelength setting of the spectrophotometer is not so critical, and (ii) the absorption due to vanadium, which can occasionally be present

up to about 0.05% of vanadium(V) oxide, is insignificant at 610 nm whereas at 585 nm it could be a considerable problem.

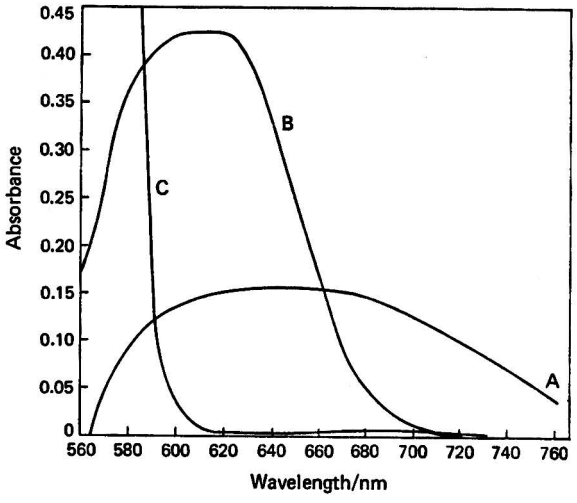

Fig. 2. Absorption spectra of carminic acid complexes: A, with 0.5% of titanium(IV) oxide; B, with 0.075% of boron oxide; and C, with 0.5% of vanadium(V) oxide (in each instance the 2-g sample procedure was used).

Correction for titanium

It can be seen from Fig. 2 that at 610 nm light absorption due to both the boron and the titanium complexes occurs, but at 730 nm the absorption is due only to the titanium complex. Hence, by measuring the absorption of a pure titanium complex at both wavelengths, a ratio can be obtained, for a given spectrophotometer, of the titanium absorption at 610 nm to the titanium absorption at 730 nm and, by applying this ratio to the measurements made at 730 nm on sample solutions that contain both boron and titanium, the absorption due to boron only at 610 nm can be calculated as described below under Calculation. The results obtained by using this method on magnesite samples containing additions of up to 0.5% of titanium(IV) oxide are shown in Table IV, and it can be seen that good correction is obtained up to this level.

TABLE IV

APPLICATION OF CORRECTION TECHNIQUES TO SAMPLES WITH ADDED TITANIUM(IV) OXIDE

Sample	Boron oxide determined, %				
	Laboratory A	Laboratory B	Laboratory C	Laboratory D	Laboratory E
AN 43	0.005	0.005	0.004	0.005	0.005
AN 43 + 0.05 % TiO_2 ..	0.005	0.005	0.004	0.006_5	0.005_5
AN 43 + 0.5 % TiO_2 ..	0.005	0.004	0.004	0.005	0.005_5
BCS 319	0.102_5	0.101	0.100_5	0.103	0.101_5
BCS 319 + 0.05 % TiO_2 ..	0.101_5	0.103	0.102	0.103_5	0.101
BCS 319 + 0.5 % TiO_2 ..	0.100_5	0.100	0.099	0.104	0.102
AN 45	0.220_5	0.223	0.220	0.217_5	0.218
AN 45 + 0.05 % TiO_2 ..	0.221	0.220	0.218	0.216_5	0.219
AN 45 + 0.5 % TiO_2.. ..	0.220_5	0.216	0.214	0.212	0.220

Time and Temperature for Complex Development

In order to determine the optimum conditions of time and temperature for the development of the boron and titanium complexes with carminic acid, development was carried out for

various times at 60, 40, 30 and 25 °C, earlier trials having shown that a crystalline precipitate, probably of magnesium sulphate, was often obtained from the more concentrated sample solutions at temperatures below 20 °C.

At 60 °C rapid fading of the boron complex occurred, the rate of fading decreasing as the temperature was reduced. The temperature must be reduced to 30 °C before a sufficiently wide plateau on the absorbance *versus* time graph is obtained. For convenience a temperature of 25 °C was decided upon, solutions being maintained at this temperature in a thermostatically controlled water-bath. Typical development graphs for the boron, titanium and vanadium complexes at 25 °C are shown in Fig. 3, from which it can be seen that a suitable development time is 3 h.

Ageing of the Carminic Acid Solution

In order to test for ageing effects on the stock carminic acid solution, absorbances were measured on the top boron oxide calibration point at regular periods for up to 53 d.

No significant long-term increase or decrease in the observed absorbances occurred; it was therefore concluded that the carminic acid solution remains stable over this period.

Effect of Excess of Water

The formation of the boron - carminic acid complex is carried out in a high concentration of sulphuric acid, and variations in the amount of water present can have a marked effect upon the final absorbance. This is illustrated in Fig. 4, in which the absorbance of one calibration standard is plotted against water present in excess of the amount stipulated in the method. Hence aqueous volumes in the final stages of the method must be accurately controlled and calibrated flasks must be dried before use.

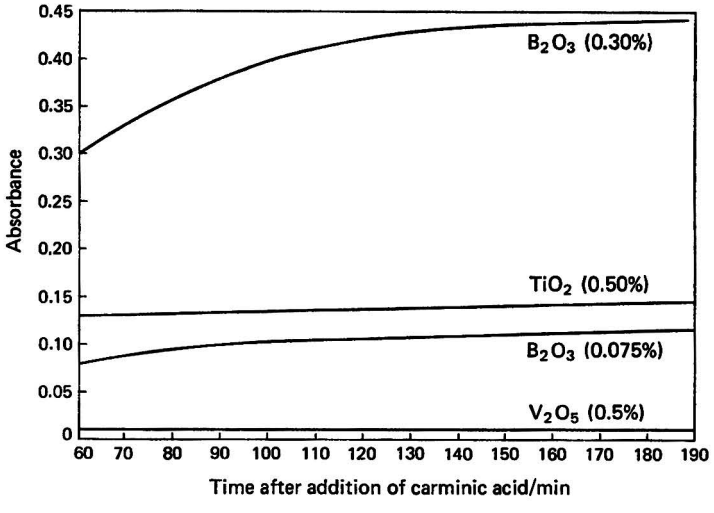

Fig. 3. Rates of development of carminic acid complexes at 25 °C.

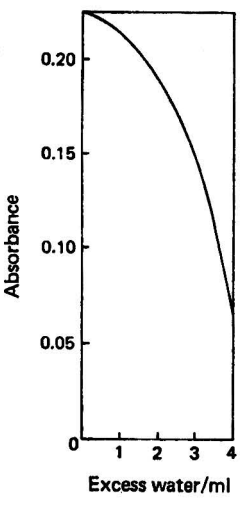

Fig. 4. Effect of excess water on colour development.

Results and Discussion

The final method recommended by the Working Group for determination of boron in magnesites and magnesia-type refractories is given in the following section and has been applied over the range 0.005–0.3% of boron oxide.

As a test of the accuracy of the method synthetic standard solutions were prepared, using Specpure magnesium oxide and making various additions of standard boron solution. The results obtained are shown in Table V. It can be seen that the Specpure magnesium oxide contained 0.002% of boron oxide (approximately 6 p.p.m. of boron) and after allowing for

TABLE V

RECOVERY OF BORON FROM SYNTHETIC STANDARDS

Composition of standard	Boron oxide, % Found	Boron oxide, % Corrected for blank
Specpure MgO (blank)	0.00_2	—
	0.00_2	—
	0.00_2	—
Specpure MgO + 0.09% B_2O_3 ..	0.09_4	0.09_2
	0.09_3	0.09_1
	0.09_3	0.09_1
Specpure MgO + 0.18% B_2O_3 ..	0.18_4	0.18_2
	0.18_4	0.18_2
	0.18_3	0.18_1
Specpure MgO + 0.30% B_2O_3 ..	0.30_3	0.30_1
	0.30_2	0.30_0
	0.30_2	0.30_0
0.30% B_2O_3 alone	0.30_0	—
	0.30_0	—
	0.30_1	—

this content the results on the synthetic standards were in very close agreement with the theoretical values.

The six co-operating laboratories used the method to analyse five magnesite samples, including one completely unknown (sample M). Triplicate results were obtained for each sample in order to allow a meaningful statistical treatment to be applied, and these are shown in Table VI. Sample AN 44 was in fact BCS 389 before final standardisation (certificate value 0.029% of boron oxide) and the results obtained for BCS 319 agree very well with the value quoted on the certificate of 0.10% of boron oxide obtained by direct reading spectroscopy.

TABLE VI

SUMMARY OF FINAL BORON OXIDE ANALYSIS BY RECOMMENDED METHOD

Sample	Boron oxide, % Laboratory A	Laboratory B	Laboratory C	Laboratory D	Laboratory E	Laboratory F	18 results Mean, %	Standard deviation, %	Coefficient of variation, %
M (unknown)	0.099	0.098	0.101	0.099	0.096	0.098	0.098	0.001 4	1.5
	0.098	0.098	0.098	0.100	0.098	0.098			
	0.098	0.101	0.098	0.095	0.098	0.099			
AN 43	0.005	0.005	0.004	0.005	0.005	0.005	0.005	0.000 3	6.5
	0.005	0.005	0.005	0.005	0.005	0.005			
	0.005	0.004	0.004	0.005	0.005	0.005			
AN 44*	0.029	0.029	0.029	0.029	0.029	0.030	0.029	0.000 7	2.4
	0.029	0.029	0.030	0.027	0.028	0.030			
	0.029	0.029	0.029	0.028	0.030	0.029			
AN 45	0.221	0.221	0.225	0.222	0.222	0.219	0.222	0.002 2	1.0
	0.221	0.222	0.222	0.227	0.219	0.220			
	0.221	0.224	0.225	0.220	0.222	0.219			
BCS 319†	0.101	0.100	0.102	0.106	0.099	0.100	0.101	0.002 2	2.1
	0.101	0.100	0.099	0.106	0.102	0.100			
	0.101	0.099	0.100	0.104	0.103	0.102			

* This was BCS 389 before final standardisation. Certificate value is 0.029% of boron oxide.
† Certificate value (by direct-reading spectroscopy) is 0.10% of boron oxide.

When a 1-g sample is used and the solution diluted to 100 ml the range of the method is from 0 to 0.3% of boron oxide and the standard deviation expected is 0.002%. For low-boron magnesites a 2-g sample should be taken and diluted to 50 ml, giving a range of 0–0.075% of boron oxide and a standard deviation of 0.000 5%.

The absorbance of the top calibration point (120 μg of boron oxide) is approximately 0.45

and the graph is linear. However, above this point the calibration graph starts to become non-linear and it is therefore not possible to extrapolate any results which may be outside the appropriate calibration range employed.

Procedure

Reagents

Unless otherwise stated, all reagents should be of analytical-reagent grade, and distilled water should be used throughout the analysis.

Carminic acid, 0.5 g l^{-1}. Dissolve 0.1 g of carminic acid in 200 ml of concentrated sulphuric acid, sp. gr. 1.84, with stirring.

Sulphuric acid, sp. gr. 1.84, *approximately* 36 N.

Titanium solution, approximately 1 mg ml^{-1} *of titanium(IV) oxide.* To 150 ml of titanium sulphate standard solution (1 mg ml^{-1} of titanium, for atomic-absorption spectroscopy) add 10 ml of sulphuric acid (1 + 3) and dilute the solution to 250 ml with water.

Standard Solutions

Boron solution A, 300 p.p.m. *of boron oxide.* Dissolve 0.532 8 g of boric acid in water and dilute the solution to 1 l in a calibrated flask. Store in a polythene bottle.

Boron solution B, 30 p.p.m. *of boron oxide.* Dilute 25 ml of boron solution A to 250 ml in a calibrated flask. Store in a polythene bottle.

Preparation of Sample

The sample prepared for analysis should be ground to pass a 120-mesh British Standard sieve. A non-metallic (*e.g.*, nylon bolting cloth) sieve is preferable.

Decomposition of the Sample

Weigh 1.000 g of the prepared sample, previously dried at 110 °C, on to a small watch-glass and transfer it into a 100-ml beaker (for samples containing less than 0.07% of boron oxide a 2-g sample should be used).

Add 20 ml of dilute sulphuric acid (1 + 3), cover the beaker with a clock-glass and heat to incipient boiling for 15–30 min. An insoluble residue, consisting mainly of silica, may remain. Cool the solution to room temperature, transfer it into a 100-ml calibrated flask, without filtering, and dilute to the mark with water. (If a 2-g sample has been used the solution should be transferred into a 50-ml calibrated flask.)

Determination of Boron

Filter a portion of the solution through a dry 110-mm Whatman No. 40 paper into a dry beaker, discarding the first few millilitres. Do not wash the filter.

Transfer a 4-ml aliquot of the filtered solution into a dry 50-ml calibrated flask (preferably polypropylene). In a second dry flask prepare a control solution by adding 4 ml of water by pipette. To both flasks carefully add 20 ml of sulphuric acid (sp. gr. 1.84) and cool them rapidly in running water. Then stand the flasks, together with the concentrated sulphuric acid and carminic acid solution, in a water-bath thermostatically controlled at 25 °C.

After about 10 min add to each flask by pipette (**Caution**: use a pipette filler) 20 ml of the carminic acid solution, allowing an appropriate drainage time. Dilute each solution to 50 ml with sulphuric acid (sp. gr. 1.84), mix and allow to stand for 3 h in the water-bath at 25 °C.

Measure the absorbance of the sample solution against the control solution in 10-mm cells at 610 and 730 nm.

Calculation

Calculate the true absorbance due to boron at 610 nm as follows:

$$A^{610}_{B_2O_3} = A^{610} - (R \times A^{730})$$

where $A^{610}_{B_2O}$ = true absorbance due to boron at 610 nm.

A^{610} = measured absorbance at 610 nm (due to boron and titanium complexes).

A^{730} = measured absorbance at 730 nm (due to titanium complex only).
R = ratio of absorbances of titanium complex at 610 nm, and 730 nm, which must be determined for the particular spectrophotometer used (see Correction for titanium content of sample).

Determine the boron oxide content of the sample by reference to the calibration graph.

Calibration

Transfer 0, 1, 2, 3 and 4 ml of the boron solution B into dry 50-ml calibrated flasks (preferably polypropylene) and dilute each solution with water to produce a volume of exactly 4 ml. This will give a calibration graph for 0–0.30% of boron oxide using a 1-g sample or 0–0.075% of boron oxide for the 2-g sample procedure. Develop the complex exactly as described above. After 3 h measure the absorbance of each solution against the zero boron oxide solution in 10-mm cells at 610 nm. From the absorbances prepare a calibration graph. A fresh calibration graph must be prepared for each new supply of solid carminic acid reagent.

Correction for Titanium Content of Sample

The presence of 1% of titanium(IV) oxide in the sample gives an apparent boron content of approximately 0.04%. In general the titanium(IV) oxide content of magnesites does not exceed 0.1% unless it has been specifically added or is a contaminant, and its effect can be allowed for without determining the titanium content of the sample.

Transfer by pipette 10 ml of the titanium solution [approximately 1 mg ml^{-1} of titanium(IV) oxide] into a 50-ml calibrated flask, add 20 ml of dilute sulphuric acid (1 + 3) and dilute to 50 ml with water. Transfer a 4-ml portion of this solution [≡0.5% of titanium(IV) oxide in the sample for the 2-g procedure] into a dry 50-ml calibrated flask (preferably polypropylene). Into a similar flask pipette 4 ml of water for preparation of the control solution. Develop the complex exactly as for boron. After 3 h measure the absorbance of the titanium-containing solution against the control solution in 10-mm cells at 610 and 730 nm. Calculate the ratio (R) of absorbances for the titanium complex at these two wavelengths.

$$R = \frac{\text{Absorbance at 610 nm}}{\text{Absorbance at 730 nm}}$$

Substitute this value (approximately 1.75) in the equation given under Calculation.

We thank Mr. A. Dinsdale, Director of Research of the British Ceramic Research Association, for permission to publish this paper. This work was carried out in the laboratories participating in the Working Group on the Determination of Boron in Magnesites, the representatives being: Mr. N. F. C. Shelton (Chairman), GR-Stein Refractories Ltd., Mr. P. G. Deane, The Associated Portland Cement Manufacturers Ltd.; Mr. B. Fletcher, Dyson Group Research Laboratories; Mr. D. Newell, Steetley Organization Research Department; Dr. G. J. Oliver (Secretary to the Group), The British Ceramic Research Association; Mr. R. A. Reed, The British Ceramic Research Association; and Mr. B. D. Summerhill, British Steel Corporation.

References

1. Taylor, M. I., Ford, W. F., and White, J., *Trans. Br. Ceram. Soc.*, 1969, **68**, 173.
2. Taylor, M. I., Ford, W. F., and White, J., *Trans. Br. Ceram. Soc.*, 1971, **70**, 51.
3. Hatcher, J. T., and Wilcox, L. V., *Analyt. Chem.*, 1950, **22**, 567.
4. Calkins, R. C., and Stenger, V. A., *Analyt. Chem.*, 1956, **28**, 399.

Received *October 17th*, 1975
Accepted *December 29th*, 1975

Elimination of Interference from Aluminium in the Determination of Total Iron in Soils and Plant Materials Using 1,10-Phenanthroline Reagent

Comment on the Paper by Jayman *et al.*[1]

R. J. Julietti

Morgan Thermic Technological Centre, Bewdley Road, Stourport-on-Severn, Worcestershire, DY13 8QR

Of the many compounds proposed for the spectrophotometric determination of iron, none has received such wide acclaim as 1,10-phenanthroline. Not only is it a sensitive and stable reagent but, by virtue of the location of the two nitrogen atoms in the molecule, it is extremely selective; all but a few elements are sterically hindered from forming complexes with it. As long ago as 1938, the interference in the determination of iron by other ions was extensively studied by Fortune and Mellon,[2] who produced a long list of ions that had no effect on the determination even when present in relatively high concentrations. Included in their list was aluminium(III).

In complete contradiction to this was the paper by Jayman *et al.*[1] published in the October, 1975, issue of *The Analyst*. The authors claimed that aluminium forms a complex with the reagent that is identical in its absorption characteristics with that of iron(II) except for its sensitivity. If this were so, then plainly it would not be feasible to determine iron in aluminous materials without prior separation. However, there is abundant evidence that no such separation is necessary.

For example, the reagent has been proposed for the direct determination of iron in aluminium and its alloys[3] and in aluminium oxide[4] and it is widely used for the analysis of a wide range of aluminosilicate materials.[5] In none of the standard methods cited is there any suggestion that aluminium forms a complex with 1,10-phenanthroline, or that it interferes in any way with the determination of iron. Hence no separation has been found necessary. My colleagues and I, working in the field of aluminous refractory materials, have determined iron by this method for many years and we have never noted any interference from aluminium. International standards for the determination of iron in aluminium[6] and aluminium oxide[7] give further support to these views.

In the light of this overwhelming evidence, it is not plausible that aluminium was responsible for the reactions that Jayman *et al.* observed. As they claim that the absorption spectrum is identical with that of iron, it is most probable that it was, in fact, due to iron and that they were misled by systematic contamination. Their results suggest that the iron was probably present as an impurity in the aluminium that they used for preparing their reference standards.

References

1. Jayman, T. C. Z., Sivasubramaniam, S., and Wijedasa, M. A., *Analyst*, 1975, **100**, 716.
2. Fortune, W. B., and Mellon, M. G., *Ind. Engng Chem. Analyt. Edn*, 1938, **10**, 60.
3. BS 1728: 1957: Part 8, "The Analysis of Aluminium and Aluminium Alloys."
4. BS 4140: 1967, "Methods of Test for Aluminium Oxide."
5. Bennett, H., and Reed, R. A., "Chemical Methods of Silicate Analysis," Academic Press, London, 1971, p. 67.
6. International Standard, ISO 793 : 1973, "Aluminium and Aluminium Alloys: Determination of Iron: Orthophenanthroline Photometric Method."
7. International Standard Recommendation, ISO/R 805 : 1968, "Aluminium Oxide Primarily Used for the Production of Aluminium: Determination of Iron Content. 1,10-Phenanthroline Photometric Method."

Received *February 4th*, 1976
Accepted *February 13th*, 1976

Book Reviews

SCANDIUM: ITS OCCURRENCE, CHEMISTRY, PHYSICS, METALLURGY, BIOLOGY AND TECHNOLOGY. Edited by C. T. HOROVITZ. Pp. xvi + 598. London, New York and San Francisco: Academic Press. 1975. Price £16; $42.25.

For a variety of reasons, scandium is undoubtedly an unusual element; for example, it has often been referred to as a rare-earth lanthanide, a pre-lanthanide triad (together with yttrium and lanthanum), and as a transitional trace element. Over the past two decades, information on the potential role of this metal in disciplines ranging from, say, metallurgy to biology, has been reliably established, largely because of its recent availability in a state of high purity.

As a direct consequence of this improved knowledge, scandium compounds, alloys, intermetallic compounds, etc., with more definitive compositions have been prepared, and in each of these developments analytical chemistry has made significant contributions. Even so, the lack of a readily available source of scandium (only a few rare minerals are known in which it is a major constituent) and its consequent high cost of production have restricted current annual consumption of the metal to the order of tens of kilograms. However, it may be that future demands for scandium and/or its associated products will significantly increase, for example, as a trace constituent. Although there appears to be little evidence to support any such speculations, it is noteworthy that so much otherwise scattered scientific information has now been collated and published under a single cover.

Of the book's 12 chapters, the two largest (about 80 pages each) are "Inorganic Compounds" by K. A. Gschneidner jun. (Iowa State University), who is also responsible for another 4 chapters, and "Analytical Chemistry" by the Editor (Stuttgart University), who also contributed 5 other chapters. The remaining chapters were prepared by G. A. Melson (Michigan State University), D. H. Youngblood (Texas A and M University) and H. H. Shock (Tübingen University). The main sub-headings in the chapter on analytical chemistry include "Separation Methods" (*e.g.*, based on precipitation and volatilisation, solvent extraction, and thin-layer, column and paper chromatography) and "Determination Methods" (*e.g.*, gravimetric, titrimetric, polarographic, spectrophotometric, fluorimetric, candoluminescence emission, radiochemical and neutron activation).

Other chapters (approximate number of pages in parentheses) include "Alloys and Intermetallic Compounds" (63), "Organic Compounds" (52), "Physical Metallurgy" (29), and "Chemical Properties" (21).

This commendable interdisciplinary publication is extensively supported with literature references, its over-all presentation is very good and, because of the present-day status of scandium, is unlikely to have a serious rival in the immediate future.

W. T. ELWELL

ANALYSIS OF WATER. By J. RODIER. Pp. xviii + 926. New York, Sydney, London, Tokyo Mexico City and Toronto. John Wiley & Sons. 1975. Price £33.

This book is the first English translation of the Fifth edition of Rodier's well established "L'Analyse de l'Eau." There are 926 pages, which contain several hundred different analytical procedures, and the reviewer must immediately confess to having read only a small fraction of the text. The aim of this review is, therefore, simply to summarise the aims and content of the book, and to provide some personal opinions on its place among the bookshelves of analytical chemists.

Rodier emphasises the great increase in interest in all aspects of water quality and the many analytical problems that ensue. The primary aim of the book is to bring together details of analytical procedures of common importance in routine measurements of water quality and in investigations of the various problems that may arise in controlling quality. Sufficient detail is given for each method to allow a laboratory to put the procedure into operation. Waters (natural and treated) and effluents are both considered, and several different methods (using different techniques and/or with different sensitivities) are given for most determinands. Thus, for all common analytical requirements, laboratories are likely to find in the book a method that could be applied to their particular needs. The contents of the book are summarised below and indicate its broad scope.

Part I: Analysis of Natural Waters
Sampling.
Tracer methods for studying sources of contamination.
Methods of analysis, including radioactivity, toxic inorganic substances and organic micro-pollutants.
Tests concerned with aspects of corrosion.
Checking and presentation of analytical results.
Tests concerned with disinfection of water.

Part II: Analysis of Residual Waters
Sampling.
Methods of analysis, inorganic, organic and radioactive substances are included.

Part III: Analysis of Sea Water (by J. Laporte and G. Kovacsik)
Sampling.
Methods for salinity, alkalinity and anions.
Methods for trace elements.
Methods for major organic groupings, *e.g.*, N and C.
Methods for special determinations.

Part IV: Bacteriological Analysis (by Ch. Geoffray and J. Vial)
General methods of sampling and measurement.
Counting aerobic mesophilic bacteria.
Counting bacteria indicative of faecal pollution.
Detection of pathogenic micro-organisms.

Part V: Biological Quality of Fresh Waters (by J. Verneaux)
Includes toxicity tests and biotic indices.

Part VI: Interpretation of Results
Includes discussion (with reference to standards for water quality) of results of physicochemical and bacteriological analyses.

Appendices
I. Series of short essays on important analytical techniques, *e.g.*, atomic-absorption spectrometry and gas chromatography.
II. Use of statistics in analysis.
III. Preparation and standardisation of standard solutions.
IV. Ion exchangers.
V. Analysis of a deposit or a sediment.
VI. Special cases of water purification.
VII. Determination of biodegradability of detergents.
VIII. Miscellaneous information, *e.g.*, buffer solutions, care of laboratory apparatus, units of measurements.

It can be seen that the book covers a very wide field, and has necessarily to be rather selective in its choice of analytical procedures. As a result, a number of procedures are not given which, in the reviewer's opinion, are worth mentioning. For example, the use of chelating ion-exchange resins for concentrating trace metals from sea water is not included and different solvent-extraction procedures are recommended for lead and cadmium prior to atomic-absorption spectrometry, even though both elements can be determined satisfactorily after the same extraction procedure. More discussion would also have been desirable on the many problems associated with sampling programmes and procedures. Notwithstanding such points, there is no doubt that the book admirably achieves its aim of providing a collection of analytical methods that would enable a water laboratory to tackle most, if not all, of the problems it is likely to meet.

"Analysis of Water" is the most comprehensive single compilation known to the reviewer, although there are, of course, many other similar collections dealing with one or more of the aspects considered by Rodier. As would be expected, the cost of the book is high (£33), and it seems dubious whether laboratories that already possess other collections of procedures will be able to justify that expenditure. However, the book would be of particular value to laboratories that are being established, and would form a useful reference work in large laboratories and organisations.

A. L. WILSON

AN INTRODUCTION TO THE FLUORIMETRIC ESTIMATION OF ENZYME ACTIVITIES. By D. H. LEABACK. Pp. ii + 49. Colnbrook: Koch-Light Laboratories Ltd. 1975. Gratis.

Koch-Light Laboratories have been performing a useful service over the last few years by producing short, readable and informative booklets on a range of topics of interest to the chemist and biochemist. The booklet by Dr. Leaback is a very good contribution to the series, and deals with a topic of considerable importance in analytical biochemistry, particularly in the clinical field.

In the space of less than 50 rather small pages the author deals with the essentials of fluorescent emission, compares fluorimetric and absorptiometric methods, surveys the instrumentation used in fluorimetry, gives a brief account of the fundamental principles of enzyme assay and describes in detail fluorimetric methods for group-specific hydrolases, the use of assays based on 4-methylumbelliferone, and gives a brief indication of other fluorimetric assays (*e.g.*, NADH- or NADPH-linked assays). The booklet includes useful tables on the properties of various chromogenic and fluorogenic systems, lists of fluorogenic substrates, and detailed properties of many fluorogenic derivatives of 4-methylumbelliferone. There are several illustrative diagrams (some unfortunately drawn with a rather shaky hand). The booklet includes a very poor quality photograph of a gel electrophoretogram, and the general layout and typesetting of the biochemical formulae are not very professional. For some reason the booklet is undated, which could cause difficulties for anyone who wanted to include it in a reference list. Its own reference list is comprehensive and constitutes a useful preliminary guide to the literature on the subject.

In general this booklet is a very successful attempt to acquaint the uninformed reader with the essentials of a most important technique. Of course, the major advantage of fluorimetry (or fluorometry in the New World) is its extreme sensitivity. This permits one to perform assays on very small amounts of enzyme. With automated analysis this results in a great saving in (often expensive) reagents. With clinical analysis it means that less biological fluid is required for the assay or that more information can be obtained from a given sample. There is therefore no doubt that we will hear far more of fluorimetric enzyme assay in the future; the second Edition of Dr. Leaback's booklet therefore appears at a most opportune time. D. B. RODYN

PRINCIPLES AND TECHNIQUES OF SCANNING ELECTRON MICROSCOPY. BIOLOGICAL APPLICATIONS. Volume 4. Edited by M. A. HAYAT. Pp. xvi + 216. New York, Cincinnati, Toronto, London and Melbourne: Van Nostrand Reinhold Company. 1974. Price £10.60.

Volume 4 of Hayat's series on "Principles and Techniques of Scanning Electron Microscopy. Biological Applications" contains six contributions.

The first concerns the preparation and examination of specimens at low temperatures and is written by an employee of a scanning electron microscope manufacturer. It does not provide a critical evaluation of the place of cryotechniques in this field. In the context of preservation of fine morphology, cryopreservation of the water content of a specimen creates more problems than it solves, but for electron-probe microanalysis it is almost sure to be regarded as practically indispensable.

The second chapter, by the founders of a valuable adjunctive technique, shows how the binding of additional osmium to osmium tetraoxide fixed tissues that have been treated with thiocarbohydrazide improves the electrical conductivity of many dried biological specimens to the point where the procedure of coating the surface with an evaporated metal layer can be omitted, even for examination at slow, recording scan speeds. They fail to point to the incredible fragility acquired by many tissues handled by this procedure. Methods for making exact copies or models of the original specimen are reviewed by Pameijer and the obvious advantages are well illustrated by examples chosen from studies of clinical dental procedures. The extremely short chapter on spermatozoa could have been greatly improved by further reduction in length, as it makes no useful contribution.

The appearance of yet another chapter on X-ray microanalysis can surely be forgiven by saying that the more places in which the biologist can read about this powerful analytical method, the more are the chances that he may be encouraged to use it in solving some real problems and this chapter, from the pen of a well trained expert, will encourage him to choose some valid procedures.

The final contribution, called "Scanning Electron Spectrometric Microscopy," claims a rosy future for the Auger electrons as carriers of invaluable information, as well as indicating that the future is a long way off. A. BOYDE

POSITIVE STAINING FOR ELECTRON MICROSCOPY. By M. A. HAYAT. Pp. xxii + 361. New York, Cincinnati, Toronto, London and Melbourne: Van Nostrand Reinhold Company. 1975. Price £13.75.

Biological electron microscopy often presents an image of an inexact art, rather than of an exact science, particularly to those with a background in analytical disciplines. The present volume goes some way towards showing how far the fixation - staining procedures used by biological electron microscope users can be explained in chemical terms. Areas that at first sight appear to be black on the magical scale can be measured as grey after reading this volume. Several procedures might be properly honoured with the term "histochemical" in that they reveal the whereabouts of particular ions or reactive groups in particular ultrastructural locations. The over-riding impression remains, however, that this is a cookery book that attempts to review other people's recipes.

Nevertheless, in terms of its potential value to the audience at which it is primarily aimed (the biological electron microscopy cooks!) it is to be recommended as an important source of information concerning what can be done to make electron opacity something which can be usefully interpreted.

A. BOYDE

NMR SPECTROSCOPY USING LIQUID CRYSTAL SOLVENTS. By J. W. EMSLEY and J. C. LINDON. Pp. xvi + 367. Oxford, New York, Toronto, Sydney and Braunschweig: Pergamon Press. 1975. Price £9.50; $22.

Liquid crystals have been much studied in recent years for two reasons. Firstly, they are finding considerable commercial use in switchable, low-power, display devices and new materials with transition points near room temperature have consequently been developed. Secondly, high-resolution nuclear magnetic resonance spectra can be obtained for a wide variety of solutes in various liquid-crystal hosts; such spectra give high-quality geometrical information or, if the geometry be known, details of the orientation of the solute in the liquid crystal. It is this second topic which is covered in this book and especially the nuclear magnetic resonance aspects. The book covers both direct spectral interpretation and the theory relating electronic structure to the various spectral parameters, anisotropic chemical shifts, anisotropic coupling constants and quadrupole couplings, especially for deuterium.

The coverage is comprehensive and the results appear to have been taken from a thorough card index supplemented by private communications so that the results are up to date. However, as regards the complex theory, the copying errors and misprints are so widespread that nothing can be trusted. Table 6.1, for instance, has two obvious misprints, q for q_{zz} twice, which prompted the reviewer to check the table, to which no reference is attached; he cannot therefore easily check his personal conviction that the four intensities, other than unity, are too small by a factor of 2 and that in the definition of R the term $-20JD$ should be $-12JD$. The next table, 6.2, also appears to have numerous mistakes so that the calculated spectrum is not symmetrical to its own centre as it should be. This table would not have passed any referee or PhD examiner and there are serious mistakes elsewhere.

Perhaps this is a consequence of direct reproduction from a typescript, so that correction of a composed proof was not required. And while the typewriter has a range of founts, the standard usage of italic for physical quantities is not followed. In this reviewer's opinion the value of this book is greatly diminished by its errors and this is sad as the topic was timely and the effort of preparation must have been great.

D. H. WHIFFEN

APPOINTMENTS VACANT

The Determination of Boron in Magnesites Using Carminic Acid

The development of a method for the determination of boron in magnesite materials as used in the refractories industry is described. The method is based on the spectrophotometric measurement of the boron - carminic acid complex, and the most probable sources of error have been investigated and either eliminated or compensated for by appropriate experimental techniques.

N. F. C. SHELTON

G.R.–Stein Refractories Ltd., Central Research Laboratories, Sandy Lane, Worksop, Nottinghamshire.

and R. A. REED

British Ceramic Research Association, Queens Road, Penkhull, Stoke-on-Trent, ST4 7LQ.

Analyst, 1976, **101**, 396–403.

Elimination of Interference from Aluminium in the Determination of Total Iron in Soils and Plant Materials Using 1,10-Phenanthroline Reagent

Comment on the Paper by Jayman *et al.*

R. JULIETTI

Morgan Thermic Technological Centre, Bewdley Road, Stourport-on-Severn, Worcestershire, DY13 8QR.

Analyst, 1976, **101**, 404.

ANALAO 101 (1202) 321–408 (1976) May 1976

ISSN 0003–2654

THE ANALYST

THE ANALYTICAL JOURNAL OF THE CHEMICAL SOCIETY

CONTENTS

Printed by Heffers Printers Ltd, Cambridge, England

Entered as Second Class at New York, USA, Post Office