The Analyst

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Information for Authors

Accurate Measurement of Ethanol in Beers, Lagers etc by Automatic Gas Chromatography

Synopsis

22 beers where examined for ethanol content using an automated GC system fitted with a column of Porapak Q*. Reproducibility of peak area was excellent, and using an internal standard method, a CV of 0.4 was obtained.

Key Words

Ethanol, Beers, Automatic, Gas Chromatography.

Introduction

Pye Unicam was recently requested by a well known organization concerned with the quality of ale to analyse a number of samples of lagers and beers for measurement of the alcohol content. This is a relatively straightforward analysis with PEG400 or Porapak Q columns. However, because of the number of samples provided, an automatic system consisting of Chromatograph, Autojector and Computing Integrator was set up and run continuously overnight.

1-Propanol was used as internal standard, a solution of 4% in water being prepared as stock. Equal volumes of sample and internal standard were mixed and diluted before injection. Dilution was necessary to ensure that the amount of analyte would fall within the linear response region of the detector. A Calibration standard of 4% of ethanol in water was treated in an identical manner to the samples.

The 22 samples were prepared and loaded on to a PU4700 Autojector. A PU4810 Computing Integrator was used to monitor each sample and label each chromatogram.

Instrumentation

Pye Unicam series 304 Gas Chromatograph, PU4700 Autojector and PU4810 Computing Integrator.

Column:	Porapak Q
Column Temperature:	175 °C isothermal
Injector Temperature:	175 °C
Detector:	FID
Detector Temperature:	200 °C
Carrier Gas:	Nitrogen 30 ml/min
Attenuation:	range 10^2 att.8
Sample Size:	0.5 µl

The system was set to carry out three analyses of each sample, and was connected so that the PU4700 could remotely start both the chromatograph and the integrator, only moving on to the next sample when the chromatograph was in the "ready" state.

Results and Discussion

The results were very repeatable for the triplicate injections although a wide range of alcohol contents over the 22 samples was indicated.

Area readout and ratio figures are summarized for three samples in the following table.

TABLE

Sample	Ethanol peak	Int. Std. Peak	% Ethanol
7	131433	147289	4.54
	131368	147736	4.52
	131610	147391	4.54
12	110569	149708	3.76
	110700	149779	3.76
	110543	149642	3.76
14	107646	146638	3.74
	107472	145835	3.75
	107353	146201	3.74

The full set of results were formulated and printed out in descending order of ethanol content. For the sake of interest these are given below though we have exercised the discretion of withholding the actual brand names of the samples involved.

Sample			% Ethanol Found		Mean	CV
beer	1	5.10	5.13	5.12	5.12	0.30%
beer	2	5.04	(4.95)	5.08	5.02	0.40
lager	3	4.99	4.97	5.01	4.99	0.40
beer	4	4.81	4.86	4.80	4.83	0.67
lager	5	4.75	4.74	4.70	4.73	0.56
beer	6	4.68	4.68	4.67	4.68	0.12
beer	7	4.54	4.52	4.54	4.54	0.25
lager	8	4.29	4.33	4.33	4.32	0.53
beer	9	4.13	4.10	4.10	4.11	0.42
lager	10	4.08	4.04	4.08	4.07	0.57
beer	11	3.87	3.87	3.89	3.88	0.30
lager	12	3.76	3.76	3.76	3.76	0.00
beer	13	3.75	3.77	3.76	3.76	0.27
beer	14	3.74	3.75	3.74	3.74	0.15
lager	15	(3.63)	3.70	3.76	3.69	0.81
lager	16	3.55	3.52	3.56	3.54	0.59
lager	17	3.48	3.50	3.49	3.49	0.29
beer	18	3.42	3.45	3.47	3.45	0.73
lager	19	3.36	3.40	3.41	3.39	0.78
lager	20	3.24	3.29	3.26	3.26	0.77
beer	21	3.23	3.25	3.25	3.24	0.36
beer	22	2.70	2.68	2.68	2.68	0.43

* Porapak Q is a trademark of Waters Associates Inc.

Further Information

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CHANNEL A     INJECT  18:12:83 20:06:39
 .88  .62
                                              2.69
               5.82

REAL ALE ANALYSIS              18:12:83 20:07:39    CH= "A"   PS=  1.
FILE  9.    METHOD  2.     RUN  70     INDEX   1
ANALYST: DAVID STOUT
SAMPLE
  SA       IS      XF
  1.       1.      4.

NAME          % VOLUME       RT      AREA BC    RF
ETHANOL           4.128     2.69  121520 01    1.272
1-PROPANOL INTERNAL STD     5.82  149790 01
TOTALS            4.128           271310
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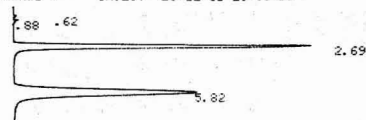
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Electrochemical Approaches to Trace Element Speciation in Waters

A Review

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Summary of Contents

1. Introduction

Speciation analysis of an element in a water sample may be defined as the determination of the concentrations of the different physico-chemical forms of the element which together make up its total concentration in the sample. The individual physico-chemical forms may include particulate matter and dissolved forms such as simple inorganic species, organic complexes and the element adsorbed on a variety of colloidal particles (Table 1). All these species can co-exist, and may or may not be in thermodynamic equilibrium with one another.[1–6] An ionic metal spike added to a filtered natural water sample may take times ranging from hours to months to equilibrate with the natural pool of metal species.[1,7–9]

For many heavy metals in sea water or river waters, the predominant physico-chemical forms are unknown. Cu in sea water, for example, is believed to exist mainly as undefined, highly stable organic complexes, the principal ligands perhaps being porphyrins, siderophores or metallothioneins.[5,10,11] In this situation, where neither the nature nor the concentrations of the dominant ligands are known, it is obviously futile to attempt to apply computer modelling techniques to determine speciation. However, for well defined experimental waters, or for elements at higher concentrations (*e.g.*, Ca and Mg in sea water), chemical modelling can be a powerful tool.[1,3,12]

There are two main reasons for studying the speciation of elements in waters—to understand either the biological or the geochemical cycling of the elements.[13] Biological cycling

Table 1. Possible physico-chemical forms of metals in natural waters

Physico-chemical form	Possible example	Diameter/ nm
Particulate	Retained by 0.45-μm filter	>450
Simple hydrated metal ion	$Cd(H_2O)_6^{2+}$	0.8
Inorganic complex	$CdCl^+$, $PbCO_3$	1
Organic complex	Cu - fulvic acid	2–4
Adsorbed on inorganic colloids	Pb^{2+}/Fe_2O_3	10–500
Adsorbed on organic colloids	Cu^{2+}/humic acid	10–500
Adsorbed on mixed organic/inorganic colloids	Cu^{2+} - humic acid/Fe_2O_3	10–500

includes bioaccumulation, bioconcentration, bioavailability and toxicity, and geochemical cycling involves the transport, adsorption and precipitation of the element in the water system. It is now well established that no meaningful interpretation of either biological or geochemical cycling can be made without speciation information.[14–16] Each different physico-chemical form of an element (Table 1) has a different toxicity, so analysis of a water sample for *total* metal concentration alone does not provide sufficient information to predict toxicity. For example, two rivers may both contain 40 μg l^{-1} of total dissolved Cu; if the first has most of the copper adsorbed on colloidal particles there will be little or no effect on aquatic life, but if the second river has free Cu(II) ion as the main species, few organisms would survive. Lipid-soluble metal complexes are particularly toxic forms of heavy metals because they can diffuse rapidly through a biomembrane and carry both metal and ligand into the cell.[15,17,18] Examples of lipid-soluble complexes are copper xanthates (from mineral flotation plants), copper 8-hydroxyquinolinate (agricultural fungicide) and alkylmercury compounds.[17,18]

Variation in the speciation of an element will also affect its degree of adsorption on suspended matter, its rate of transfer to the sediment and its over-all transport in a water system. Speciation analysis will therefore assist in the prediction of the distance over which a river will be affected by effluent discharged from a point source.[19]

Speciation measurements have been made by a variety of techniques, including electroanalysis, ion exchange, dialysis, ultrafiltration, solvent extraction and computer modelling.[1–3] If the measurements are made to study aquatic toxicity, then the aim is to determine the *toxic fraction* of the element, *i.e.*, the fraction of its total concentration in the water sample that is toxic to aquatic organisms. For a metal complex, this aim will be realised if the fraction of total metal that is reactive at a mercury electrode, adsorbed by an ion-exchange resin or measured by some other technique, is similar to the fraction that is dissociated at, and transported across, a biomembrane.[10,14–16]

Electroanalysis is a powerful technique for the study of trace element speciation, and has been applied to (or is potentially applicable to) about 30 elements: Ag, As, Au, Bi, Br, Cd, Cl, Co, Cr, Cu, Eu, Fe, Ga, Hg, I, In, Mn, Mo, Ni, Pb, S, Sb, Se, Sn, Tl, U, V, W, Yb and Zn.[1,20,21] Four metals of prime environmental concern, Cu, Pb, Cd and Zn, can be determined simultaneously and with great sensitivity. Moreover, the redox potential of an electrode can be varied accurately, precisely and continuously over a wide potential range, and the study of the kinetics of metal complex dissociation at an electrode is supported by well established theory.[22–28] Of all trace element speciation methods available at present, electroanalysis appears to provide the best opportunity for experimentally modelling the bioavailability of elements and their complexes with organic and inorganic ligands.

Trace element speciation in natural waters requires specialised techniques for the collection, filtration, storage and analysis of the samples, because there is a constant risk of contamination or trace element losses when working with such low concentrations of analyte.[1,3,29] A clean room, or at least a laminar flow clean-air cupboard, is essential for this type of work. Electrochemical techniques have an important advantage in that the sample requires much less handling and is in contact with fewer potential sources of contamination than when other speciation methods, such as solvent extraction, dialysis or ultrafiltration are used.

2. Range of Applicability of Electrochemical Speciation Methods

Electrochemical techniques can be used to provide speciation information based on labile/inert discrimination, redox state and half-wave potential measurements. The techniques are applicable to metals, non-metals, colloidal particles and organic compounds.

2.1. Labile/Inert Discrimination

The determination of labile (*i.e.*, reactive) metal involves the measurement of the concentration of metal in the water sample that can be reduced at, and deposited into, a mercury electrode from a stirred solution. Labile metal is usually expressed as a percentage of total dissolved metal, and the difference between total and labile metal is termed "inert" or "unreactive" metal. Some electrochemical parameters that affect the percentage of labile metal are deposition potential, electrode rotation (or stirring) rate, mercury drop diameter, pulse frequency, pH, temperature and buffer composition. Under certain conditions, labile metal has been found to correlate well with the toxic fraction of metal.[10,30]

Labile metal consists of free metal ion and metal that can dissociate in the double layer from complexes or colloidal particles, and hence be deposited in the mercury electrode.[4,5,31] For natural waters, anodic stripping voltammetry (ASV) is the technique usually used, and it has been applied to labile/inert measurements of Cu, Pb, Cd, Zn, Mn, Cr, Tl, Sb and Bi.[1] Heavy metal "pseudo-colloids," *i.e.*, colloidal particles of Fe_2O_3, MnO_2, humic acid, etc., with adsorbed heavy metal ions,[32] can be treated as a special type of metal complex, and may contribute significantly to some labile metal measurements.

Labile/inert discrimination for some elements may also be made by chemical, rather than electrochemical, exchange. This approach is particularly advantageous for metals such as Fe, which are difficult to determine by direct ASV, and where concentrations are too low for polarography. In one procedure,[33] labile Fe was determined by treating the sample with bismuth - EDTA, and the bismuth, liberated by chemical exchange [reaction (1)], was measured with high sensitivity by ASV.

$$Fe^{3+} + BiY^- \rightarrow FeY^- + Bi^{3+} \quad \ldots \quad \ldots \quad (1)$$
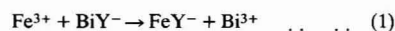
Total Fe was then determined in the same way, but after heating the acidified water sample to convert all iron into a reactive state. Another unusual measurement is the determination of labile (or solvent-accessible) sulphur in proteins by

Table 2. Toxicity and electrochemical lability of some species in natural waters[1,3,10]

Species						Toxicity	Electrochemical lability
As(III) ..	..	..	..	..	..	High	High
As(V) ..	..	..	..	..	..	Low	Low
Cr(III) ..	..	..	..	..	..	Low	Low
Cr(VI) ..	..	..	..	..	..	High	High
Tl(I)	..	..	..	..	..	High	High
Tl(III) ..	..	..	..	..	..	Low	Low
Cu^{2+} ..	..	..	..	..	.. ·	High	High
$CuCl_2$ ..	..	..	..	..	..	High	High
$CuCO_3$ ..	..	..	..	..	..	Low	High
Cu^{2+} - fulvic acid	..	..	..	..	..	Low	Low
Cu^{2+}/humic acid - Fe_2O_3			..	..	Medium	Medium
Cu^{2+} - DMP*	..	..	..	..	High	Low

* DMP = 2,9-dimethyl-1,10-phenanthroline.
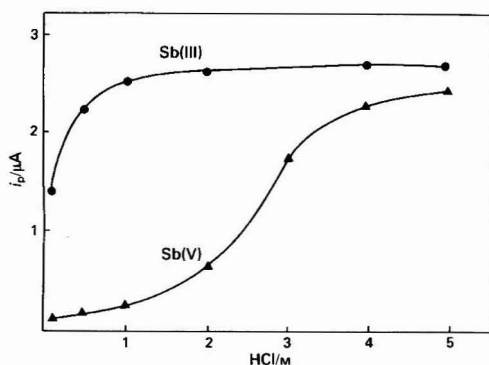
Fig. 1. Effect of HCl concentration on the ASV peak heights of Sb(III) and Sb(V)

the cathodic stripping voltammetric determination of sulphide ion liberated from protein disulphide bonds [reaction (2)].[34]

$$HC{-}CH_2{-}S{-}S^- + OH^- \rightleftharpoons HC{-}CH_2{-}S{-}O^- + HS^- \quad (2)$$
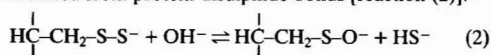
2.2. Redox State

Determination of the redox state of an element in solution is an important speciation measurement because it can drastically affect toxicity, adsorptive behaviour and metal transport (Table 2). Polarography and/or ASV have been used to distinguish between Fe(III)/(II),[35] Cr(VI)/(III),[36] Tl(III)/(I),[25,37] Sn(IV)/(II),[25,38] Mn(IV)/(II),[39] Sb(V)/(III),[25] As(V)/(III),[40] Se(VI)/(IV),[41] V(V)/(IV),[42] Eu(III)/(II),[42] U(VI)/(IV)[43] and I(V)/(−I).[44] Whereas Cr(VI) is anionic (chromate) and highly toxic, Cr(III) is non-toxic and may exist as anionic or cationic hydrolysed or organic species.[45] For some other elements, however, including Tl, As and Sb, the lower valency state is the more toxic.[46] The Mn(IV)/(II) discrimination[47] measurement is important because fine MnO_2 particles cause problems in water supply treatment plants by clogging filters.[39]

For several elements, redox state speciation is actually a special case of labile/inert discrimination, as one valency state is electrochemically active and the other inactive within the potential range of the electrode. Unreactive valency states of some elements under certain conditions are As(V), Cr(III), Mn(IV), Sb(V), Sn(IV) and Tl(III). Determination of the labile valency state of the element in the presence of these unreactive forms can be made by a simple ASV or polaro-

graphic measurement.[25] Total metal can then be determined after chemical treatment of the sample (e.g., chemical reduction) and the concentration of the inert valency state determined by difference. Some metal ions that are electrochemically inert because they are extensively hydrolysed in most media, e.g., Sb(V) and Sn(IV), become labile when the sample is made strongly acidic.[25] Fig. 1 shows the ASV behaviour of Sb(III) and Sb(V) as a function of acidity. Sb(III) can be determined in 0.2 M HCl, and total antimony in 6–8 M HCl, then Sb(V) by difference. Alternatively, and preferably, total Sb can be measured after reduction of Sb(V) to Sb(III) using hydrazine hydrochloride.[25]

Electrochemical methods for measuring valency state produce redox numbers directly, which is preferable to using ion-exchange methods to determine ionic charge. This latter technique may often lead to erroneous interpretations. A commonly used method for distinguishing Cr(VI) from Cr(III) is to pass the sample through a column of anion-exchange resin. Cr(VI) as CrO_4^{2-} is adsorbed, whereas Cr(III) is assumed to pass through the column as cationic species.[36,48] It has been suggested, however, that anionic $Cr(OH)_4^-$ is the most common form of Cr in natural waters.[49] In the polarographic method for Cr speciation,[36] Cr(VI) and total Cr are determined sequentially in acetate buffer with half-wave potentials of −0.3 and −1.8 V vs. SCE, respectively.

2.3. Half-wave Potential Shifts

Shifts in the polarographic half-wave potential or ASV peak potential of metal ions in the presence of complexing agents can provide information about the thermodynamic stability of complexes in solution.[42] However, quantitative deductions from these shifts, which have a sound theoretical basis for well defined experimental solutions containing one, or at the most two, ligands, are inapplicable to natural or polluted waters, which may have many unknown ligands and several metals. Under these conditions, quantitative interpretation of the shift is impossible, although some qualitative deductions can sometimes be made. For example, the ASV peak potential of Cu in sea water is about 0.2 V more negative than the peak in nitrate or acetate media. This shift reflects the relatively high stability of Cu(I) chloro complexes compared with those of Cu(II).[50] In high-chloride media Cu is stripped from the electrode in a one-electron reaction to form Cu(I) chloro complexes, whereas in nitrate solution Cu(II) is produced in a two-electron stripping step.

2.4. Limitations of Electrochemical Speciation Techniques

One of the main limitations of electrochemical speciation methods, the inability to measure the concentrations of individual ionic species, is common to most speciation techniques. Ion-selective electrode potentiometry (ISE) is the only method that can measure the activity of an individual ion, but the applicability of ISE to water analysis is severely limited by its poor sensitivity. Other electrochemical techniques, such as polarography and ASV, are dynamic systems that draw current through the solution and disturb ionic equilibria. It is not possible, for example, to use ASV to distinguish between labile cadmium species such as Cd^{2+}, $CdSO_4$, $CdCl^-$ and $CdCO_3$, which may coexist in a river water sample. A single ASV peak is obtained for a mixture of these Cd species. However, other speciation methods, including ion-exchange chromatography, solvent extraction, dialysis and ultrafiltration, also disturb the natural ionic equilibria in a water sample during the separation process, and all suffer from the same lack of specificity.[1]

Direct electrochemical speciation procedures are limited to measuring gross behavioural differences of groups of species (Table 3). This applies to the usual labile/inert discrimination,

Table 3. Electrochemical lability classification of metal species

Degree of lability	Examples[10,22–24,71,80]
Labile	$CdCO_3$, $CuCO_3$, $PbCl_2$, Cd‑NTA, Cu‑glycine, Zn‑cysteine, Cu‑citrate
Quasi‑labile	$PbCO_3$, $ZnCO_3$, Cu‑cysteine, Zn‑fulvic acid, Cd‑tannic acid, Cu^{2+}/humic‑Fe_2O_3, Cu‑NTA
Inert	Pb‑EDTA, $Pb_2(OH)_2CO_3$, Cu‑tannic acid,* Cu‑APDC,† Cu‑fulvic acid,* Zn‑tannic acid

* Sea water, pH 8.2.
† APDC = ammonium pyrrolidinedithiocarbamate (ammonium tetramethylenedithiocarbamate).

and to the effect of deposition potential on ASV peak height.[26,51] Other groups of species can be determined by ASV after chemical treatment of the sample (e.g., UV irradiation, acidification),[1–3,52] after physical separations (ion exchange, ultrafiltration, etc.)[53,54] or after chemical exchange reactions.[33] Although some deductions can be made about the nature of the species that are likely to occupy these behavioural "boxes," exact conclusions cannot be drawn.[55] As results from all these speciation procedures are *operationally defined*, it is most important when publishing speciation methods to report all details of the analysis so that results from different laboratories can be compared.

There has been considerable confusion in the literature over the ability of ASV to measure the "existing" or "natural" trace element speciation in a water sample. It is often specified[56–59] that buffer should not be added to a water sample before measurement of speciation by ASV, so as to avoid disturbing the natural ionic equilibria. However, because ASV is a dynamic technique, it cannot possibly measure the "natural" speciation, as the very act of measurement disturbs the equilibrium.[3,60] If the aim of the determination is to estimate the bioavailable fraction of the metal, some pH other than the natural pH of the water may well give the best correlation between ASV-labile metal and bioavailability (Section 7). The *purpose* of the measurement should always be kept in mind when designing a speciation procedure. For some purposes, however, it may be desirable to avoid changing the pH of the sample.[59] Sea water can be analysed without the addition of buffer, but some fresh waters have too low an ionic strength, and are too poorly buffered to be analysed directly by ASV.[24,61] It is possible to buffer the water and maintain its original pH by bubbling N_2 - CO_2 mixtures of controlled composition through the sample.[62,63] This is inconvenient, and hydrogen carbonate buffers are poorly poised and contribute little to the sample's conductivity.

An important potential interference in ASV, polarography and other electrochemical techniques is the adsorption of organic matter on the mercury electrode.[3,58,64] An adsorbed layer of organic matter may hinder the diffusion of metal ions, and thus diminish or eliminate the diffusion current and cause a non-linear relationship between stripping current and deposition time.[65] Alternatively, adsorption - desorption processes by organic dipoles on the mercury surface can yield "tensammetric" peaks when high frequency (a.c. or pulse) voltammetric techniques are used.[66,67] These tensammetric adsorption waves have no faradaic component, but in ASV are often mistaken for metal stripping peaks, as in natural waters they may appear at potentials similar to those found for Cd, Pb or Cu.[67] They can be readily distinguished from metal peaks because they are absent if a simple d.c. scan is used, their peak height is seldom proportional to deposition time, their peak potential is very sensitive to pH, and they disappear when the sample is UV irradiated.[67] Fortunately, tensammetric peaks

are uncommon in water analysis, but analytical chemists need to be aware of their existence.

Interference by adsorbed organic matter may be a more frequent problem in ASV speciation analysis, although it is often difficult to determine if a metal wave is diminished because of physical interference to diffusion, by formation of an inert organo complex or by a combination of the two processes. For a particular sample, the measured concentration of ASV-labile metal can only be operationally defined by the instrumental and solution conditions used and, in most instances, little information can be deduced about the electrode processes involved.

For standardising ASV-labile metal measurements, it is important to use an ionic metal peak-height calibration graph, rather than to attempt to quantify the results by standard additions ("spiking") of ionic metal to the sample. A metal spike may equilibrate only very slowly with the natural pool of physico-chemical species of the metal in the sample[7] and, even when equilibrated, the spiking experiment would give total, rather than labile, metal. The water blank used to construct the calibration graph should have an ionic composition similar to that of the sample.

A special type of interference occurs in ASV as a result of intermetallic formation in the mercury electrode.[1] These intermetallic compounds cause depression of the stripping peaks and shifts in peak potential. The most common interference is the depression of the zinc wave by an excess of Cu. In practical water analysis, however, this is rarely a problem, because metal concentrations are low, and Zn is usually present in excess of Cu.[68]

2.5. Speciation Schemes Combining Electrochemical and Other Techniques

Speciation information obtained from direct electrochemical analysis (e.g., labile/inert discrimination) can be supplemented by ASV or other measurements after various preliminary treatments of the sample. In this case, electroanalysis is simply used as a highly sensitive method of analysis. Some important preliminary speciation steps are as follows. (a) UV irradiation to destroy organic matter.[7,68–70] If the sample is irradiated at natural pH, only metal associated with organic matter will be liberated, and the increase in labile metal compared with the unirradiated sample represents metal bound in inert organic complexes or to organic colloids.[60,62,71,72] When the sample is acidified (0.02 M HNO_3) before irradiation, all forms of metal, including inorganic colloids, are converted into labile species and total metal is obtained.[69,71] (b) Determination of lipid-soluble complexes. Lipid-soluble metal species are likely to be highly toxic.[10,15,17,73] Extraction of a water sample with octan-1-ol or 20% butan-1-ol in hexane, or passage of the water through a column of Bio-Rad SM2 resin, will remove the lipid-soluble fraction of the metal.[71] Analysis of the aqueous phase or column effluent by ASV and subtraction from total metal gives lipid-soluble metal. (c) Chelating resin separation. Metal that cannot be removed from a water sample by a column of Bio-Rad Chelex-100 chelating resin represents metal bound in highly stable or inert complexes, or associated with colloidal particles.[70,71,74,75] However, the resin may remove some metal from colloidal particles.[71] (d) Ultrafiltration and dialysis. These techniques separate species on the basis of molecular size, and both can provide useful information about the size distribution of metal complexes and colloids,[1,3] although contamination can be a problem. In general, the smaller a metal complex, the higher is its biological activity.

Several comprehensive speciation schemes combining ASV and these preliminary treatments have been proposed.[1–3,7,20,76] The scheme used at present in this laboratory is shown in Table 4.

Table 4. Speciation scheme for copper, lead, cadmium and zinc in waters

Sample (*unacidified*):

Filter through a 0.45-μm membrane filter. Reject particulates and store filtrate unacidified at 4 °C.

Filtrate analysis:

Aliquot No.	Volume/ml	Operation	Interpretation
1	20	Acidify to 0.05 M HNO₃, add 0.1% H₂O₂ and UV irradiate for 8 h, then ASV*	Total metal
2	10	ASV at natural pH for sea water. Add 0.025 M acetate buffer (pH 4.7) for fresh waters	ASV-labile metal
3	20	UV irradiate with 0.1% H₂O₂ at natural pH, then ASV†	(3)−(2) = organically bound labile metal
4	20	Pass through small column of Chelex 100 resin. ASV on effluent‡	Very strongly bound metal
5	20	Extract with 5 ml of hexane - 20% butan-1-ol. ASV on acidified, UV-irradiated aqueous phase§	(1)−(5) = lipid-soluble metal

* Adjust to pH 4.7 with acetate buffer.
† Not valid if [Fe] >100 μg l⁻¹.
‡ Optional step.
§ Dissolved solvent in aqueous phase must be removed first.
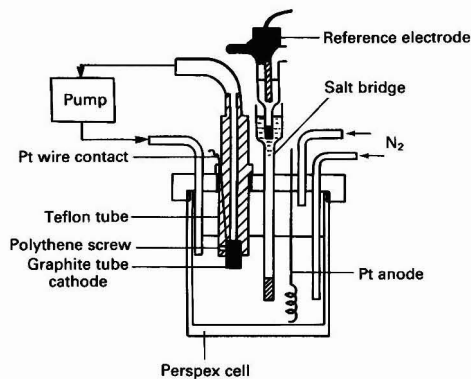
Fig. 2. Flow-through cell for electrodeposition on an AAS graphite furnace tube. Reproduced with permission from *Anal. Chem.*, 1980, **52**, 1570
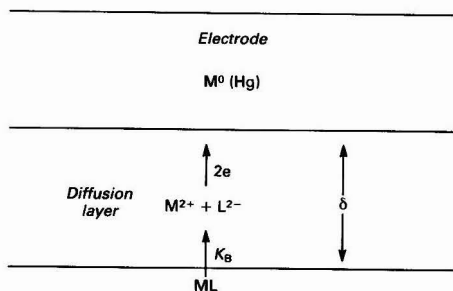
Fig. 3. Diagrammatic representation of the reduction of a metal complex at a mercury electrode. The degree of dissociation of the metal complex, ML, at the electrode (and hence the lability of the complex) increases with increasing K_B and increasing δ

2.6. Electrodeposition Prior to Carbon Furnace Atomic Absorption Spectrometry

A novel application of electrochemical techniques to speciation studies is the controlled-potential electrodeposition of

trace metals on to graphite furnace tubes, which are then transferred to an atomic absorption spectrometer for electrothermal measurement.[36,77,78] The advantage of this technique is that elements such as Cr, Ni and Co, which are difficult to determine by direct ASV, can be concentrated from solution by electrolysis using labile/inert discrimination, and determined with good precision. The furnace atomisation simply replaces the ASV stripping step. The flow-through electrolysis cell used by Batley and Matousek[78] is shown in Fig. 2; it was successfully applied to the discrimination of Cr(VI) and Cr(III) and the labile/inert forms of Ni and Co in natural waters; Another type of flow-through cell using a graphite furnace tube was described for the *in situ* determination of lead and cadmium in sea water.[79]

3. Theory of Labile/Inert Discrimination

3.1. The Electrodeposition Step

The dissociation of a 1 : 1 complex formed between a divalent metal ion, M, and a ligand, L, and the subsequent reduction of M^{2+} at a mercury electrode may be represented by the following equilibria:

$$ML \underset{k_f}{\overset{k_d}{\rightleftharpoons}} M^{2+} + L^{2-} \quad \ldots \quad \ldots \quad \ldots \quad (3)$$

$$\beta_1 = [ML]/[M][L] = k_f/k_d \quad \ldots \quad \ldots \quad (4)$$

$$M^{2+} + 2e^- \rightarrow M^0(Hg) \quad \ldots \quad \ldots \quad \ldots \quad (5)$$
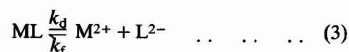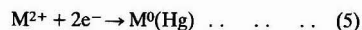
These reactions are shown diagramatically in Fig. 3. When the complex, ML, is not itself directly reducible, the electrolysis (faradaic) current is due solely to the reduction of M^{2+} ions dissociated from ML [reactions (3) and (5)]. This process leads to a *kinetically controlled* current, and i_k/i_d, the ratio of the kinetic current, i_k, to the diffusion current, i_d, is an index of the *lability* of the complex. The diffusion current is the current observed for the same concentration of metal ion, but in the absence of ligand. In the absence of kinetic control, $i_k/i_d = 1$. Turner and Whitfield[22] calculated that, for ASV at a thin mercury film electrode (TMFE),

$$i_k/i_d = (1 + \sigma\eta^{-1}\tanh\eta)^{-1} \quad \ldots \quad \ldots \quad (6)$$

and at a hanging mercury drop electrode (HMDE)

$$i_k/i_d = [r_0(r_0 + \delta)^{-1} + \sigma r_0(r_0\eta \coth \eta + \delta)^{-1}]^{-1} \quad .. \quad (7)$$

where $\sigma = k_f[L]/k_d$; $\eta = \delta\, D^{-\frac{1}{2}}(k_d + k_f[L])^{\frac{1}{2}}$; δ = diffusion layer thickness (cm); D = diffusion coefficient of the metal ion (cm^2 s^{-1}); and r_0 = radius of HMDE (cm). When $i_k/i_d > 0.99$ (i.e., a highly labile complex), it can be shown that[80]

$$\sigma/\delta[D/k_d(1 + \sigma)]^{\frac{1}{2}} < 10^{-2} \quad .. \quad .. \quad (8)$$

Turner and Whitfield[80] suggested the criteria shown in Table 5 for the definition of labile, quasi(or partially)-labile and inert (non-labile) complexes in ASV analysis at a rotating disc TMFE. The calculations assume that the ligand, L, is in large excess.

Davison[23] calculated the following criterion for a labile complex using ASV at a rotating disc TMFE, assuming a diffusion coefficient of 1×10^{-5} cm^2 s^{-1} for the metal ion: labile ($i_k/i_d > 0.90$):

$$\beta_1^{3/2}[L]/\delta k_f^{\frac{1}{2}}(1 + \beta_1[L])^{\frac{1}{2}} < 2.85 \times 10^3\, \text{cm}^{-1}\, \text{s}^{\frac{1}{2}} \quad .. \quad (9)$$

For both ASV and polarography, the lability of a complex depends not only on its dissociation kinetics, but also on the *effective measurement time*, which, with the constant electrolysis time of ASV, depends on the time the complex molecule is *resident* in the diffusion (or reaction) layer, and this resident time depends in turn on δ, the thickness of the diffusion layer. The larger the value of δ, the longer is the residence time of the complex in the diffusion layer, the greater is the opportunity for dissociation and deposition of metal in the electrode and hence the higher is the fraction of labile metal.[23] The thickness of the diffusion layer is governed principally by rotation rate for a rotating disc electrode (RDE) and by the rate of solution stirring for a HMDE. The Levich equation can be used to calculate δ (cm) at an RDE[23]:

$$\delta = 1.62\, D^{1/3}\, \omega^{-\frac{1}{2}}\, \nu^{1/6} \quad .. \quad .. \quad .. \quad (10)$$

where D is the diffusion coefficient (cm^2 s^{-1}), ω is the electrode rotation rate (rad s^{-1}) and ν is the kinematic viscosity of the electrolyte (Stokes). For rotation speeds in the range 10^2–10^4 rev min^{-1}, values for δ of 5×10^{-3}–5×10^{-4} cm are obtained. Kinetic control in ASV can be studied by measuring the effect of ω on i_k. A constant value of $i_k\omega$ is obtained in the absence of kinetic control, but decreases with increasing ω if kinetic effects are significant. Calculation of δ at an HMDE using a magnetic stirring bar for solution stirring is difficult because of the ill-defined hydrodynamic conditions. However, δ can be determined experimentally for an HMDE by measuring the d.c. diffusion current, i_d, in the stirred solution[23]:

$$i_d = nFADC/\delta \quad .. \quad .. \quad .. \quad (11)$$

where n is the number of electrons involved in the electrode reaction, F is the faraday, A is the electrode area and C is the concentration of electroreducible species. A typical value for δ at an HMDE is 2×10^{-3} cm. The diffusion layer thickness at

a dropping (or static) mercury electrode can be estimated from[81]

$$\delta = (\pi D t_e)^{\frac{1}{2}} \quad .. \quad .. \quad .. \quad (12)$$

where t_e is the effective measurement time, i.e., the drop time for maximum current d.c. polarography or, for pulse techniques, the duration of the applied pulse before the current is sampled plus the mean of the sampling interval.[23] For example, if the current is sampled for 20 ms, 40 ms into the life of the pulse, the average measurement time is 50 ms. With a.c. modulation, t_e is the inverse of the frequency (Hz). The reaction layer thickness, μ, which may be less than the diffusion layer thickness, is given from reaction layer theory as[26]

$$\mu = (Dk_f^{-1})^{\frac{1}{2}} \quad .. \quad .. \quad .. \quad (13)$$

Van Leeuwen[26] calculated from reaction layer theory, assuming a large excess of ligand, that the following conditions apply for polarographic lability measurements:

labile complex:	$k_d k_f^{-\frac{1}{2}} t_e^{\frac{1}{2}} \gg 1$	..	..	(14)
quasi-labile complex:	$k_d k_f^{-\frac{1}{2}} t_e^{\frac{1}{2}} \approx 1$	..	..	(15)
inert complex:	$k_d k_f^{-\frac{1}{2}} t_e^{\frac{1}{2}} \ll 1$	..	..	(16)

For polarographic conditions, it can also be shown that[23]

$$i_k/i_d = \pi k_f t_e/\beta_1^2[L] \quad .. \quad .. \quad .. \quad (17)$$

It is apparent that ASV lability, determined during the deposition step, depends solely on the kinetic parameters of the metal complex dissociation, the concentration of excess ligand and the diffusion layer thickness, which in turn is a function of the rate of stirring of the solution or the rotation speed of the electrode. In polarography the diffusion (or reaction) layer thickness is governed by the drop time or, in pulse techniques, by the pulse width and current sampling times. The value of i_k/i_d is *not* affected by factors such as deposition time, sample volume or cell volume. Deposition time may only affect i_k/i_d in the special case where an adsorbed substance interferes in a non-linear manner with the rate of electrodeposition.

An implicit assumption in the preceding discussions is that the metal complex, ML, is not *directly* reducible. However, where electrons are added directly to the complex without its initial dissociation in the diffusion layer, direct electrochemical reduction of some complexes is known to occur, especially at very negative potentials.[5,51,71,82] The presence of such complexes in a sample can be detected from the effect of ASV deposition potential, E_d, on peak current; the peak current will increase continuously with increasing E_d (Fig. 4) instead of increasing from zero to a limiting value over a small range of E_d (Section 5.5). To minimise the chance of directly reducible complexes contributing to the ASV-labile measurement, the deposition potential should be just sufficiently negative to yield the maximum peak current for the free metal ion in that medium, i.e., just on the plateau of the relevant pseudo-polarogram (Section 5.5). For this reason it is preferable in speciation analysis to determine each element separately, using the minimum deposition potential, rather than, e.g., measuring Cu, Pb, Cd and Zn simultaneously with a deposition potential of -1.3 V vs. SCE.

Reducible metal ions adsorbed on colloidal particles of humic acid, hydrated iron oxide, etc., can be treated as a special type of metal complex in the preceding theoretical discussions, and metal ions dissociated from pseudo-colloids at the solution/diffusion layer boundary may contribute to kinetic currents.[1,70,71] Although the involvement of pseudo-colloids in metal deposition has been questioned,[59] there seems no reason why ions could not dissociate from these

Table 5. Electrochemical lability criteria for lead complexes[80]

Description	Lability criterion*	Lead complexes concerned
Labile	$i_k/i_d > 0.99$	$PbCl^+$, $PbSO_4$
	$\log(\beta_1[L]^{\frac{1}{2}}) < 2$	
Quasi-labile ..	$i_k/i_d < 0.99$	$PbCO_3$, $PbOH^+$
	$i_k/i_d > (1 + \sigma)^{-1}$	Pb - humic acid
Inert	$i_k/i_d = (1 + \sigma)^{-1}$	Pb - EDTA

* β_1 = stability constant for $1:1$ complex; $[L]$ = concentration of ligand; i_k = kinetic current; i_d = diffusion current; $\sigma = \beta_1[L]$.
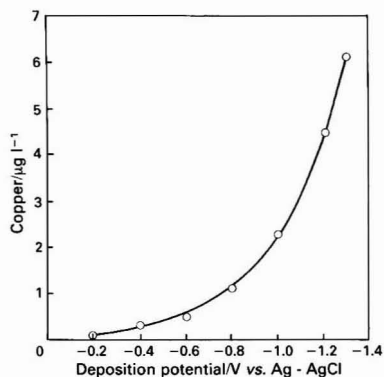
Fig. 4. Pseudo-polarogram of copper in sewage plant effluent water

Fig. 5. Diagrammatic representation of the transport of metal complexes through a biomembrane

particles at the diffusion layer boundary under the influence of the potential gradient. Metal ions are known to dissociate from colloidal particles as a result of the concentration gradient across a dialysis or ultrafiltration membrane,[34,83] and metal bonding to the particle is unlikely to be stronger than that involved in some ASV-labile molecular complexes such as Cu - NTA.[10,71] Pseudo-colloids, however, cannot act as *directly* reducible metal complexes, because the diffusion coefficients of colloidal particles are so small (10^{-7} cm^2 s^{-1}) that they would not contribute significantly to the peak current.[5,34]

3.2. The ASV Stripping Step

The preceding discussion has considered only the effect of deposition parameters on the amount of deposited metal and on kinetic currents in ASV speciation analysis. Ideally, the relative heights of the stripping peaks for labile and total metal in the sample, and hence the calculated percentage of labile metal, should be controlled solely by the preliminary electrodeposition step, *i.e.*, by the amount of metal deposited in the electrode. However, under certain circumstances the kinetics of the stripping process (electrooxidation), especially when pulse techniques are used, may have a significant, even dominant, effect on the stripping peak height. This can occur if a complexing agent present in the sample solution, but not in the standard, affects the stripping chemistry or kinetics.[22,27] This situation could arise from a number of causes. If a ligand in the sample solution stabilises an intermediate valency state of the metal, leading to a smaller number of electrons being involved in the electrochemical oxidation, lower stripping peaks will result:

$$\text{Standard:} \qquad Cu^0 \rightarrow Cu^{2+} + 2\,e^- \qquad \cdots \quad \cdots \quad \cdots \quad (18)$$

$$\text{Sample:} \qquad Cu^0 + 2\,Cl^- \rightarrow CuCl_2^- + e^- \quad \cdots \quad \cdots \quad \cdots \quad (19)$$
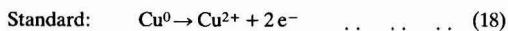
This has been observed for the ASV determination of Cu in the presence of chloride and some other ligands.[50,64,84]

The presence of complexing agents or surface-active substances may also affect the kinetics of stripping and lead to a change in peak height, especially when pulse techniques are used.[85] Buffle[27] showed how the large surface excess of the oxidised metal ion (compared with the bulk solution), present during the initial stages of stripping, can cause precipitation and other chemical reactions at the electrode surface that might affect the stripping peak current.

Perhaps the most useful method for ensuring that the ASV-labile measurement is controlled only by the deposition step is to use medium exchange where the sample solution,
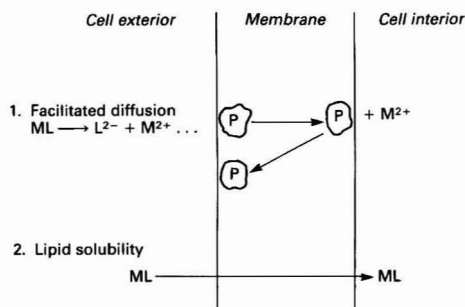
after electrodeposition, is replaced with a new supporting electrolyte in which stripping is carried out.[86,87] The new electrolyte would be chosen to yield reversible, reproducible stripping peaks for the element under study.

3.3. Comparison of Kinetics of Dissociation of Metal Complexes at an Electrode and a Biomembrane

For the study of aquatic toxicity by metals, the electrochemical and solution parameters should be chosen so that the ASV-labile fraction of total dissolved metal is similar to the toxic fraction.[10,88] Hydrophilic heavy metal ions are believed to be transported across the hydrophobic space of a biomembrane by the "shuttle" process of facilitated diffusion (or "host-mediated transport"), where a receptor molecule (*e.g.*, a protein) on the outer membrane surface binds a metal ion.[89,90] The hydrophobic metal - receptor complex then diffuses to the interior of the membrane and releases the metal ion into the cytosol where it is trapped, perhaps by reaction with a thiol compound.[91] The receptor then diffuses back to the outer surface of the membrane, ready to collect another metal ion (Fig. 5).[10,14-17] Alternatively, if the metal complex is lipid soluble, the much more rapid process of direct diffusion can take place (Fig. 5). Direct diffusion is basically different from facilitated diffusion, not only because it is faster, but because the ligand is also transported into the cytosol.[10]

The fraction of total metal in solution that can be transported across a membrane surface is equivalent to the bioavailable or toxic fraction. This in turn depends on the relative affinity of the metal for solution ligands and the receptor molecule (Fig. 5), or on the solution - membrane partition coefficient for a lipid-soluble complex.

The process of metal accumulation in an organism by dissociation of a metal complex at a membrane surface, facilitated diffusion of the metal through the membrane and deposition in the cytosol (Fig. 5), has obvious similarities to the process of ASV electrodeposition (Fig. 3), where the metal complex dissociates at the diffusion layer boundary and the metal ion travels through the diffusion layer to the electrode where the metal is deposited.

4. Electrodes for Speciation Measurements

The electrodes used most often for routine speciation measurements in natural waters are the hanging mercury drop electrode (HMDE), the thin mercury film (on glassy carbon) electrode (TMFE), and the dropping (or static) mercury electrode (DME). Other electrode systems have been used mainly in research and for special applications. Ion-selective electrodes will not be reviewed here because they are usually insufficiently sensitive for speciation analysis in natural waters, although they may have application to polluted waters.

4.1. Hanging Mercury Drop Electrode (HMDE)

The HMDE is widely used in ASV and speciation analysis. Use of simple d.c. techniques leads to stripping peaks with a drawn-out shape, owing to the slow diffusion of metal from the interior to the surface of the mercury drop. For this reason it is necessary to use high-frequency (pulse or a.c.) waveforms for ASV at the HMDE.[21] The high-frequency techniques respond only to dissolved metal at the surface of the mercury drop, and so the stripping peaks have a sharp, theoretical shape. With a 15-min deposition time, the limit of detection for Pb using differential pulse ASV (DPASV) at the HMDE was found to be 5×10^{-11} M, based on noise levels in the Princeton Applied Research (PAR) Model 174 voltammeter.[25] However, reagent blanks usually increase this limit substantially.

The use of a static mercury drop electrode (SMDE, Section 5.1) instead of the older micrometer screw-type hanging mercury electrode system greatly improves the reproducibility, reliability and simplicity of ASV at an HMDE, because the mercury drop is formed automatically and its size is very reproducible.[81]

4.2. Thin Mercury Film Electrode (TMFE)

While the use of a HMDE or a SMDE may offer better reproducibility than a TMFE, especially for Zn,[25] the rotated TMFE is much more sensitive (Table 6). This higher sensitivity is essential for high-purity samples such as open-ocean sea water, which cannot be analysed at the HMDE.[92,93] Whereas pulse techniques are essential for an HMDE, ASV at a TMFE can conveniently be carried out using a simple d.c. scan, because the mercury film is so thin that dissolved metal is stripped from the film very quickly. Use of differential pulse modulation at a TMFE decreases the limit of detection by a factor of about five over a d.c. scan.[94] The glassy carbon TMFE can be rotated (1000–3000 rev min⁻¹) or the solution stirred, although rotation gives more precise results.[5]

The substrate used for a TMFE is nearly always glassy carbon, polished to a mirror finish with diamond or alumina dust.[33,95] Glassy carbon is a commercially available, synthetic substance, almost as hard as diamond, but with good electrical conductivity and a wide potential range.[94,96] Like all forms of carbon, however, glassy carbon is rapidly attacked by free halogens,[97] so the electrode should never be polarised in the positive region when the solution contains halide ions. In chloride media, a film of mercury(I) chloride (calomel) forms on a TMFE if the electrode potential, E, is more positive than

$$E = +0.026 - 0.0296 \log[Cl^-]^2 \text{ V } vs. \text{ SCE} \quad .. \quad (20)$$

Table 6. Relative sensitivity of some electrochemical techniques

Electrochemical technique*				Limit of detection for lead/M[25,81,135]
D.c. polarography (DME)	..	..	..	2×10^{-6}
D.c. polarography (SMDE)	..	..	..	1×10^{-7}
D.p. polarography (DME)	..	..	..	8×10^{-8}
D.p. polarography (SMDE)	..	..	..	1×10^{-7}
D.p. anodic stripping voltammetry (HMDE)	..			2×10^{-10}
S.w. anodic stripping voltammetry (HMDE)	..			1×10^{-10}
D.c. anodic stripping voltammetry (TMFE)	..			5×10^{-11}
D.p. anodic stripping voltammetry (TMFE]	..			1×10^{-11}
S.w. anodic stripping voltammetry (TMFE)	..			5×10^{-12}

* D.c. = direct current; D.p. = differential pulse; S.w. = square wave; DME = dropping mercury electrode; SMDE = static mercury drop electrode; HMDE = hanging mercury drop electrode; TMFE = thin mercury film electrode.

This film of calomel seriously degrades the performance of the electrode, and is difficult to remove (ethanol is the best solvent).[94] Because even low chloride concentrations lead to calomel formation, in general a TMFE should not be polarised at potentials more positive than 0 V vs. SCE.[94] When first prepared, a glassy carbon electrode should be polished metallographically (diamond dust) and thereafter should only require polishing with wet and dry filter-paper (e.g., Whatman No. 541) after each analysis. If the electrode becomes contaminated with organic matter or metal hydroxides, wiping with filter-paper soaked in ethanol or 2 M HNO₃, respectively, will usually restore the surface. The mercury film should be removed by wiping with filter-paper, and not by anodic polarisation,[98] as this will degrade the surface if chloride is present.[94]

The mercury film may be electrodeposited on the glassy carbon substrate by two methods—pre-formed or *in-situ* deposition. The pre-formed method consists of electrodepositing a film of mercury from a stirred mercury(II) nitrate solution (1×10^{-4} M, pH 3–5, -0.6 V vs. SCE for 5–10 min). The plated electrode is then washed briefly and immediately used for analysis of the deaerated sample. A fresh film must be deposited for each sample. The *in situ* technique simply involves adding an aliquot of 1×10^{-2} M Hg(NO₃)₂ [kept in a dark bottle at pH 3 to prevent autoreduction to Hg(I)] to each sample to give a final concentration of $2 \times 10^{-5} - 4 \times 10^{-5}$ M Hg²⁺. During the deposition step of ASV, trace metals and Hg(II) are reduced simultaneously and codeposited, forming a very thin film of a dilute amalgam on the electrode. Measurements are usually made on the second or third deposition - stripping cycle, as the first deposition is needed to condition the electrode.[95] The mercury film thickness, l (cm), may be calculated from[25]

$$l = 2.43 \times 10^{-11} \, it/r^2 \quad .. \quad .. \quad (21)$$

where i is the limiting mercury(II) ion deposition current (μA), t is the deposition time (s) and r is the radius of the electrode surface (cm). Typical mercury film thicknesses used in the *in situ* technique are $5 \times 10^{-6} - 10 \times 10^{-6}$ cm. The *in situ*
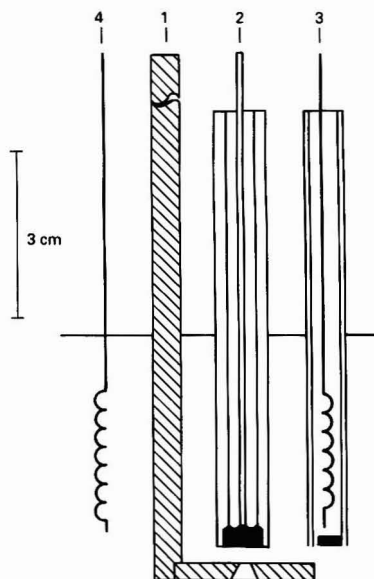

Fig. 6. Jet stream electrode. Reproduced with permission from *Croat. Chem. Acta*, 1977, **49**, L1

deposition method is much simpler than pre-forming a new film for each sample, and avoids the danger of oxidation of the pre-formed film before it can be transferred to the deaerated sample. Oxidised films give erratic results, especially for Cu.

It has often been claimed[56-58] that the *in situ* mercury film cannot be used for speciation studies because the addition of mercury(II) ions to the sample will change the "natural" speciation and cause an increase in labile metal as a result of Hg^{2+} exchanging with a metal complex, ML, and liberating free metal ion, M^{2+} (Section 2.4):

$$ML + Hg^{2+} \rightarrow HgL + M^{2+} \quad .. \quad .. \quad (22)$$
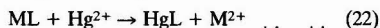
Certainly, mercury(II) forms very stable complexes with many ligands, and the exchange reaction [reaction (22)] may readily occur with labile metal complexes in natural waters. However, it is unlikely that this exchange reaction would significantly affect many ASV speciation results.[60] If the complex, ML, is sufficiently labile to undergo significant chemical exchange with Hg^{2+} during the period of the analysis (10–20 min), then it may also dissociate at the electrode surface and yield labile metal. If this occurred, the addition of Hg^{2+} would have no effect on the measured concentration of ASV-labile metal. Recent research using natural waters and synthetic waters containing various ligands showed that Hg^{2+} rarely has any effect on the ASV determination of labile Cu, Pb, Cd and Zn if only natural ligands are present.[99]

Stewart and Smart[100] showed that a glassy carbon TMFE covered with a dialysis membrane gave excellent results for the ASV determination of Cd. Wang and Hutchins[101] used a cellulose acetate film to cover a glassy carbon electrode, and found that electrode fouling by protein adsorption was greatly minimised. It would be most interesting to apply these covered electrodes to the determination of ionic metals in the presence of large organometallic complexes.

A special application of a glassy carbon TMFE is its use in a micro-cell using a 13 mm diameter membrane disc to adsorb the sample (15 µl).[102] The membrane disc with absorbed sample (containing Hg^{2+}) is dropped into the cavity of a Perspex block. The base of the cavity has flush-fitting platinum and silver discs acting as auxiliary and reference electrodes, respectively. The glassy carbon working electrode is mounted in a Teflon rod, which is made a sliding fit in the Perspex block. When the cell is screwed together, the membrane disc is compressed between the glassy carbon electrode and the other two electrodes. An O-ring seals the cell. Oxygen is removed by applying a potential of -1.4 V vs. Ag - AgCl for 20 s. Conventional ASV-labile measurements can then be made. Discs of an ultrafiltration membrane or Chelex-100 paper can be placed between the glassy carbon electrode and the sample disc to provide additional speciation measurements.[102] A similar filter-paper ASV cell using a mercury pool electrode has also been described.[103]

4.3. Jet Stream Mercury Film Electrode

A new method for transporting sample to the surface of a glassy carbon TMFE was described by Magjer and Branica.[104] In this technique, instead of rotating the electrode, or stirring the solution, a flat disc having a conically shaped hole is positioned below the glassy carbon electrode (Fig. 6). The disc is then vibrated at high frequency in a vertical plane, forcing solution on to the electrode surface in a jet stream. The sensitivity of the electrode is critically dependent on the geometries of the vibrating disc and the conical hole, but if these parameters are optimised, higher sensitivity than rotation or stirring can be achieved.[105]

4.4. Flow-through Cells

A variety of flow-through cells designed for on-line stripping analysis have been described.[106-110] These cells often have dual mercury-plated glassy carbon or reticulated vitreous carbon[108] electrodes with independent potential control for removal of dissolved oxygen or interfering elements at the upstream electrode.[110] Labile/inert speciation measurements are possible with these electrodes, although they have seldom been used for this purpose. Flow-through cells used for high-performance liquid chromatography could also be used for speciation measurements in a closed-loop system.[78] The wall-jet electrode, in which a jet of the sample impinges on the working electrode,[111] should provide excellent sensitivity and, because of its rapid hydrodynamic characteristics, could yield data on the dissociation kinetics of metal complexes.

4.5. Streaming Mercury Electrode

The streaming mercury electrode (SME), first used by Heyrovsky and Kuta[42] for oscillographic polarography, involves forcing a thin stream of mercury through a short path of the test solution, using a mercury reservoir to provide the necessary pressure. The electrode thus consists of a short, rapidly changing cylinder of mercury. A modified SME that uses less mercury and gives more reproducible results has been described by Florence and Farrar.[112]

A unique characteristic of the SME is that the electrode is being constantly and very rapidly renewed, so that only fast electrochemical reactions are registered and, most important, substances that adsorb on mercury have little or no effect.[112] Although the use of the DME with high-frequency techniques can also discriminate against slow electrode reactions, organic matter (e.g., humic acid) adsorbed on the electrode can seriously affect the results. However, with the SME the electrode is being renewed so rapidly that adsorption has little chance to occur and so has a negligible effect on electrode kinetics.[112] The SME has not yet been applied to speciation measurements, but it may prove especially useful, in conjunction with differential pulse or square-wave modulation, for measuring free metal ion in the presence of metal complexes and surface-active substances.

4.6. Carbon Fibre Electrodes

Electrodes consisting of minute carbon fibres (5–10 µm diameter, 0.1–0.3 cm length), either bare or mercury coated, are finding increasing use in electrochemistry. The electrodes exhibit low background current and, because of the extremely low cell current, the *iR* drop in the solution is negligible.[113-115] These low cell currents provide an analysis that is essentially non-destructive, so *in vivo* analysis, e.g., in the brain, can be made without damage to the animal.[116] Analysis can be carried out in the absence of supporting electrolyte and in aprotic organic solvents.[114] A two-electrode system may be used, thus avoiding the need for a potentiostat, which is expensive and is a major source of electronic noise.[114] Carbon fibre electrodes have considerable potential for speciation analysis *in vivo*.[117]

4.7. Chemically Modified Electrodes

Electrodes that have been coated with a chemical that alters their characteristics are now widely used in electrochemistry, and some systems have been applied to electroanalysis.[118] The substrate may be platinum,[119] carbon paste[120] or glassy carbon.[121] Polymers such as poly(vinylbipyridine) and vinyl-ferrocene can be electrodeposited on a platinum electrode, or groups such as $-Si(CH_2)_3NHCOCOOH$ directly bonded to oxide groups on glassy carbon.[121]

The use of surface-active metal complexes in cathodic stripping voltammetry has produced extremely sensitive methods for some metals. One of the early applications of this technique was to the determination of total and reactive Al in waters using linear scan voltammetry and the di-*o*-hydroxyazo

dye Solochrome Violet R5.[122] Very sharp, peak-shaped voltammograms were obtained with a limit of detection of 0.2 μg l^{-1} of Al as a result of adsorption of the aluminium - dye complex on the mercury drop.[123] More recent applications have involved the use of adsorbed films of dimethylglyoxime for Ni and Co,[124] ammonium tetramethylene dithiocarbamate for Zn,[125] catechol for Cu, Fe, U and V[126-130] and 8-hydroxy-quinoline for Mo,[131] all using cathodic stripping voltammetry at an HMDE. Metal concentrations as low as 10^{-10} M can be determined with a short deposition time,[5] and labile and inert metal species in a water sample can be determined on the basis of their reactivity with the organic ligand.[132]

5. Electrochemical Techniques for Speciation

5.1. Polarography

In natural waters, even using differential pulse or square-wave modulation, polarography is not sufficiently sensitive for speciation measurements of most elements. Many of the elements of interest (*e.g.*, the toxic elements) are present in the range 10^{-10}–10^{-8} M, whereas polarography is limited to concentrations above 10^{-7} M. For iodine speciation in sea water, however, polarography is an ideal technique for determining iodate to iodine ratios.[44] Iodate is usually present in sea water at concentrations of about 3×10^{-7} M and, because its reduction involves six electrons, a large and sharp polarographic peak is produced. Polarography may also find application for speciation studies of polluted waters that have much higher metal concentrations. The technique is especially useful for valency state discrimination (Section 2.2).

The development of the static mercury drop electrode (SMDE) has greatly simplified and improved polarographic analysis (Section 4.1).[133] Whereas the conventional (Hey-rovsky) dropping mercury electrode produces a gravity-fed mercury drop of continuously changing area, the SMDE has a constant area when the current - voltage curve is recorded, thus essentially eliminating charging current due to drop growth.[81] This advantage of the SMDE is achieved by using a wide-bore capillary, through which the mercury flow is controlled by a valve that can be opened for variable times. This allows drops of different size to form very quickly. The voltage scan is applied after the valve has been closed and the drop is stationary. After completion of the scan, the drop is mechanically detached. Because the charging current is so small with an SMDE, the advantages of pulse techniques over a simple d.c. scan are only marginal (Table 6).[81] The Metrohm SMDE has outstanding performance and uses inexpensive, wide-bore capillary tubing for the electrode. Bond *et al.*[134] designed an efficient, high-capacity flow-through cell for use with the EG and G PAR Model 303 SMDE.

5.2. Anodic Stripping Voltammetry

Anodic stripping voltammetry is the most widely applicable electrochemical technique for trace element speciation in waters.[21] Because of the "built-in" concentration step in ASV, extremely high sensitivity can be obtained. At present, the most sensitive commercially available ASV technique is square-wave stripping at a glassy carbon TMFE (Table 6).[135] In an unmodified (*i.e.*, no pre-treatment) sample such as open-ocean sea water, metal concentrations below 10^{-11} M can be determined although, for many analyses, the limit of detection is set by the blank, and not by the intrinsic sensitivity of the technique.[25] Differential pulse voltammetry is a factor of two or three less sensitive than the square-wave method,[135] and an HMDE is 5–10 times less sensitive than a TMFE.

In most instances, ASV calibration for labile metal is best carried out by the use of measurements on separate standard solutions, rather than by making standard additions ("spik-ing") to the test solution. In many natural waters, excess of

organic matter (*e.g.*, fulvic acid) in the sample will complex the metal spike, and the increase in peak height will be related to total, rather than labile, metal. If the concentration of the spike is high, *i.e.*, at least 20 times that of the complexing agents in the sample, then the peak-height difference between the first and second spikes can be used to calculate labile metal. These high spikes, however, may lead to metal contamination of the cell. It is more accurate to use a matched matrix with standard metal concentrations similar to that of the sample.

Dissolved oxygen is a serious interferent in ASV, and care must be taken to remove it completely. Ideally, the ASV cell should be under a positive pressure of oxygen-free nitrogen but, if this is not possible, the cell should be sealed as well as possible, and a rapid flow of inert gas maintained at all times. If the gas flow is too vigorous, however, solution spray in the cell may cause memory effects. Mechanically detached DMEs pose a special problem, because the cell must have a slot to allow for movement of the electrode. It is better to use a high-quality grade of oxygen-free nitrogen than to complicate the system (with the possibility of air leaks through the tubing connectors) by installing an oxygen scrubbing system. Oxygen contamination is much more likely to originate from air ingress into the cell or through tubing than from impurity in the sparging gas.

Dissolved oxygen can cause an apparent increase in the Cu and Pb stripping peaks[79,103] and, in unbuffered solutions, a decrease in the Cd peak as a result of the consumption of hydrogen ion at the electrode surface:

$$O_2 + 4 H^+ + 4 e^- \rightarrow 2 H_2O \quad .. \quad .. \quad (23)$$
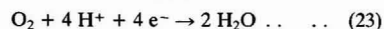
In many supporting electrolytes, oxygen contamination is manifested by a broadening of the copper stripping peaks.

5.3. Cathodic Stripping Voltammetry

Cathodic stripping voltammetry (CSV) involves the cathodic stripping of an insoluble film of the mercury salt of the analyte (H_2L) deposited on the working electrode:

$$\text{deposition: } Hg + L^{2-} \rightarrow HgL + 2 e^- \quad .. \quad (24)$$

$$\text{stripping: } HgL + 2 e^- \rightarrow Hg + L^{2-} \quad .. \quad (25)$$
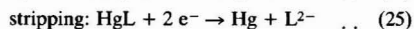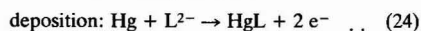
CSV has not yet found a great deal of application in trace element speciation. As(III) and Se(IV) can be determined in the presence of their higher valency states,[1,3] sulphide can be measured in a large excess of other inorganic or organic sulphur compounds[34,136] and the recently developed adsorp-tion - CSV technique can be used to determine free metal ion plus labile complexes for Ni, Co, Cu, Zn, Fe, V, U and Mo (Section 4.7).

5.4. Potentiometric Stripping Analysis

Potentiometric stripping analysis (PSA), largely developed by Jagner[137] in Sweden, uses the same initial step as ASV, *i.e.*, metal is deposited into a TMFE at a controlled potential. However, instead of applying a voltage ramp to oxidise and strip the metal, a chemical oxidant (O) in solution is allowed to diffuse to the electrode to oxidise the deposited metal, and the potential of the working electrode is followed as a function of time[138]:

$$\text{deposition: } M^{2+} + 2 e^- \rightarrow M^0(Hg) \quad .. \quad (26)$$

$$\text{oxidation: } M^0(Hg) + O \rightarrow M^{2+} + R^{2-} \quad .. \quad (27)$$
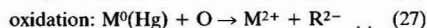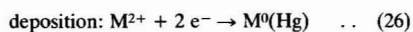
Well separated potential - time steps are obtained as the metals are successively oxidised by oxidants such as dissolved oxygen or mercury(II) ion. PSA is much less affected by adsorbed organics than is ASV,[137,139] and redox compounds do not interfere with the analysis.

As(III) has been determined in the presence of As(V) by PSA,[140] but there has been little other interest in applying the technique to speciation analysis.

5.5. Pseudo-polarography

A pseudo-polarogram is a plot of ASV stripping peak current *versus* deposition potential. The half-wave potential (E_c) of a pseudo-polarogram of a metal is related to (but not identical with) the polarographic half-wave potential ($E_{\frac{1}{2}}$). The value of $E_{\frac{1}{2}} - E_c$ becomes increasingly positive as the rate constant of the electrochemical reaction increases.[141]

Pseudo-polarograms may have a classical polarographic shape, or the peak height may increase continuously with deposition potential (Fig. 4). This latter behaviour implies that metal complexes are present that are directly reduced, *i.e.*, they diffuse intact to the electrode surface without first dissociating in the diffusion layer to metal ion and ligand.[71]

Brown and Kowalski[141] demonstrated the application of pseudo-polarography to a study of the speciation of As, Cd and Pb in various natural waters. Valenta[142] used pseudo-polarography to identify Pb carbonate complexes in sea water, while Bubic and Branica[143] used the same technique to study the ionic state of Cd in seawater.

5.6. Modulation Waveforms

Modulating the d.c. voltage ramp with various waveforms provides increased sensitivity in ASV, especially when a mercury drop electrode is used.[135] At present, the most commonly used modulation waveforms in stripping analysis are differential pulse and square wave. However, a.c. and staircase waveforms have also been used.[144,145] The use of microcomputers[21,146,147] in electrochemical instrumentation allows a wide range of waveforms to be applied to the cell to optimise the analysis, in terms of sensitivity and selectivity, for a particular sample type. Square-wave and staircase voltammetry have the advantage over the differential pulse technique that much faster scan rates can be used,[145] up to 2 V s⁻¹ with a square-wave frequency of 200 Hz, so that a complete voltammogram can be obtained in less than 1 s, and on a single drop in polarography.[135,148] Differential pulse voltammetry cannot utilise scan rates in excess of 5 mV s⁻¹, so that scanning from the Zn to the Cu ASV peaks takes at least 4 min. A disadvantage of pulse and square-wave techniques is that they are more affected than linear scan voltammetry by substances that adsorb on the mercury electrode.[25] Adsorbed layers interfere seriously in differential pulse ASV because of the multiple redox reactions that occur at the electrode during deposition.[149] It must be appreciated that different modulation waveforms will give different results in labile/inert ASV determinations.

6. Some Speciation Results Using Electrochemical Techniques

6.1. Collection and Preservation of Water Samples for Speciation Measurements

Detailed instructions have been given for the contamination-free collection of water samples for trace element speciation analysis.[1,3,29,150] In general, samples should be collected in linear polyethylene bottles, which are initially acid cleaned, then reserved for collecting the same type of water. Special procedures are required for some elements such as mercury and iodine.[1] The collected water sample cannot, of course, be preserved by adding acid, as this alters the element speciation. Freezing of water samples is also prohibited for trace heavy metal speciation, because concentration of the solutes during the freezing process may cause hydrolysis of metal ions and other reactions that are irreversible, or only slowly reversible, on thawing.[3] The safest preservation procedure is to filter the

sample immediately after collection and store the filtered sample at 4 °C. The concentrations of Cu, Pb, Cd and Zn in both fresh water and sea water samples remained unchanged for several months under these conditions.[3,70,151] Reports of serious adsorption of these metals on to polyethylene containers can be traced to the use of ionic spikes (either stable or radioactive) in the water samples to measure such losses.[3] Whereas ionic metal rapidly partitions to the walls of the plastic container, the naturally present metal in pristine water samples, very little of which is in the ionic form, has a low affinity for both polyethylene and glass.[3,70] In polluted waters, however, ionic metal may persist close to the source of pollution or when the complexing capacity of the water is exceeded. In such instances, losses may occur on storage. On the other hand, storage of ultra-pure waters in polyethylene containers may lead to zinc contamination from the plastic.[3,152]

Filtration and any other manipulation of a water sample should be carried out in a clean room or a clean air cupboard. Electroanalysis should also be carried out in a clean room, glass electrolysis cells should be siliconised and the cell and electrodes should be rinsed copiously with high-purity water.[153]

6.2. Selected Electrochemical Speciation Results for Some Trace Metals in Waters

6.2.1. *Copper*

Computer chemical models for the speciation of inorganic Cu in sea water predict that the carbonato and hydroxy complexes are the dominant species.[1,3,154] The computed distribution of these complexes varies widely with the models used,[3,155] but the latest calculations[153] indicate that in sea water at pH 8.2, 25 °C and a total alkalinity of 2.3 mequiv. kg⁻¹, inorganic Cu exists as $CuCO_3^0$ (82%), $CuOH^+ + Cu(OH)_2^0$ (6.5%), $Cu(OH)(CO_3)^-$ (6.3%), $CuHCO_3^+$ (1.0%) and Cu^{2+} (2.9%). These species are all believed to be ASV labile.[24,155] In a typical fresh water, more than 90% of inorganic Cu should be present as $CuCO_3$, although some is likely to be associated with colloidal particles of hydrated iron oxide.[1,3,50,156]

Coastal surface sea water usually has 40–60% of total Cu (the total copper concentration in surface Pacific water off Sydney is 0.3–0.8 μg l⁻¹ [151]) present as inert organic complexes.[3,11] These complexes are so stable that they pass essentially unchanged through columns of iminodiacetate (Chelex 100) or thiol resins.[71] It has been suggested that the Cu-binding ligands are siderophores, metallothioneins or porphyrins.[15,71] In unpolluted sea water, ASV-labile copper usually comprises less than 50% of total dissolved Cu, even at a pH as low as 4.7 (Table 7).[3,71,151] Most of the inert Cu is organically bound, but a significant fraction is inorganic, probably adsorbed on colloidal particles of hydrated iron oxide, which are perhaps coated with humic acid.[7,84,157,158] Most fresh water streams also have little ASV-labile Cu, and the percentage of organically bound Cu is usually high.[70,159–161]

Industrially polluted waters sometimes exhibit Cu pseudo-polarograms (Section 5.5) that do not have a plateau, but which give continuously increasing peak currents with increases in deposition potential (Fig. 4). Such behaviour indicates the presence of directly reducible copper complexes.

The determination of the activity of free copper(II) ion using the Cu ion-selective electrode is unreliable in chloride media.[162]

6.2.2. *Lead*

Computer modelling of fresh waters suggests that carbonato species, *e.g.*, $PbCO_3$ and $Pb_2(OH)_2CO_3$, are the main (*ca.* 90%) inorganic Pb species,[1,3] whereas in sea water speciation is divided between carbonato complexes (83%) and chloro species (11%).[163] Calculations by Turner and Whitfield[80] and

Table 7. Concentrations and ASV-labile fractions of dissolved metals in surface sea water

Metal	Concentration (ng l^{-1}) and labile fraction (%) in parentheses[1,3]	
	Open ocean	Near shore
Cu	120	350 (45)
Pb	14	250 (25)
Cd	15	75 (85)
Zn	10	1500 (50)
Ni	150	500 (70)
Fe	750	3500 (<20)
Mn	60	1500 (20)

Valenta[142] suggest that in sea water the carbonato and hydroxy complexes are only partially ASV labile.

Unlike Cu, Pb has a stronger affinity for some inorganic adsorbents, especially iron oxide, than for organic ligands, and it is likely that in most natural waters with pH above 7 a significant fraction of the Pb is associated with hydrated Fe_2O_3.[1,3] Batley and Gardner[151] found that in sea water 40–80% of dissolved Pb was present in the inorganic colloid fraction, whereas in some low pH (pH 6.0) fresh waters most of the Pb appeared as an electroinactive inorganic molecular species, possibly $Pb_2(OH)_2CO_3$.[70] Most natural waters have little ASV-labile Pb (Table 7).[31,164]

Alkyllead species in natural waters may be determined by ASV[165] after selective organic phase extraction.[166]

6.2.3. *Cadmium*

In sea water, Cd is computed to exist as the CdCl$^+$ and CdCl$_2{}^0$ complexes (92%), whereas in river water the dominant inorganic forms are Cd^{2+} and CdCO$_3$, depending on pH.[1,3,163] A high proportion (>70%) of Cd is ASV labile in both sea water[151] and fresh waters (Table 7).[70] Because Cd ions are adsorbed on colloidal particles at only relatively high pH,[1,3] very little Cd is present as pseudo-colloids. In anoxic waters, Cd may exist as non-labile CdHS$^+$.[3,151,163]

Cd contamination during analysis can occur via rubber O-rings or seals and colour-code markings on pipettes.[1,3]

6.2.4. *Zinc*

The main Zn species computed to be present in sea water are Zn^{2+} (27%), chloro complexes (47%) and ZnCO$_3$ (17%), whereas in fresh waters the dominant inorganic forms are Zn^{2+} (50%) and ZnCO$_3$ (38%).[1,3,163] The carbonate complexes of Zn, and especially the basic carbonates, may have low ASV lability.[70,167,168] Only about 50% of the total Zn in sea water and river water is ASV labile (Table 7) or extractable by ammonium tetramethylenedithiocarbamate, even though added ionic Zn spikes are completely extractable.[7,70,168] Open ocean water contains as little as 10 ng l^{-1} of Zn at the surface,[169] although coastal sea water usually contains 0.5–2 µg l^{-1} of Zn as a result of river inputs and sewage outfalls.[3,168,170]

Zn determinations at the sub-µg l^{-1} level are extremely difficult because of contamination problems, which may originate from a variety of sources including paint, skin, clothing, plastics, rubber, filter membranes, reagent chemicals and vapour from copying machines.[1,168] The HMDE generally produces more precise results for Zn than does a TMFE, because small changes in hydrogen overpotential on a Hg-coated glassy carbon electrode affect the efficiency of Zn electrodeposition.

High Cu concentrations depress the Zn ASV stripping peak as a result of the formation of intermetallic compounds in the Hg.[171] This interference, however, is rare in natural water analysis.

6.2.5. *Manganese*

The natural water chemistry of Mn is dominated by non-equilibrium behaviour.[3] Oxidation of Mn(II) to Mn(IV), *i.e.*, to MnO$_2$, is thermodynamically favoured in sea water and high pH fresh waters, but the oxidation is extremely slow unless catalytic bacteria are present.[172] Colloidal MnO$_2$ is troublesome in water treatment plants because it blocks filters and causes discolouration. Both polarography[173] and ASV[47] have been used to determine labile Mn [Mn^{2+} and Mn(II) complexes] in the presence of electroinactive MnO$_2$. Mn(III), formed from the oxidation of Mn(II) by the algae-produced superoxide radical (O$_2{}^-$), may also be present.[172]

Knox and Turner[39] found that in samples from the Tamar Estuary (S.W. England), the polarographically detectable Mn level varied, over a 6-month period, from <10% up to 100% of total manganese (31–252 µg l^{-1}) (Table 7).

7. Correlation Between ASV-labile Measurements and Toxicity

Variation in the speciation of trace elements can dramatically change their toxicity. Most studies of the toxicity of heavy metals to fish and other aquatic organisms have shown that the free (hydrated) metal ion is the most toxic form, and that toxicity is related to the activity of free metal ion rather than to total metal concentration.[3,10,13,15,16,174–179] Toxicity usually decreases with increasing water hardness or salinity, presumably because of increased metal complexing by inorganic ligands.[14,180] Nature has provided aquatic animals with effective defences against ingested heavy metals, which are eliminated via the gut,[89,181] or detoxified in the liver, kidneys and spleen by a group of high-sulphur proteins, the metallo-thioneins, which are synthesised in these organs in response to a heavy metal challenge.[15] These defences allow the animal to cope with fairly high levels of heavy metals in the food chain and sediment; toxicity occurs only with "spillover," *i.e.*, when the metal intake exceeds the body's ability to synthesise metallothionein. Evolution has not, however, equipped animals to tolerate free metal ion in the water that contacts their gills or other exposed biomembranes.[15] Unpolluted sea water or river water contains very little free metal ion, most of the dissolved metal being present as non-toxic complexes (*e.g.*, with fulvic acid) or adsorbed on colloidal particles (*e.g.*, humate-coated Fe$_2$O$_3$ or fibrils[182]). Natural waters use these detoxification mechanisms to convert free metal ions into non-toxic forms, but considerable damage can be caused close to the source of pollution if the complexing capacity (Section 8) of the water is exceeded.

Cu(II) ions bind initially to marine phytoplankton with a stability constant, log β$_1$, in the range 10–12, complexing apparently occurring via protein amino and carboxylic acid groups.[183] Cu is then transported across the membrane by a carrier protein (facilitated diffusion),[184,185] where it reacts with a thiol (possibly glutathione) in the cytosol or on the interior surface of the membrane, and is reduced to Cu(I).[183] Reaction with thiols and thiol-containing enzymes may be a common toxic effect of heavy metals, although deactivation of enzymes such as catalase by metal substitution may also be involved.[183,184]

Although there is considerable evidence that free metal ion is the most toxic metal form, the situation is not completely clear.[14,16] Some studies suggest that other species, such as the Cu hydroxy complex[175] and the Cu complexes of citrate and ethylenediamine,[186] are also toxic. In addition, lipid-soluble Cu[10,15,73] and Hg[15] complexes are extremely toxic, and a step to measure lipid-soluble metal complexes should be included in all trace element speciation schemes for polluted waters (Section 2.5).

Attempts to use ASV-labile measurements to determine the toxic fraction of a metal have met with varied success.[3,10,30,176,187,188] Young *et al.*,[30] using larval shrimp as a test

Table 8. Correlation between ASV-labile and toxic fractions of copper in sea water using the marine diatom *Nitzschia closterium*

Ligand[10]*	Concentration	Copper/ M × 10⁷	ASV-labile fraction,† %		Toxic fraction,‡ %
			−0.6 V	−1.3 V	
Fulvic acid	1×10^{-5} M	3.2	1.5	2.9	7.5
Tannic acid	6×10^{-7} M	3.2	5.5	10.5	12.5
Iron - humic acid colloid§	$1.0 + 5.3$ mg l⁻¹§	3.2	70	74	60
NTA	2×10^{-5} M	3.2	100	100	20
LAS	0.5 mg l⁻¹	3.2	65	100	25
8-Hydroxyquinoline	5×10^{-8} M	0.32	64	100	>100
DMP	5×10^{-8} M	0.32	2.5	—	>100
Ethyl xanthogenate	2×10^{-6} M	3.2	10.5	48	>100

* NTA = nitrilotriacetic acid; LAS = linear alkylbenzene sulphonate; DMP = 2,9-dimethyl-1,10-phenanthroline.
† pH 8.2, with deposition potential of −0.6 or −1.3 V *vs.* Ag - AgCl.
‡ Fraction of added Cu appearing toxic compared with ligand-free solution.
§ 1.0 mg l⁻¹ of Fe + 5.3 mg l⁻¹ of humic acid.

Table 9. Complexing capacity of some natural waters

Source of water	Complexing capacity[1,191]† M × 10⁶ Cu²⁺	pH‡	Log *K§
Lake Ontario	0.34	7.4	8.6
Chapel Hill Lake	31	6.0	5.0
Swiss Lakes	2.7	8.8	10.9
Newport River	0.87	7.0	9.7
Neuse River	0.21	6.8	9.5
Magela Creek, Australia	0.10–0.46	6.0	7.6
Pacific Ocean, coastal	0.02–0.2	4.8	—

† ASV titration with Cu²⁺.
‡ pH of titration.
§ Conditional stability constant of Cu complex.
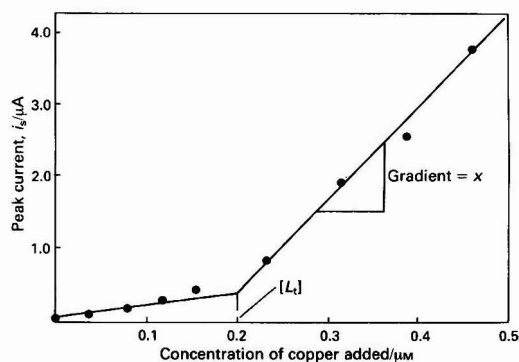
Fig. 7. Complexing capacity titration of a natural fresh water

species, found a good correlation between ASV-labile Cu and toxicity, whereas Srna *et al.*[187] reported that ASV gave values that were only half those measured by bioassay. Florence *et al.*[10] found that ASV-labile Cu, determined in sea water using a low deposition potential, correlated well with Cu toxicity towards the marine diatom *Nitzschia closterium* when natural complexing agents, including fulvic, humic, tannic and alginic acids, and hydrated iron oxide, were present in the growth medium. However, when synthetic ligands such as nitrolotriacetic acid (NTA), 8-hydroxyquinoline or ethyl xanthogenate were present, there was no sensible correlation (Table 8). The fraction of total dissolved Cu removed by a column of Chelex 100 resin grossly overestimated the toxic fraction.[10] ASV-labile metal might therefore be a simple and reasonable method for measuring the toxic fractions of metals in natural

waters, but could be inapplicable if synthetic ligands are present.

8. Complexing Capacity

Natural waters contain a variety of metal complexing agents, including fulvic, humic and tannic acids, lignin and colloidal particles of Fe_2O_3, Al_2O_3 and MnO_2.[1,189] Polluted waters may contain additional natural and synthetic compounds. The concentrations of these ligands are usually well in excess of those of the metals present, and the determination of this excess "metal complexing capacity" is an important water quality parameter because it is a measure of the concentration of heavy metal that can be discharged to a waterway before free metal ion appears.[1,3,190-202]

Complexing capacity is determined by titrating the water sample with a heavy metal ion; Cu(II) is usually chosen as the titrant because it is a common heavy metal ion, highly toxic to aquatic organisms.[65] Complexing capacity is then defined as the concentration of Cu(II) ion (mol l⁻¹) that must be added to a water sample before free Cu²⁺ appears. It reflects the concentration of organic and inorganic substances in the water sample, both molecular and colloidal, that bind (and detoxify) Cu ions. Near-shore surface sea water has a Cu-complexing capacity of about 2×10^{-8} M, whereas that of river waters ranges from 1×10^{-8} to 50×10^{-8} M (Table 9).

Methods used to measure complexing capacity include bioassays, ion exchange on resins or MnO_2, ion-selective electrode potentiometry, Cu salt solubilisation, chemical exchange, amperometry and voltammetry.[1,3,191,192,203-205] Of these methods, voltammetry, using an ASV titration, has been most widely applied. ASV titration consists of adding aliquots of a standard Cu solution to the sample and measuring the Cu ASV peak until the slope of the peak current - Cu concentration graph increases to that found for ionic Cu (Fig. 7).

Assuming a 1 : 1 Cu - ligand complex, the complexing capacity (C) and the apparent stability constant ($^*K$) can be found from a plot of the relationship[191]

$$[Cu]/(Cu_T - [Cu]) = [Cu]/C + 1/^*KC \quad .. \quad (28)$$

where [Cu] is the concentration of free Cu(II) ion and Cu_T is the total Cu concentration. Some typical values for C and $^*K$ are shown in Table 9.

There are several problems associated with the ASV titration method for determining complexing capacity. (i) Some Cu complexes, such as Cu - NTA, although thermodynamically stable, are kinetically labile and dissociate extensively in the diffusion layer, the complex appearing as free metal ion. These kinetic currents can be corrected for to some extent,[156] but the procedure required is fairly complex.[16] (ii) Organic matter adsorbed on the electrode may cause a depression of the metal ASV peak by hindering electrodeposition, even though no actual complex formation takes place. (iii) Formation of the Cu complex may be slow, and several hours may need to be allowed between the additions of Cu titrant. It is often better to add increasing aliquots of standard Cu solution to a series of flasks containing a fixed volume of sample and to allow it to stand overnight before ASV measurement.

The problem of electrode fouling by organics could be minimised by the use of linear scan voltammetry at a rapidly dropping mercury electrode or a streaming mercury electrode, provided that the samples have a sufficiently high complexing capacity. Interference from adsorbed organic matter increases in the order differential pulse polarography (DME) < linear scan ASV (TMFE) < differential pulse ASV (HMDE).[149] In ASV the electrode is exposed to the organic matter for the period of the deposition time, whereas in polarography exposure lasts only for the drop time. In linear scan voltammetry, the metal ion has to cross the adsorbed organic layer only once during deposition, whereas in differential pulse techniques, where multiple redox reactions are involved, many crossings of the adsorbed layer must occur (Section 5.6).

Van den Berg[201] has described a ligand-exchange CSV method for measuring complexing capacity, based on competition between the natural ligands and catechol for Cu ions, followed by cathodic stripping of the adsorbed Cu - catechol complex (Section 5.3). An estimate of the Cu - ligand conditional stability constant can also be obtained. This procedure has the advantages that the problem of dissociation of the Cu complex in the double layer is eliminated, and interference by adsorption or organics may be less severe if the Cu - catechol complex is preferentially adsorbed. However, because of the high stability constant of the Cu - catechol complex,[149] the method would measure only those ligands which form relatively stable complexes with Cu. Waite and Morel[200] described a novel amperometric titration method for complexing capacity using Cu(II) as titrant and a high chloride media to stabilise Cu(I).

Ion-selective electrodes measure the activity of free, hydrated metal ion, and no other species. If a Cu ion-selective electrode is calibrated with a standard $CuSO_4$ solution in non-complexing media (e.g., nitrate or perchlorate), then even simple complexes such as $CuCl^+$, $Cu(OH)_2$ and $CuCO_3$ will be included in the complexing capacity measurement, as they are not sensed by the electrode. This explains why literature results for complexing capacity determined by Cu(II) titrations using an ion-selective electrode for end-point detection are often much higher than ASV values for similar waters.[1,191]

Before any method for measuring complexing capacity is chosen over others, it should be shown that it gives a reasonable correlation with bioassay techniques, otherwise it will have little value for ecotoxicological studies. The ideal method for measuring complexing capacity would be one

where the affinity of the analytical probe for the metal ion titrant would be the same as that of a biomembrane, e.g., the gill of a fish, for the metal ion. If this ideal situation could be achieved, the equilibrium constant for the reaction

$$ML + P \rightleftharpoons MP + L \quad .. \quad .. \quad (29)$$

where P is the analytical probe and ML is the complex formed between the metal titrant and the natural ligand, would be the same as the constant for the reaction

$$ML + B \rightleftharpoons MB + L \quad .. \quad .. \quad (30)$$
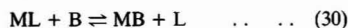
where B is a biomembrane.

9. Conclusions and Recommendations for Future Research

Speciation analysis is essential for an understanding of the biological and geochemical cycling of trace elements; simple total element analysis provides little information about these processes. Dividing trace elements in waters into different behavioural classes (speciation "boxes") is a difficult task when the total concentration is at, or below, the $\mu g \, l^{-1}$ level. Electroanalysis, especially anodic stripping voltammetry (ASV), is perhaps the most powerful technique available for this exacting branch of analytical chemistry. It must be appreciated, however, that ASV and polarography are dynamic techniques, and cannot possibly measure the "natural" speciation of a trace element in a water sample, because the measurement itself disturbs the equilibrium. All electrochemical speciation results are, therefore, operationally defined. This characteristic of electroanalysis may actually be an advantage, as the interaction of a trace metal with a biomembrane is also a dynamic process, and it should be possible to choose the solution and electrochemical parameters so that the kinetics of electrodeposition are similar to the rate of uptake of a trace metal by a biological system. An important point here is that the effective measurement time of different electrochemical techniques (ASV, polarography, linear scan, pulse) varies considerably, and hence the kinetic contribution of metal complexes to the analytical signal will also vary with the method used. Full analytical details (including calculation of the diffusion layer thickness) must therefore be reported in all published research on trace element speciation.

Two broad areas of the methodology of electrochemical speciation analysis of waters would benefit from further research, as follows.

1. The relationship between trace element speciation and aquatic toxicity. Much of the interest in speciation stems from the knowledge that the toxicities of different physico-chemical forms of an element vary enormously, and that speciation analysis could possibly be used to determine the potential toxicity of a water system. There is little point in developing, from a purely chemical viewpoint, "new" speciation schemes without consideration of their application. If the ultimate aim is directed towards ecotoxicology, then development of the speciation method should be carried out in parallel with bioassays in an attempt to achieve the best correlation. On the other hand, if the research aim is to study geochemical cycling, then the speciation procedure should be tailored to mimic, as closely as possible, the relevant adsorption and precipitation processes. These remarks also apply to the development of new methods for measuring complexing capacity; bioassays must be carried out hand-in-hand with the chemistry to ensure the relevance of the data.

2. Electrochemical speciation measurements may often be affected by extraneous substances, especially surface-active compounds, in solution. In ASV trace metal speciation, the interpretation of the results is greatly simplified if it can be assumed that the deposition step alone controls the results,

i.e., that the kinetics of metal deposition controls the magnitude of the stripping peak, and that stripping kinetics are unaffected by ligands that are present in the sample, but not the standard, solution. Perhaps the best way to ensure that this situation exists is to use a medium exchange technique. This involves depositing metals from the sample solution, but replacing it with a simple electrolyte (*e.g.*, acetate buffer) before the stripping step. Research is required to design better cells for medium exchange, and to determine the usefulness and application of the procedure.

Adsorption of surface-active substances (*e.g.*, humic matter) from the sample solution on to the mercury electrode is one of the most serious complications in electrochemical speciation measurements. Both deposition and stripping currents may be decreased in an unpredictable manner, and there is often a non-linear relationship between peak current and deposition time. Because the build-up of an adsorption layer on an electrode is a relatively slow process, adsorption has less influence when short ASV deposition times (and short drop times in polarography) are used. To overcome the problem of adsorption, the streaming mercury electrode (SME) should be investigated for speciation analysis when total metal concentrations are sufficiently high to allow its use. Because of the extremely rapid renewal of the electrode in a SME, adsorptive processes and metal complexes with slow dissociation kinetics usually have little effect on the diffusion current. The SME may be especially useful for complexing capacity titrations. Another promising technique for the elimination of interference by adsorption is to cover the thin mercury film electrode with an ultrafiltration or cellulose acetate membrane. This type of covered electrode may be particularly useful in flow-through cells for continuous monitoring of waters where electrode fouling is a vexing problem.

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Determination of Ethanol in Alcoholic Beverages Using a Headspace Procedure and Fuel Cell Sensor*

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A procedure is described that allows the rapid determination of ethanol in a wide variety of alcoholic beverages. Dynamic headspace analysis is employed and a fuel cell sensor is used for the quantitative determination of ethanol. The method is rapid and compares favourably in accuracy with distillation and gas-chromatographic procedures.

Keywords: *Ethanol determination; alcoholic beverages; headspace analysis; fuel cell sensor*

The approved method of determining the ethanol content of a beverage in the UK and most other countries dates back to the early 1900s, and is based essentially on studies made by Thorpe and Brown.[1] Tables of data were established that related the specific gravity of distillates to original gravity and thus the ethanol content of the final product. Although improvements have been made over the years in distillation apparatus and in procedures for measuring specific gravity, the method is essentially unchanged. However, certain disadvantages are inherent in the procedure, including a substantial skill element on the part of the operator carrying out the analysis and the excessive time required for each determination. Cost can also be a factor when expensive products are being examined.

In more recent times, gas chromatography has become increasingly used for ethanol assay[2] and in some countries is accepted as a standard procedure. However, gas chromatographs are relatively expensive and require the usual back-up facilities normally associated with a well equipped laboratory, as does the distillation procedure. Other techniques are available that depend either on density measurements[3,4] or headspace analysis (Alcoltrol Ac3, L. H. Engineering Co., Stoke Poges, Buckinghamshire), but both suffer from high cost and the need for a laboratory environment.

In this paper we describe a prototype instrument and associated procedure that can be used to determine ethanol in alcoholic beverages with an accuracy comparable to those of the above-mentioned methods and that hopefully will provide the basis of a commercial, portable and simple to use instrument for ethanol determination.

Experimental

Instrument Construction

The instrument consists essentially of two main components, *viz.*, the headspace generation element and the electronic ethanol detection system. These two components will be considered separately.

Headspace ethanol generation

Two specially designed and calibrated (100 cm^3 at 20 °C) vessels were constructed incorporating inlets and outlets for liquids, air and a thermocouple inlet as shown in Fig. 1. The

procedure for charging the vessels was as follows. The standard vessel was half filled with water and a standard volume of an aqueous ethanol solution (2.0 cm^3, 5.0% V/V; 1.0 cm^3, 10.0% V/V; 0.5 cm^3, 20.0% V/V; 0.25 cm^3, 40.0% V/V) was added to the vessel using a digital pipette, *e.g.*, Boehringer BCL 1000 DG, and the solution was made up to the mark with water. All of the standard solution was removed from the pipette tip by injecting the sample below the water level and depressing the pipette plunger three or four times. After the addition of two drops of Dow Corning 1520 silicone antifoaming agent, the sample vessel was filled identically using the appropriate volume of beverage. An air supply (*ca.* $300 \text{ cm}^3 \text{ min}^{-1}$) was generated using a small air pump, and passed alternately through the two vessels (isolated using the taps shown in Fig. 2), thus generating a dynamic headspace containing ethanol. The ethanol content was measured as described below.

Headspace ethanol analysis

The air emerging from the vessel was passed through a short polypropylene T-piece pierced by a microlance hypodermic needle fitted to a standard Lion Laboratories (Barry, South Glamorgan) fuel cell sensor head. Sampling of the flowing air - ethanol stream was achieved by first pressing the RESET button and then the READ button. This resulted in a standard volume of ethanol - water vapour being drawn into the fuel cell, where an electrical potential was developed. This potential was amplified and used as a measure of the ethanol concentration in the vapour.
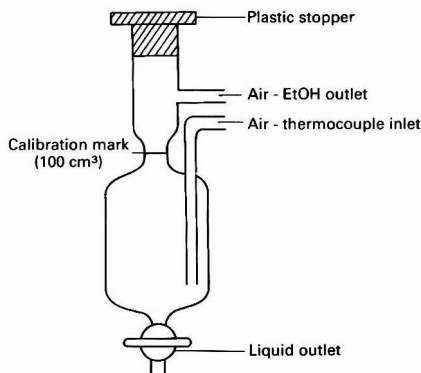

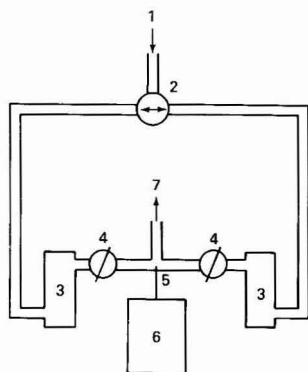Fig. 1. Diagrammatic representation of a headspace generating vessel

Fig. 2. Schematic flow diagram of the ethanol analyser. 1, Regulated air supply; 2, two-way tap; 3, headspace generating vessel; 4, isolating taps; 5, polypropylene T-piece; 6, sampling valve, fuel detector unit and microlance hypodermic needle; and 7, air - ethanol outlet

Table 1. Determination of the slope (m) of the linear logarithmic plot of temperature *versus* displayed ethanol concentration

Ethanol concentration, % V/V	Slope	Correlation coefficient
0.05	0.0291	0.998
0.08	0.0285	0.997
0.08	0.0286	0.999
0.10	0.0272	0.998
0.10	0.0290	0.997
0.10	0.0279	0.999
0.10	0.0265	0.999
0.13	0.0275	0.999
0.15	0.0279	0.999
0.20	0.0290	0.998

Statistical analysis:
$n = 10$; $\bar{x} = 0.0281$; S.D. $= 8.27 \times 10^{-4}$; C.V. $= 2.94\%$

Data Analysis

The signal developed in the fuel cell was analysed using a Lion AE-D3 unit, which was specially built for this study, and derived from the company's microprocessor-controlled Auto Alcolmeter.

In the AE-D3 four range controls are utilised, for 5.0, 10.0, 20.0 and 40.0 V/V. Depending on the nature of the unknown sample, *i.e.*, whether beer, wine, etc., the appropriate selector button is activated and the signal from the fuel cell obtained using the standard vessel is displayed (LCD). Note that the concentration of ethanol in the standard is always constant, but will be displayed as one of the four values indicated above. After calibration, the air stream is passed through the sample vessel, the new air - ethanol stream is sampled (after *ca.* 2 min to allow flushing of the previous sample) and the new signal is displayed on the LCD. As the fuel cell output is directly proportional to the ethanol concentration over a wide range of concentrations, it is a simple matter to display the unknown ethanol concentration.

Temperature Correction

The temperatures of both sample and standard were monitored continuously using an RS Components Type 610–067 digital thermometer fitted with a Type K thermocouple and any difference in temperature was compensated for mathematically (see Discussion).

In order to establish the relationship between the temperatures of the vessels and the instrument readings, both vessels were filled with standard ethanol solutions (100 cm³, 0.05–0.20% V/V) and the reference vessel was maintained at 25.0 °C in a thermostatically controlled bath while the temperature of the sample vessel was varied in the approximate range 10–35 °C. The AE-D3 was calibrated in the usual way using the reference vessel and the apparent ethanol content of the sample vessel was recorded using the 5.00% V/V range.

Determination of Ethanol in Various Beers, Wines and Spirits

The ethanol contents of a wide range of alcoholic beverages were determined using the procedure described above with the appropriate volume of the various beverage types, *viz.*, beers (2.00 cm³), table wines (1.00 cm³), fortified wines (0.50 cm³) and spirits (0.25 cm³).

Fig. 3. Graphical representation of the mathematical process involved in temperature compensation

Gas-chromatographic Analysis of Reference Samples

The ethanol contents of a wide variety of samples were determined using a Perkin-Elmer 8310 gas chromatograph fitted with a 3 m × 3 mm i.d. stainless-steel column containing 10% Carbowax 20M on 60–80 mesh Celite and a flame-ionisation detector. The column was operated isothermally at 80 °C using helium as the carrier gas (15 cm³ min⁻¹) and propan-1-ol as an internal standard.

Analysis of Reference Samples by Distillation

The ethanol contents of all reference samples were also determined by the distillation procedure approved by HM Customs and Excise, *i.e.*, using gravimetric density measurement.[5]

Results and Discussion

The technique of headspace analysis, particularly in the context of alcoholic beverages, has one major advantage over most other analytical procedures, *viz.*, that the number of possible interfering substances in the headspace is greatly reduced, *i.e.*, many substances present in beverages are non-volatile and would not contribute to the headspace composition. However the procedure does have two inherent

Table 2. Determination of ethanol in various alcoholic beverages

		Ethanol content, % V/V		
Classification	Type	Headspace analysis	Gas chromatography	Distillation
Beers·	Light ale	3.10	3.11	3.12
	Bitter ale	2.90	2.91	2.91
	Lager	4.18	4.16	4.17
	Chinese beer	4.85	4.84	4.94
Table wines	Moselle	8.5	8.5*	8.4
	Claret	12.2	12.2*	12.1
Fortified wines	Fino sherry	17.6	17.7*	17.6
	Cream sherry	17.7	17.7*	17.8
	Amontillado sherry	17.8	17.8*	17.7
	White port	20.2	20.3*	20.5
	Ruby port	20.2	20.3*	20.2
Spirits	Whisky	40.6	40.0	40.1†
	Rum	40.5	40.0	40.1
	Vodka	38.0	37.5	37.4
	Vodka	46.4	45.8	45.6
Liqueurs	Irish whiskey cream liqueur	17.0	16.9	16.9†
	Coconut liqueur	27.7	27.8	27.9†

* Independently determined by J. Harvey & Sons Ltd.
† Independently determined by International Distillers and Vintners Ltd.

Table 3. On-site results of ethanol determination in 1984 port wine vintage compared with laboratory distillation values. Samples 1–30 are red and 31–36 are white port wine

	Ethanol concentration, % V/V	
Sample No.	Distillation	Analyser
1	20.0	20.0
2	19.8	19.8
3	20.3	20.2
4	20.2	20.4
5	20.0	20.1
6	19.6	19.6
7	18.8	18.9
8	18.9	19.0
9	17.5	17.9*
10	17.3	17.2*
11	18.3	18.9*
12	19.3	19.6
13	19.9	20.1
14	18.5	18.3
15	19.6	19.9
16	20.0	20.0
17	17.8	18.1
18	19.6	19.6
19	16.0	16.0
20	16.8	16.5
21	19.7	19.7
22	19.7	19.6
23	19.1	19.0
24	19.8	20.0
25	18.7	18.6
26	19.7	19.6
27	20.1	20.0
28	20.1	20.0
29	19.8	20.0
30	18.2	17.9
31	17.7	17.3
32	16.1	16.8
33	16.3	16.5
34	17.6	17.3*
35	19.3	19.7*
36	19.3	18.9

* Sample diluted using river water.

disadvantages, *viz.*, extreme temperature sensitivity, the concentration of ethanol in the vapour phase increasing exponentially with increase in temperature, and the effect of dissolved solids on the composition of the vapour phase, *i.e.*, the well known "salting-out" effect.

Considering the latter problem, no great difficulty was experienced in overcoming this. The simple act of dilution, necessary in any event to produce a headspace ethanol concentration suitably low for use with the fuel cell sensor, overcame the problem, the "salting-out" effect being undetectable at the drink dilutions used.

Compensation for temperature variation between the standard and sample solutions is, however, more complex. As indicated above, the concentration of ethanol in a headspace, dynamic or static, is exponentially dependent on temperature, the slope of the linear logarithmic plots being independent of concentration in the range 0.05–0.28% V/V. To avoid the necessity for thermostating the two vessels, it is necessary to compensate mathematically for any variation. Interestingly, however, it is not necessary to know the actual temperatures of the liquids, but only the temperature difference between them. The following mathematical treatment illustrates this point. Table 1 shows the slopes of such plots, the mean slope of these lines being 0.0281. A graphical representation of the process involved with temperature correction is given in Fig. 3, which shows logarithmic plots for the variation of headspace ethanol (as measured on the AE-D3) for standard and unknown samples.

If we let S be the AE-D3 response to a standard at a temperature T_s (note that S is therefore the value of the range selected), R be the AE-D3 response to an unknown sample at a temperature T_u and U be the AE-D3 response to the unknown solution at the temperature of the standard solution T_s, *i.e.*, the required corrected response value, then from Fig. 3

$$\text{Log } S = mT_s + C_1 \quad .. \quad .. \quad .. \quad (1)$$

and

$$\text{Log } R = mT_u + C_2 \quad .. \quad .. \quad .. \quad (2)$$

where m is the slope of the logarithmic plots and C_1 and C_2 are the intercepts for the standard and unknown lines, respectively.

From equation (2),

$$C_2 = \log R - mT_u \quad .. \quad .. \quad .. (3)$$

and

$$\text{Log } U = mT_s + C_2 \quad .. \quad .. \quad .. \quad (4)$$

i.e.,

$$\text{Log } U = mT_s + \log R - mT_u$$
$$= m(T_s - T_u) + \log R \quad .. \quad (5)$$

It follows that only the difference in temperature is involved in correction, log R and m being determined experimentally.

Results obtained using this correction were excellent, and data obtained for actual determinations of ethanol (under laboratory conditions) in a wide variety of drinks are shown in Table 2. The agreement between standard (*i.e.*, gas-chromatographic and distillation) procedures and the head-space procedure described here shows clearly that the method is applicable to most beverages and has a precision of the same order as those of gas chromatography and distillation. The analysis times are considerably shorter if times are measured from the initial preparation of samples and standards.

It is worth noting that data obtained for the analysis of high-ethanol beverages (spirits) are less satisfactory in absolute terms than for the other beverages studied. We attribute this to difficulties in reproducibly dispensing the small (0.25 cm^3) sample volume associated with the analysis of spirits. However, for general screening purposes, the accuracy achieved, coupled with the rapidity of the determination, may make the procedure of some use to analysts in the spirits industry. If a precision similar to that obtainable by gas chromatography or distillation is required, we do not recommend the headspace procedure as described in this paper.

Extensive field trials of the determination of ethanol under non-laboratory conditions were undertaken during the port wine vintage of 1984, where a large number of wines were analysed on site in Portugal under the adverse conditions that operate in most port wine-producing farms. Water taken directly from the Douro River was sometimes used for dilution with no adverse affects, as the results in Table 3 show. Under these conditions power was provided by the integral, sealed, re-chargeable lead - acid battery in the AE-D3 unit, which provided power for up to 8 h of continuous use. We would particularly recommend the use of digital pipettes for field use as their plastic construction makes them extremely robust.

Turning to the fuel cell and its role as the sensor in this analytical study, its mode of operation is such that a two-stage oxidation of ethanol occurs, giving ultimately acetic acid as the final product.[6] It is apparent, therefore, that compounds other than ethanol may be involved in similar oxidation reactions, and thus interfere quantitatively with data obtained. This is clearly true for most alcoholic beverages, which contain a wide variety of volatile, oxidisable organic species, notably acetaldehyde, methanol and primary and secondary alcohols up to C_6. However, the occurrence of these substances is such that in total they are not likely to exceed 500 mg l^{-1} in beer, wine or spirits and can therefore be discounted as interfering at a measurable level. The same argument is used to justify the distillation procedure, where volatile components would affect the final distillate density, and we would concur with this line of thought.

It is worth noting the modification to the normal use of digital and similar pipettes. Standard usage was found to give erratic delivery of standard volumes, depending on the nature of the beverage used. Certain of the more viscous liquids (port wines, liqueurs, etc.) often left small globules in the disposable pipette tips, thus affecting the precisely delivered volume. To overcome this problem, the total contents of the tip were removed as described under Experimental. Although the precisely displayed volume may not therefore be delivered, reproducible volumes certainly were. Bearing in mind that a comparison procedure is involved in the analytical method described here, approximate but reproducible volumes are the basic requirement, not absolutely precise volumes, as is usually the case.

The use of an antifoaming agent is recommended for eliminating the carry-over of liquid from the calibrated vessel to the sampling chamber. Some alcoholic beverages, particularly beer, give solutions that show severe frothing and, if no antifoaming agent is added, bubbles form in the neck of the calibrated vessels and are carried to the sampling chamber. This clearly must be avoided and the recommended antifoaming agent is extremely efficient in eliminating this effect.

Whilst considering the possible carry-over of liquid from the vessels to the sampling chamber, it should be noted that the bubbles of air passed through the solution are coarse, and sinters are not used to produce fine bubbles, as might be expected in an analytical procedure of this type. Sinters were tested in the first instance but it was found that a fine, almost invisible spray was produced that was carried to the sampling chamber by the air flow. This resulted in the solution finding its way into the fuel cell, with the obvious disastrous results. Removal of the sinter eliminated this effect, and it should be noted that bubbles of large dimensions equilibrate very rapidly with a water - ethanol system.[7]

It would appear that the headspace procedure compares in precision with both gas-chromatographic and distillation methods for ethanol determinations. It should be pointed out that the determination of ethanol by either of the above established procedures requires a high degree of practical analytical expertise. In addition, neither procedure can be conveniently operated outside a laboratory environment. We feel, therefore, that the procedure described in this paper has much to commend it in terms of simplicity of operation, *i.e.*, a relatively low skill requirement, and its ability to be operated virtually anywhere and with very short warm-up periods. On these grounds alone, the procedure scores well against the established procedures.

In conclusion, the prototype instrument described in this paper offers a rapid, inexpensive (below £3000) and portable means for the determination of ethanol in most alcoholic beverages. It is hoped that it will, in due course, and with appropriate packaging and automation, appear as a commercially available instrument for the alcoholic beverage industries.

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Piezoelectric Quartz Crystal Detection of Ammonia Using Pyridoxine Hydrochloride Supported on a Polyethoxylate Matrix*

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The use of a nonylphenoxypolyethoxylate (Antarox CO-880) as a support polymer is confirmed as a means of prolonging the life (to >50 d) of pyridoxine hydrochloride as a sensitive sorbent coating during the piezoelectric crystal detection of ammonia. However, the matrix system incurs possible interference from hydrogen chloride gas although, except for triethylamine, the other gases studied at high levels (sulphur dioxide, nitrogen dioxide, carbon dioxide and hydrogen sulphide) give only a small piezoelectric signal. The extreme sensitivity of the piezoelectric crystal detection of ammonia to below the µg dm^{-3} range is inconsistent with the Sauerbrey equation, which normally applies to straightforward deposition on piezoelectric transducers. This is a consequence of the low slopes of the log(frequency decrease) *versus* log(concentration) graphs. Such slopes can be increased by modifying the syringe dilution procedure, but there are other more intransigent factors involved.

Keywords: *Ammonia detection; flow injection analysis; polyethoxylate support; piezoelectric quartz crystal detection; gas sensor*

Suitably coated piezoelectric quartz crystal detectors form a highly sensitive technique for the detection of trace amounts of atmospheric pollutants.[1,2] The analyte is selectively sorbed by the coating, thereby increasing the mass on the crystal and decreasing the frequency of vibration. The frequency change, ΔF (Hz), is linearly related to the mass sorbed according to the Sauerbrey equation,[3,4] which for AT-cut crystals takes the form

$$\Delta F = -2.3 \times 10^6 \, F^2 \, \frac{\Delta m}{A} \qquad (1)$$

where F is the initial frequency of the quartz plate (MHz), Δm is the mass sorbed (g) and A is the area of the coating (cm^2). Thus, for a particular experimental set-up, the change in frequency can be expressed as

$$\Delta F = KC \qquad (2)$$

where C is the analyte concentration (mg dm^{-3} or µg dm^{-3} or ng dm^{-3}) and K is a constant that includes the basic frequency of the quartz crystal, the area coated and a factor to convert the mass of analyte sorbed into its gas-phase concentration.

Ucon 75-H-90 000 and Ucon LB-3000X were first used for detecting ammonia in air and were found to have good sensitivity.[5] These were followed by coatings of extracts of *Capsicum annuum* pods and ascorbic acid, with and without silver nitrate,[6] and later by L-glutamic acid hydrochloride and pyridoxine hydrochloride (vitamin B$_6$ hydrochloride),[7] which showed exceptional sensitivity. Supporting the pyridoxine hydrochloride on a matrix of a high relative molecular mass polyethoxylate, Antarox CO-880 (nonylphenoxypolyethoxylate with 30 ethoxylate units) helps to extend considerably the useful lifetime of the detector.[8]

This paper describes further studies on the detection of ammonia using an AT-cut quartz crystal of 9-MHz resonant frequency coated with pyridoxine hydrochloride in a matrix of Antarox CO-880. The parameters studied include coating methods for applying the sorbent to the electrode surfaces of the crystal, the effect of interferences, procedural steps in the syringe dilution method for obtaining low concentrations of ammonia test gas, and tests of the Sauerbrey equation.

Experimental

Apparatus and Detector Design

A laboratory-constructed piezoelectric apparatus[9] was assembled as shown in Fig. 1, and the operating conditions were optimised. The measuring unit consisted of a frequency oscillator with a buffered output powered by a Weir 400 power supply set at 9 V d.c. The frequency output from the oscillator was measured by a Marconi Type 2431A 200-MHz digital frequency meter. A digital to analogue converter selected the last two digits of the frequency meter output for conversion into an analogue signal to a Bryans Model 28000 chart recorder, reading to ±1 Hz.

The AT-cut quartz crystal with gold electrode (Fig. 1, inset) (Quartz Crystal Co., Wellington Crescent, New Malden, Surrey) had a resonant frequency of 9 MHz. The detector cell incorporating this crystal was based on the double impinger cell design of Karmarker and Guilbault[10] wherein the gas sample was split into two streams impinging directly on opposite faces of the coated crystal. The glass-encased cell detector was immersed in a water-bath at 25 ± 0.1 °C.

Pyridoxine Hydrochloride Coatings

Coating solutions consisted of mixtures (1 + 1 V/V) of a 0.2% m/V solution of pyridoxine hydrochloride in ethanol and water (1 + 1 V/V) and a 0.2% m/V solution of Antarox CO-880 in acetone. For the capillary coating approach, the mixture was applied to the electrodes of the quartz crystal with a fine drawn-out capillary tube (melting-point tube).

For the brush-coating approach, a tiny brush was used to apply the mixture to the electrode surfaces in the manner described by Hlavay and Guilbault.[11] In each instance the coated crystal was then placed in the oven at 80 °C to evaporate the solvent, and crystals were stored overnight or between measurements at this temperature. The coating applied in each instance corresponded to a decrease of about 9.5 KHz in the basic frequency of the crystal, and was readily removed with ethanol and water (1 + 1 V/V). The crystal was dried before re-coating.

Ammonia Test Samples

The ammonia gas test samples were obtained with a 10-cm^3 gas-tight Perspex syringe from ammonia vapour over the headspace of dilute (2 M) ammonium solution equilibrated at
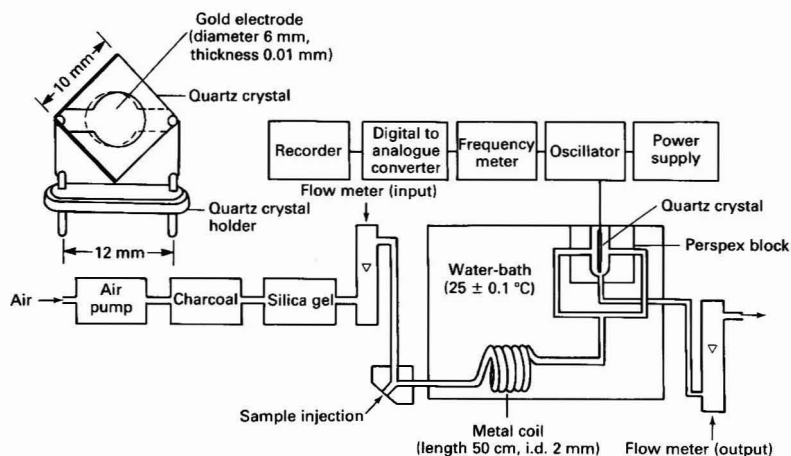
Fig. 1. Schematic diagram of piezoelectric quartz crystal detection apparatus with (inset on left) detail of quartz crystal

25 °C. Serial dilution of the headspace gas was effected by syringe dilution[12] with ambient air (dry air gave $\Delta F = 0$). Successive dilutions were delayed by 30–60 s in order to allow ammonia to diffuse throughout the air in the syringe.

The concentration of ammonia in the headspace was checked by titration. Thus, 10-cm³ samples were slowly injected from the syringe into 20 cm³ of 0.025 M sulphuric acid. The excess of sulphuric acid was titrated with 0.1 M sodium hydroxide solution using methyl orange as an indicator. Thirty replicate samples of the headspace gas contained 32.0 mg dm⁻³ (s.d. 0.25 mg dm⁻³) of ammonia. Interfering gases were analysed in a similar way with appropriate absorbents and titrants.

For the syringe dilution procedure, 9 cm³ of the test gas were expelled from the syringe and air (9 cm³) was sucked into a total volume of 10 cm³ plus the volume of needle and syringe connector (0.23 cm³, s.d. = 0.01). The tip of the syringe needle was closed by piercing into a rubber bung. The mixture in the syringe was allowed to stand for 0.5–1 min in order to allow it to become homogeneous by diffusion. The original test gas was thus 10.23/1.23-times diluted. In the second dilution stage, 1.23 cm³ of the first mixture was diluted to 10.23 cm³ giving a mixture of $(10.23/1.23)^2$ times dilution over the original concentration. By repeating the procedure, mixtures of low concentrations could be obtained.

Samples for Testing the Sauerbrey Equation

For testing the Sauerbrey equation, serially diluted samples were taken as above. Additionally, sample dilutions were prepared by a procedure involving replacing the syringe needle with a clean one in between each dilution. Thus, commencing with a 10-cm³ syringe-full of headspace ammonia standard (32.0 mg dm⁻³) [actually 10.23 cm³ after allowing for the volumes of the needle (0.033 cm³) and the connector (0.197 cm³)], the following procedure was adopted for serial dilution:

(a) expel 9 cm³ of the ammonia standard and replace the needle with a new one; (b) draw in 9 cm³ of air and allow to mix; (c) expel 9 cm³ of the diluted sample from (b), again replacing the needle with a new one; (d) repeat stage (b) and the concentration of ammonia standard should be 100 times diluted [actual concentration is $32 \times (1.197/10.197)^2 = 0.441$ mg dm⁻³ = 441 µg dm⁻³ for a syringe needle connector volume of, for example, 0.197 cm³]; (e) expel 1 cm³ in order to fill the needle with sample; (f) inject 5 cm³ of the sample into the piezoelectric crystal detector; (g) expel 3 cm³ of the remaining sample from (f), then replace the needle with a new one; (h) pull in 9 cm³ of air and allow to mix; and (i) repeat stages (e), (f), (g) and (h).

Operation of Piezoelectric Quartz Crystal Detection Apparatus

The responses of coated piezoelectric quartz crystals were tested on 5-cm³ samples of appropriate dilutions of the headspace ammonia test samples, and the mean decrease in frequency for replicate samples was measured. The diluted samples were injected into a carrier stream of dry (silica gel) air and passed through the quartz crystal compartment (at 25 °C) at a rate of 20 cm³ min⁻¹ by the pump of a Pitman Instruments Model 7069 air sampler (Fig. 1). As stated, the power supply was set at 9 V d.c.

Results and Discussion

The manner of operation of the piezoelectric quartz crystal detection system is essentially a gas-phase mode of flow injection analysis. Sensitivity is helped by the highly commended[12,13] double impinger detector cell design. This is facilitated by the sorption and subsequent desorption of ammonia by the pyridoxine hydrochloride:
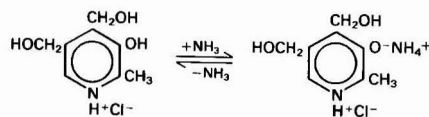
Fig. 2 illustrates recorder responses for serially diluted ammonia samples, while Fig. 3 confirms the previously reported[8] role of Antarox CO-880 as a matrix for prolonging the life of the piezoelectric detector.

The typical responses shown in Fig. 2 illustrate that although the response of the detecting system is fast, the return to the base-line frequency takes several minutes because of the relatively slow desorption of the ammonia. However, as mentioned previously,[8] fresh samples may be injected before returning to the base-line frequency, for it is the immediate decrease in frequency caused by the injected sample that is analytically significant.
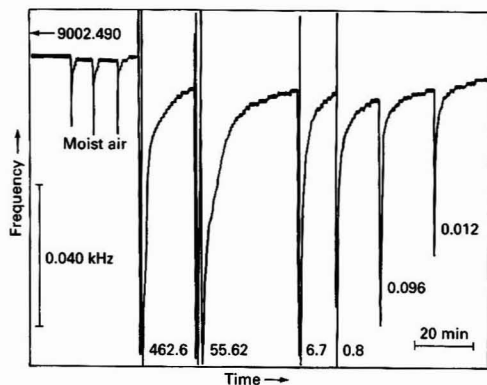
Fig. 2. Typical recorder trace of a calibration of ammonia gas using a quartz crystal coated with pyridoxine hydrochloride and Antarox CO-880. Sample size: 5 cm^3. Numbers on peaks are NH$_3$ concentration (μg dm^{-3})
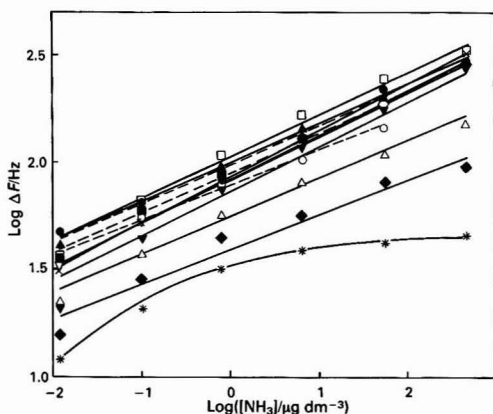
Fig. 3. Calibrations over several days of a piezoelectric quartz crystal for ammonia coated with pyridoxine hydrochloride and Antarox CO-880 and illustrating long functional lifetime. Day: ▲, 1; ○, 2; ■, 4; ▽, 10; □, 15; ●, 25; ×, 30; ◇, 36; ▼, 46; △, 57; ◆, 61; and *, 67

It has previously been shown[8] that Antarox CO-880 coated on the quartz crystal without pyridoxine hydrochloride was not significantly involved in ammonia sorption, but that it did sorb water. The extent of water sorption (only the carrier air stream was dried) for the larger samples used here (5 cm^3 compared with 1 cm^3 in earlier studies[8]) is shown in Table 1 for a pyridoxine hydrochloride - Antarox CO-880 coated crystal.

As expected, the larger volume samples used in this study (5 cm^3) produced larger frequency changes [ca. 350 Hz for 463 μg dm^{-3} (Fig. 2)] than were observed in the previous study[8] for pyridoxine hydrochloride in an Antarox CO-880 matrix for 1 cm^3 samples (215 Hz for 30 mg dm^{-3}). These data compare with 1190 Hz for 1 mg dm^{-3} and a surprising 386 Hz for 10 ng dm^{-3} reported by Hlavay and Guilbault[4] for pyridoxine hydrochloride alone on the quartz crystal. Good linearity of calibration was obtained (Fig. 3) with correlation coefficients of 0.99 when log ΔF was plotted against log [NH$_3$].

Sorbent Coating Methods

Capillary-tube and brush-coating approaches were compared for the application to the quartz crystal of pyridoxine hydrochloride alone and of hydrochloride in Antarox CO-880. For the pyridoxine hydrochloride alone, the capillary-tube approach gave an uneven coating as the hydrochloride was concentrated in certain areas, thereby reducing the reacting surface area. The brush-coating approach gave a more even coating. Both approaches gave visually even coatings for pyridoxine hydrochloride in Antarox CO-880, although the brush-coating approach produced larger frequency changes (Table 2). The brush-coating approach was used for all other results discussed here.

Tests of reproducibility of the brush-coating technique were carried out for later coatings (Table 3). The five coatings shown were made on the same quartz crystal, the previous coating being removed after each set of calibrations by brushing the crystal surface gently with ethanol - water (1 : 1 m/V). In each instance, the crystal was oven dried at 80 °C for 30 min after coating as stated in the experimental procedure.

The relative standard deviations for the frequency decreases (Table 3) were generally low, being less than 4% for the various concentrations of ammonia, with 4.0% for 0.012 μg dm^{-3} of ammonia and 0.9% for the 463 μg dm^{-3} ammonia sample.

The area of coating (A) was measured with a Quantitiet 800 Image Analyser (Cambridge Instruments Ltd.). The amount of coating material was calculated from Sauerbrey's equation (1).

Table 1. Effect of moisture on the quartz crystal brush coated with pyridoxine hydrochloride and Antarox CO-880

Dilution stage	0	1	2	3	4	5	6	7	Laboratory air
ΔF/Hz	60	57	47	35	27	23	22	19	21

Table 2. Comparison of the capillary tube and brush-coating methods of coating pyridoxine hydrochloride with Antarox CO-880 on quartz crystal electrodes. All results are given as ΔF (Hz); sample volume of ammonia standard = 5 cm^3

Coating method	Ammonia standard (μg dm^{-3})						Moist laboratory air
	463	56	6.7	0.80	0.096	0.012	
Day 1:							
Capillary tube	278	176	110	63	37	24	28
Brush	392	200	142	101	66	41	25
Day 5:							
Capillary tube	185	134	96	59	30	23	20
Brush	248	139	104	66	50	33	25
Day 10:							
Capillary tube	164	117	94	56	34	22	24
Brush	278	192	130	84	52	31	25

Table 3. Tests on the reproducibility of the brush-coating technique with relative standard deviation data for another five coatings by capillary tube coating

| [NH₃]/μg dm⁻³ | Response (ΔF/Hz) to NH_3 with piezoelectric quartz crystal coated with pyridoxine hydrochloride and Antarox CO-880 | | | | | | | Relative standard deviation of similar data for capillary tube coating, % |
	1st coating	2nd coating	3rd coating	4th coating	5th coating	Mean	Relative standard deviation, %	
462.6	339	333	332	337	332	334.8	0.90	6.4
55.62	189	195	198	182	194	191.6	2.9	3.4
6.70	125	140	136	133	138	134.4	3.9	6.1
0.80	87	88	94	90	90	89.8	2.7	5.5
0.096	60	62	65	58	61	61.2	4.0	9.9
0.012	35	33	36	37	34	35.0	4.0	9.9
Area of coating/cm²	0.56	0.51	0.58	0.53	0.51	0.533	5.6	11.7
Amount deposited/μg	28.0	24.3	35.9	25.7	22.8	27.3	19.0	21.4
ΔF due to coating/Hz	9334	8905	11576	9042	8334	9438.2	12.0	10.7

Table 4. Interferences in the piezoelectric crystal detection of ammonia

| Gas | Concentration/ mg dm⁻³ | ΔF/Hz | | |
		Vitamin B₆ coating	Antarox CO-880 coating	Vitamin B₆ + Antarox CO-880 coating
NH₃	3.85	408	37	—
NH₃	0.46	261	23	320
SO₂	101	21	34	44
NO₂	75	47	32	35
HCl	109	45	2674	1496
HCl	13.2	—	255	143
CO₂	1477	19	30	32
H₂S	116	27	41	43
TEA*	5.3	211	43	383
Dry lab. air	—	0	0	0
Moist lab. air	—	10	11	16

* TEA = triethylamine.

The mass of coating material deposited on the crystal surface varied from 22.8 to 35.9 μg. As the coating material was a 1 + 1 mixture of pyridoxine hydrochloride and Antarox CO-880, the amount of pyridoxine hydrochloride was deemed to be half of the mean value of 27.3 μg, that is, 13.7 μg with a relative standard deviation of 19%. The frequency decrease due to the coating was 9.44 kHz, that is, slightly more than the 9.24 kHz for a similar analysis of the capillary tube coating method, for which relative standard deviation data are presented in Table 3. The two sets of relative standard deviation data show that the brush-coating approach is more reproducible.

Chemical Interferences

Interferences in the piezoelectric crystal assay of ammonia from other gases are listed in Table 4 for the pyridoxine hydrochloride - Antarox CO-880 coating and for the separate materials. With a few exceptions, these confirm previous observations.[7]

The generally low interferences from acidic gases on pyridoxine hydrochloride alone are expected as the concentrations of the interferents are much higher than the concentration of ammonia gas injected, and the observed frequency decreases are much less than those for 3.85 mg dm⁻³ of ammonia (Table 4). However, 5.3 mg dm⁻³ of triethylamine caused a frequency change of 211 Hz. This is not unexpected, because amines have similar structures and properties to ammonia, though Hlavay and Guilbault[7] found that trimethylamine had no effect.

Interference profiles for the mixture of pyridoxine hydrochloride and Antarox CO-880 as coating material are generally

similar to responses recorded for pyridoxine hydrochloride alone, except for hydrogen chloride gas (109 mg dm⁻³) with a frequency decrease of 1496 Hz. This response is due to the reaction between hydrogen chloride and Antarox CO-880 and is even more marked when the crystal is coated with Antarox CO-880 alone. Such sensitivity can be attributed to hydrogen bonding between the hydrogen chloride and the ethoxylate oxygen, as confirmed by broadening of the ethoxylate infrared absorption band at 3500 cm⁻¹ (ν_{O-H} stretching) for Antarox CO-880 in the presence of hydrogen chloride. The piezoelectric interference reaction is reversible as shown by the fast return (ca. 5 s) of the frequency decrease to the base line. This is now being evaluated for hydrogen chloride sensing[14] as the frequency decrease for 109 mg dm⁻³ of ammonia is a much greater response than 400 Hz for 100 mg dm⁻³ using trimethylamine hydrochloride as substrate coating.[11]

Application of the Sauerbrey Equation

The analytical utilisation of coated piezoelectric quartz crystal detectors has been based on the assumption that Sauerbrey's equation[1] is valid, i.e., that the mass increase caused by sorption is directly related to the concentration of the sample in the flowing gas stream and is proportional to the decrease of the resonance frequency.[1,2] However, all analysis of previous published data by Beitnes and Schrøder[15] shows that the sensitivity of piezoelectric crystal detectors for flowing gas streams does not obey the Sauerbrey equation. Thus, as previously indicated,[16] despite the poorer sensitivities that would be expected from incomplete sorption on the crystal coating, mixing effects with carrier gas, etc., the observed decreases in frequency are often greater than the value

Table 5. Comparison of ammonia present in 5 cm³ of sample according to dilution calculations (for 3rd coating data of Table 3) and ammonia calculated [from the frequency change by the Sauerbrey equation (1)] to be sorbed on the piezoelectric crystal coating

Concentration of NH_3 in sample/µg dm⁻³	ΔF/Hz	Ammonia present in 5 cm³ of sample/ng	Ammonia calculated to be sorbed on coating (for $A = 0.58$ cm²)/ng
463	332	2320	1030
56	198	280	620
6.7	136	34	420
0.80	94	4	290
0.096	65	0.48	202
0.012	36	0.06	112
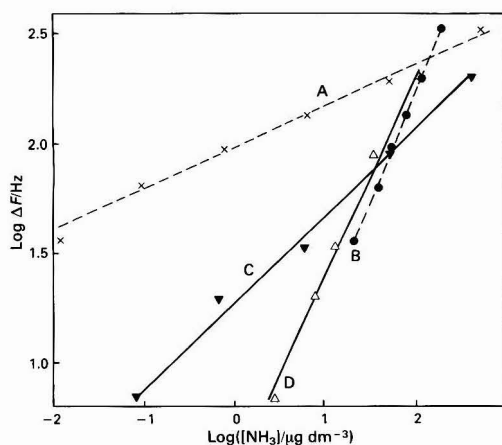
Fig. 4. Ammonia content of standards deduced by syringe dilution procedures related to frequency decrease (A for Table 5 data and C) compared with ammonia content (assuming 100% absorption) calculated from Sauerbrey equation (1) using observed frequency decrease for the corresponding syringe diluted standards (B for Table 5 data and D). Lines A and B are for the ordinary syringe dilution method (with allowance for needle and connector volume) and lines C and D are for the alternative dilution method with needle replacement (with allowance for connector volume)

Table 6. Frequency decrease (ΔF) data for the removal of 463 µg dm⁻³ of ammonia from a 10-cm³ syringe by successive evacuation and re-filling with dry air after replacing the needle at the end of each evacuation. (The 5-cm³ samples examined for piezoelectric frequency changes corresponded to the volume fraction between 1 and 6 cm³)

Re-fill No.	ΔF/Hz for different tests			
	1	2	3	4
0	323	316	320	320
1	28	25	16	28
2	7	7	6	6
3	0	0	0	0

Data such as the above have led Beitnes and Schrøder[15] to investigate systematic errors in the syringe dilution method, but the likelihood of sorptions on syringe walls between dilutions is demonstrated (Table 6) to be an incomplete explanation of the anomalies, and suggest that other factors are involved.

The problem revolves around the better than predicted sensitivities, and Beitnes and Schrøder[15] found that alternative dilution methods, such as bottle dilution, also give sensitivities that are better than predicted. Although not a complete solution, a change in the syringe dilution procedure, as discussed above, brings the experimental response nearer to the predictions of the Sauerbrey equation (Fig. 4, C) but there could be other factors not recognised here.

deduced from Sauerbrey's equation. Conversely, observed decreases in frequency relate, according to the Sauerbrey equation, to mass changes that are greater than the amount of the sought component actually present in the sample (see Table 5).

A graph of the data of columns 1 and 2 of Table 5 (A of Fig. 4) has a much lower slope than that of unity expected for log ΔF versus log C according to the logarithmic form of equation (2). By changing the dilution procedure from the ordinary serial dilution with allowance for needle and needle connector volumes to one where the needle is changed for each dilution stage (detailed in the Experimental section relating to testing the Sauerbrey equation), the slope of log (sample ammonia concentration) versus log ΔF is steeper (Fig. 4, C) and nearer to the expectation of the Sauerbrey equation (Fig. 4, D).

Even greater anomalies than exist in the above data occur in other reported work, e.g., for the 0.01 µg dm⁻³ ammonia sample of Hlavay and Guilbault[7] with a ΔF of 386 Hz, the ammonia calculated to be sorbed by the pyridoxine hydrochloride coating is about 1 µg compared with the 0.000 05 µg deemed to be present in the 5-cm³ sample used.

The anomalies are related to the gentle slopes observed for the log ΔF versus log C graphs, and some previously reported[7] slopes are very gentle, e.g., 0.0615 for the coating of L-glutamic hydrochloride and 0.0978 for a coating of pyridoxine hydrochloride. In this study, the slopes of the graphs of Fig. 3 are between ca. 0.15 and ca. 0.2 while that of C in Fig. 4 improves to >0.4.

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Correlation Between Fluorescent Polarisation Immunoassay and Enzyme Immunoassay of Anticonvulsant Drugs, and Stability of Calibration Graphs*

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Quality control materials and serum samples from patients on long-term drug therapy were analysed for anticonvulsant drugs by enzyme immunoassay (EMIT) and fluorescence polarisation immunoassay (TDX). The accuracy and precision of the two procedures were studied and the stability of calibration graphs was evaluated over a 30-d period. The accuracy and precision of both assays were satisfactory over the therapeutic ranges of phenobarbitone, primidone, phenytoin and carbamazepine and there was a good correlation between the results obtained by EMIT and TDX; for sodium valproate the accuracy and precision of the EMIT assay were poor. Calibration graphs generated by the TDX procedure were found to be stable, but with the EMIT procedure, calibration graphs for phenobarbitone, carbamazepine and sodium valproate showed considerable drift.

Keywords: *Enzyme immunoassay; fluorescence polarisation immunoassay; calibration graph stability; anticonvulsant drugs*

Over the last few years the concept of therapeutic drug monitoring (TDM) has become widely accepted as a valuable aid in the care and management of patients with epilepsy.[1] Well established techniques used for measuring concentrations of anticonvulsant drugs in serum include UV spectrophotometry,[2-6] gas - liquid chromatography (GLC)[7-14] and high-performance liquid chromatography (HPLC),[13-17] but as a result of the increase in the numbers of determinations carried out, new methods of analysis have been developed that emphasise speed in addition to precision and accuracy.[13] The introduction of radioimmunoassay[18,19] and its adaptation for the analysis of anticonvulsant drugs[20] was followed by the development of the related techniques of enzyme immunoassay, fluoroimmunoassay and substrate-labelled fluorescence immunoassay.[21-29] These methods have proved to be particularly well suited for the production of commercial kits and hence for automation under microprocessor control, and have enabled the drug monitoring units to keep abreast of a rapidly increasing work load.[13]

The technique of enzyme immunoassay (EIA) has had an enormous impact in the field of TDM,[21] mainly because of the development of EMIT, a homogeneous immunoassay system that was introduced by the SYVA Company in 1974. Using a discrete analyser, samples can be processed at a rate of between 60 and 100 per hour and the results obtained correlate well with results from standard methods, including GLC and HPLC.[24,30-34] In 1981, as a further development in the field of TDM, the Abbott Company introduced the TDX, which employs the principle of fluorescence polarisation.[35,36] Although the principle was first described as early as 1926,[37] and adapted for drug determinations in 1973,[38] the TDX was the first commercial application of fluorescence polarisation immunoassay (FPIA). Results obtained using this method correlate well with those obtained using other procedures.[39,40]

Both the EMIT and TDX procedures are now widely used in the monitoring of anticonvulsant drugs in the routine situation, and the accuracy and precision of both methods are acceptable in the context of routine clinical chemistry, which commonly works to a 95% confidence limit. Both methods have been designed to take full advantage of modern instrument technology, which is increasingly based on microprocessor control of the function and sequence of the analysis; sampling and dilution procedures can thus be automated by

the use of robotic arms and probes. Further, introduction of EPROM (erasable program read-only memory) has the advantage that calibration graphs can be stored in memory and their stability monitored over a long period, so that a fresh calibration graph does not have to be generated for each run. In order to establish limits for the stability of the EMIT and TDX reagents in the routine analysis of anticonvulsant drugs, we now present a comparison of the two techniques in respect of the stability of calibration graphs over a period of 30 d. We also present data on accuracy and precision obtained by analysing quality control materials and pooled patients' sera for anticonvulsant drugs by both methods.

Experimental

Patients' Samples

Blood samples collected without anticoagulant from patients at the National Hospitals and Chalfont Centre for Epilepsy for the routine measurement of anticonvulsant drugs were used; all the patients were on two or more drugs. After centrifugation the sera were stored at −20 °C.

Quality Control Sera

Quality control sera prepared by pooling patients' sera known by previous analyses to contain the drugs required were used to establish the within- and between-batch precision of the assays and the stability of the calibration graphs. The patients' sera were divided into two groups. One group was pooled, filtered twice with thorough mixing, aliquoted into bottles and deep frozen at −20 °C (QCRa). The other group was subdivided into three batches of sera, with subtherapeutic (SL), therapeutic (TL) and toxic (TO) levels of each drug, aliquoted and stored deep frozen (Table 1).

In addition, a commercial quality control material, Ortho Bi-Level Assayed Anticonvulsant/Antiasthmatic Control Set I (Corning Medical), was used to investigate the accuracy of the assays and the stability of the calibration graphs.

Methods

Kits for the determination of phenobarbitone, primidone, phenytoin, carbamazepine and sodium valproate by the EIA (EMIT) procedure (SYVA) and FPIA (TDX) procedure (Abbott) were used according to the manufacturers' instruc-

Table 1. Therapeutic ranges of anticonvulsant drugs

Drug			Therapeutic range*/ μmol l^{-1}
Phenobarbitone	..	..	20–130
Primidone	..	..	15–60
Phenytoin	..	..	28–67
Carbamazepine	..	..	12–50
Sodium valproate	..	..	360–600

* Values adapted from the literature and used routinely at The National Hospital.

tions and the analyses carried out using an EMIT Auto-Carousel with CP5000 EMIT Clinical Processor (SYVA) and a TDX Analyser (Abbott).

Calibration graphs were generated on the EMIT Auto-Carousel and Abbott TDX, according to the manufacturers' instructions.

Correlation between the EIA and FPIA methods was examined by analysing randomly chosen patients' samples for the five anticonvulsant drugs in duplicate on the same day and carrying out regression analysis, and the accuracy and precision of the two procedures and the stability of the calibration graphs were assessed using the quality control sera Ortho I and QCRa and the pooled patients' sera SL, TL and TO as follows.

The accuracy of the EIA and FPIA methods was investigated by analysing samples of the commercial quality control serum Ortho I in duplicate by both procedures.

The within-batch precision of the EIA and FPIA methods was determined by analysing 40 samples of the quality control serum QCRa in duplicate by both procedures on the same day, and the between-batch precision was assessed by analysing samples of QCRa in duplicate by both procedures in separate routine runs, using newly generated calibration graphs for each run.

The stability of the calibration graphs for the five anticonvulsant drugs over a 30-d period and the precision of the assays under these conditions were determined by the following procedure. Using freshly prepared calibration graphs samples of QCRa and Ortho I and one sample from each of the groups of pooled patients' sera SL, TL and TO were determined by both methods. The calibration graphs for the EIA and FPIA procedures were stored in the memories of the CP5000 and TDX, respectively, and the analyses of the quality control samples and pooled patients' sera were repeated at 3-d intervals for 30 d, using the stored calibration graphs which were retrieved from memory as required.

Results and Discussion

Figs. 1–5 show the results obtained from the analysis of random patients' samples by EIA and FPIA. It can be seen that the correlation between the two methods is acceptable over a wide range of concentrations; the correlation coefficient lies between 0.98 and 0.99.

Results of the within-batch analysis of samples of Ortho I by the two immunoassay procedures are shown in Table 2. It can be seen that the accuracy of the two immunoassay procedures is acceptable for phenobarbitone, primidone, phenytoin and carbamazepine, although with sodium valproate the mean result of 40 analyses of Ortho I using the EMIT procedure barely falls within the range for the analysis quoted by the manufacturer. On the other hand, it can be seen from Table 3 that the accuracy of the EMIT assay is unacceptable for phenobarbitone and sodium valproate, with the mean result of 40 analyses of Ortho I falling well outside the manufacturer's quoted range, when between-batch analyses were carried out using the same calibration graphs for a period of 30 d. The

Fig. 1. Correlation of TDX with EMIT assay of phenobarbitone. Analysis of randomly collected patients' samples: $n = 40$; $r = 0.99$; slope = 1.07; and intercept = -5.27

Fig. 2. Correlation of TDX with EMIT assay of primidone. Analysis of randomly collected patients' samples: $n = 40$; $r = 0.98$; slope = 1.01; and intercept = -0.99

Fig. 3. Correlation of TDX with EMIT assay of phenytoin. Analysis of randomly collected patients' samples: $n = 50$; $r = 0.99$; slope = 0.99; and intercept = 0.76

accuracy of the TDX procedure is acceptable under these conditions (Table 3).

Table 4 shows the results of the within-batch analysis of QCRa by both immunoassay procedures. It can be seen that the within-batch precision of both immunoassay procedures measured by the analysis of QCRa is acceptable for phenobarbitone, primidone, phenytoin and carbamazepine with a standard deviation of less than 2.5 µmol l⁻¹ and a coefficient of variation of less than 5%. For sodium valproate, however, whereas the coefficient of variation for the analysis of 40 samples by both procedures is less than 5%, the standard deviation for the EMIT assay is as high as 15.37 µmol l⁻¹, and for the TDX assay the standard deviation is 9.13 µmol l⁻¹.

Only for phenobarbitone and primidone in this experiment are the standard deviation and coefficient of variation lower for the EMIT assay than for TDX. Similar results were obtained from the between-batch analysis of the five drugs by the TDX and EMIT procedures, as shown in Table 5. The performance of both assays is satisfactory for phenobarbitone, primidone, phenytoin and carbamazepine, but less so for sodium valproate with a standard deviation of 6.35 µmol l⁻¹ for the TDX assay and 12.64 µmol l⁻¹ for EMIT under the conditions of the experiment. In the EMIT assay, these results are in marked contrast to the results shown in Table 6, obtained from the between-batch analysis of QCRa for all five drugs using the same calibration graphs throughout the 30-d

Fig. 4. Correlation of TDX with EMIT assay of carbamazepine. Analysis of randomly collected patients' samples: $n = 40$; $r = 0.98$; slope = 0.96; and intercept = −1.41

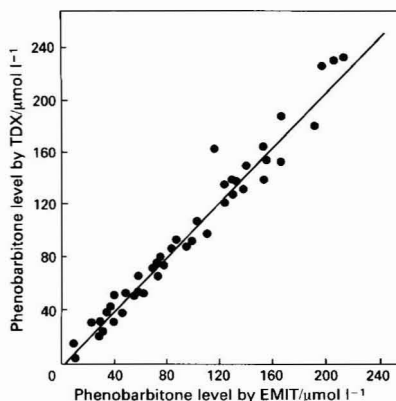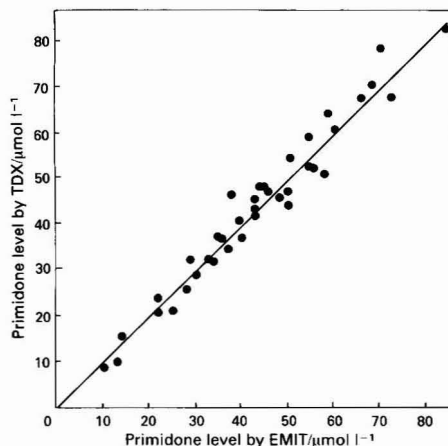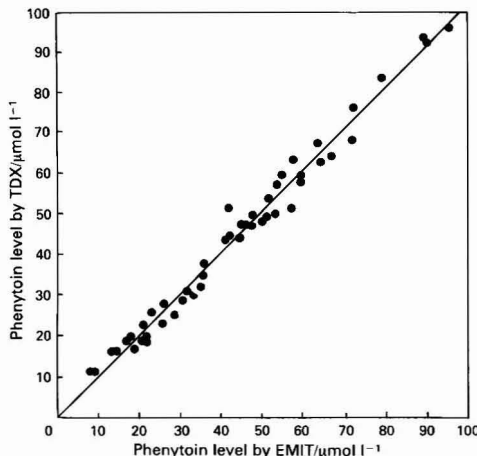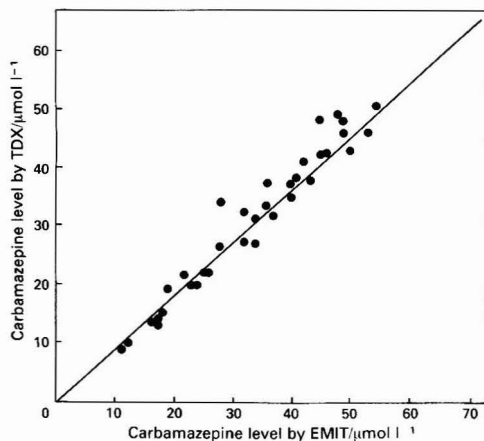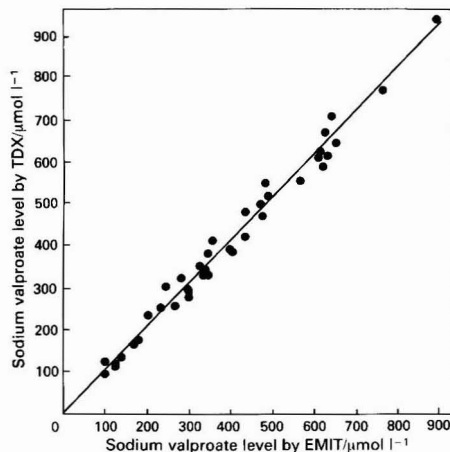**Fig. 5.** Correlation of TDX with EMIT assay of sodium valproate. Analysis of randomly collected patients' samples: $n = 40$; $r = 0.99$; slope = 1.02; and intercept = 4.70

Table 2. Results of within-batch analysis of Ortho Control Set I by TDX and EMIT

Drug	Method	No. of samples	Mean/ µmol l⁻¹	Standard deviation/ µmol l⁻¹	Coefficient of variation, %	Mean concentration quoted by manufacturer/ µmol l⁻¹
Phenobarbitone	TDX	40	65.24	1.10	1.38	59 ± 12
	EMIT	40	76.88	2.86	4.28	65 ± 13
Primidone	TDX	40	33.82	0.98	2.64	33 ± 6
	EMIT	40	32.24	1.89	4.68	33 ± 7
Phenytoin	TDX	40	28.24	0.99	2.86	25 ± 5
	EMIT	40	29.45	1.24	4.28	28 ± 6
Carbamazepine	TDX	40	17.28	0.78	2.69	17 ± 3
	EMIT	40	19.16	1.38	4.88	18 ± 3
Sodium valproate	TDX	40	330.42	10.24	4.08	326 ± 62
	EMIT	40	420.87	20.38	9.85	354 ± 69

Table 3. Results of between-batch analysis of Ortho Control Set I by TDX and EMIT using the same calibration graph over a 30-d period

Drug	Method	No. of samples	Mean/ µmol l⁻¹	Standard deviation/ µmol l⁻¹	Coefficient of variation, %	Mean concentration quoted by manufacturer/ µmol l⁻¹
Phenobarbitone	TDX	11	62.09	1.58	2.45	59 ± 12
	EMIT	11	80.73	5.00	6.20	65 ± 13
Primidone	TDX	11	33.64	1.03	3.05	33 ± 6
	EMIT	11	30.64	2.11	6.89	33 ± 7
Phenytoin	TDX	11	26.64	1.03	3.86	25 ± 5
	EMIT	11	27.45	1.44	5.24	28 ± 6
Carbamazepine	TDX	11	16.73	0.65	3.87	17 ± 3
	EMIT	11	20.64	1.43	6.95	18 ± 3
Sodium valproate	TDX	11	321.55	11.41	3.55	326 ± 62
	EMIT	11	547.64	113.08	20.65	354 ± 69

Table 4. Results of within-batch analysis of quality control serum QCRa by TDX and EMIT

Drug				Method	No. of samples	Mean/ $\mu mol\,l^{-1}$	Standard deviation/ $\mu mol\,l^{-1}$	Coefficient of variation, %
Phenobarbitone ..	..	..		TDX	40	77.63	2.00	2.67
				EMIT	40	76.32	1.83	2.40
Primidone ..	..	..	..	TDX	40	22.03	1.07	4.88
				EMIT	40	22.20	0.41	1.82
Phenytoin ..	..	..	..	TDX	40	44.00	0.99	2.24
				EMIT	40	40.82	1.80	4.40
Carbamazepine ..	..	..		TDX	40	34.40	0.50	1.44
				EMIT	40	36.97	1.58	4.26
Sodium valproate		..	..	TDX	40	375.88	9.13	2.43
				EMIT	40	360.35	15.37	4.27

Table 5. Results of between-batch analysis of quality control serum QCRa by TDX and EMIT

Drug				Method	No. of samples	Mean/ $\mu mol\,l^{-1}$	Standard deviation/ $\mu mol\,l^{-1}$	Coefficient of variation, %
Phenobarbitone ..	..	..		TDX	40	75.15	2.55	3.44
				EMIT	40	78.70	3.60	4.57
Primidone ..	..	..	..	TDX	40	22.03	0.62	4.81
				EMIT	40	21.38	0.74	3.46
Phenytoin ..	..	..	..	TDX	40	43.38	1.76	4.07
				EMIT	40	42.38	1.27	4.01
Carbamazepine ..	..	..		TDX	40	33.65	1.12	3.30
				EMIT	40	36.70	1.11	3.04
Sodium valproate		..	..	TDX	40	368.80	6.35	1.75
				EMIT	40	378.40	12.64	4.62

Table 6. Results of between-batch analysis of quality control serum QCRa by TDX and EMIT using the same calibration graph over a 30-d period

Drug				Method	No. of samples	Mean/ μmol^{-1}	Standard deviation/ $\mu mol\,l^{-1}$	Coefficient of variation, %
Phenobarbitone ..	..	..		TDX	11	71.27	2.15	3.02
				EMIT	11	86.18	7.85	9.10
Primidone ..	..	..	..	TDX	11	21.00	1.00	4.76
				EMIT	11	19.18	2.32	12.07
Phenytoin ..	..	..	..	TDX	11	42.09	1.22	2.90
				EMIT	11	40.36	3.98	9.86
Carbamazepine ..	..	..		TDX	11	33.55	0.82	2.44
				EMIT	11	40.91	4.68	11.44
Sodium valproate		..	..	TDX	11	350.73	10.46	2.98
				EMIT	11	557.91	111.36	19.96

period. Whereas the standard deviation for the EMIT procedure is satisfactory for primidone, phenytoin and carbamazepine under these conditions (less than 5 $\mu mol\,l^{-1}$ in all instances), it is 7.85 $\mu mol\,l^{-1}$ for phenobarbitone and 111.36 $\mu mol\,l^{-1}$ for sodium valproate; the range of the coefficient of variation lies between 9.10% for phenobarbitone and 19.96% for sodium valproate, which is unsatisfactory. On the other hand, it can be seen that the results obtained from the between-batch analysis of QCRa for all five anticonvulsant drugs by the TDX assay under these conditions (shown in Table 6) are comparable to those obtained from the within-batch analyses (Table 4) and the between-batch analyses where a fresh calibration graph was generated for each run (Table 5).

Tables 7–11 show the results of the analyses of the pooled patients' sera SL, TL and TO for the five drugs by TDX and EMIT using the same calibration graph over a 30-d period. It can be seen that the variation in the results for the pooled sera containing drugs below the therapeutic range (SL), within the therapeutic range (TL) and above the therapeutic range (TO) is random for primidone and phenytoin (Tables 8 and 9). For phenobarbitone and carbamazepine the results of the analysis of the sera TL and TO by EMIT, unlike the results obtained by TDX, show a pronounced high bias particularly between day 18 and day 30 (Tables 7 and 10). For sodium valproate (Table

11) the variation in the results obtained for the sera SL, TL and TO by TDX is apparently random, but it can be seen that the results obtained for SL using the EMIT assay increase by a factor of 480 $\mu mol\,l^{-1}$ between day 1 and day 30, and that the test ceases to give a result for serum TL after day 24 and for serum TO after day 3 because the apparent increase goes above the upper limit for the test quoted by the manufacturer (1145 $\mu mol\,l^{-1}$).

The results in Tables 7–10 show that the between-batch precision for the TDX method is acceptable for phenobarbitone, primidone, phenytoin and carbamazepine for the sera TL and TO with a standard deviation of less than 5.5 $\mu mol\,l^{-1}$ and a coefficient of variation of less than 7%. For the EMIT assay the corresponding values, in particular for the coefficient of variation, are poor except for phenytoin (Table 9). In the sub-therapeutic range, it can be seen from Tables 7–10 that both assays yield comparable results for the determination of phenobarbitone, primidone, phenytoin and carbamazepine, but that the mean of the 11 results from the determination of phenobarbitone by EMIT is considerably higher than the mean of the 11 results from the TDX assay (Table 7). For sodium valproate, the between-batch precision of the TDX assay is less satisfactory in terms of standard deviation than for the other drugs analysed under these conditions. Although the coefficient of variation is less than

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Table 7. Analysis of pooled patients' sera for phenobarbitone by TDX and EMIT using the same calibration graph over a 30-d period

	Phenobarbitone/μmol l⁻¹*					
	Sub-therapeutic level (SL)		Therapeutic level (TL)		Toxic level (TO)	
Day	TDX	EMIT	TDX	EMIT	TDX	EMIT
1	16	18	107	105	224	216
3	15	24	105	116	227	232
6	13	21	104	121	221	242
9	13	19	100	119	222	254
12	13	19	100	112	215	244
15	14	21	99	115	233	245
18	14	21	103	128	225	269
21	13	21	102	130	229	265
24	13	20	102	127	219	263
27	14	23	103	119	228	258
30	15	20	103	132	226	291
Mean	13.91	20.64	102.55	120.36	224.45	252.64
Standard deviation	1.04	1.75	2.34	8.30	5.03	20.11
Coefficient of variation, % ..	7.51	8.47	6.89	6.89	2.24	7.96

* Mean of three determinations.

Table 8. Analysis of pooled patients' sera for primidone by TDX and EMIT using the same calibration graph over a 30-d period

	Primidone/μmol l⁻¹*					
	Sub-therapeutic level (SL)		Therapeutic level (TL)		Toxic level (TO)	
Day	TDX	EMIT	TDX	EMIT	TDX	EMIT
1	10	13	47	50	71	69
3	10	12	47	48	70	68
6	11	11	48	46	70	66
9	11	9	48	40	68	55
12	9	10	46	43	65	56
15	11	10	47	41	65	56
18	11	10	46	41	68	57
21	11	10	46	44	67	59
24	12	9	48	38	70	54
27	10	11	44	41	69	58
30	12	10	48	40	67	55
Mean	10.73	10.45	46.82	42.91	68.09	59.36
Standard deviation	0.90	1.21	1.25	3.73	1.92	5.55
Coefficient of variation, % ..	8.43	11.61	2.67	8.69	2.82	9.36

* Mean of three determinations.

Table 9. Analysis of pooled patients' sera for phenytoin by TDX and EMIT using the same calibration graph over a 30-d period

	Phenytoin/μmol l⁻¹*					
	Sub-therapeutic level (SL)		Therapeutic level (TD)		Toxic level (TO)	
Day	TDX	EMIT	TDX	EMIT	TDX	EMIT
1	19	20	49	48	96	96
3	16	20	44	48	96	96
6	19	20	48	48	85	89
9	18	22	48	51	93	97
12	18	19	46	43	95	82
15	18	17	48	44	95	89
18	16	18	47	44	91	85
21	16	18	47	45	93	85
24	16	19	47	48	91	90
27	17	20	46	51	92	93
30	16	17	43	45	90	85
Mean	17.18	19.09	46.64	46.82	92.45	89.73
Standard deviation	1.25	1.51	1.80	2.79	3.24	5.20
Coefficient of variation, % ..	7.28	7.93	3.87	5.95	3.50	5.79

* Mean of three determinations.

5% for the analysis of all three sera, the standard deviation ranges from 17.25 to 26.66 μmol l⁻¹ (Table 11). However, the performance of the EMIT assay in the analysis of serum SL is extremely poor with a standard deviation of 132.72 μmol l⁻¹ and a coefficient of variation of 22.15%, and no calculations of

any kind could be made on the results of the analysis of the sera TL and TO by this method (Table 11).

The results obtained from the within and between-batch analysis of quality control samples with freshly generated calibration graphs show that the performance of both immuno-

Table 10. Analysis of pooled patients' sera for carbamazepine by TDX and EMIT using the same calibration graph over a 30-d period

| | Carbamazepine/μmol l^{-1} | | | | | |
| | Sub-therapeutic level (SL) | | Therapeutic level (TL) | | Toxic level (TO) | |
Day	TDX	EMIT	TDX	EMIT	TDX	EMIT
1	7	8	22	22	50	55
3	7	8	21	22	50	55
6	7	7	19	22	50	54
9	7	7	19	22	49	50
12	5	8	19	24	52	69
15	5	8	19	23	49	75
18	5	7	18	23	48	60
21	6	7	19	22	48	63
24	6	7	19	24	53	75
27	6	8	19	26	49	89
30	6	8	19	27	50	93
Mean	6.09	7.55	19.27	23.36	49.82	67.09
Standard deviation	0.83	0.52	0.90	1.75	1.54	14.50
Coefficient of variation, % ..	13.65	6.92	4.69	7.48	3.09	21.61

* Mean of three determinations.

Table 11. Analysis of pooled patients' sera for sodium valproate by TDX and EMIT using the same calibration graph over a 30-d period

| | Sodium valproate/μmol l^{-1}* | | | | | |
| | Sub-therapeutic level (SL) | | Therapeutic level (TL) | | Toxic level (TO) | |
Day	TDX	EMIT	TDX	EMIT	TDX	EMIT
1	354	397	590	643	923	937
3	354	474	604	732	923	1139
6	389	516	590	748	923	>1145
9	368	541	569	805	854	>1145
12	368	541	569	805	854	>1145
15	361	621	618	922	902	>1145
18	382	643	590	679	951	>1145
21	382	643	597	878	902	>1145
24	410	569	625	937	902	>1145
27	354	733	604	>1145	923	>1145
30	382	877	625	>1145	909	>1145
Mean	382.18	599.18	600.18		914.82	
Standard deviation	17.80	132.72	17.25		26.66	
Coefficient of variation, % ..	4.78	22.15	2.87		2.91	

* Mean of three determinations.

assay procedures is satisfactory in the context of a clinical chemistry laboratory, where the standard of a 95% confidence limit is widely accepted. The results of the analysis of the pooled patients' sera, however, show that the calibration graphs generated by the TDX procedure largely remain stable over that period, whereas the calibration graphs for EMIT exhibit a considerable drift in the determination of sodium valproate and to a lesser extent phenobarbitone and carbamazepine, marked by a high bias in the results for the determination of these drugs. The fact that the standard deviation and coefficient of variation for the between-batch analysis of quality control sera by the EMIT assay are acceptable when a fresh calibration graph is generated for each run is a further indication that the results shown in Tables 7, 10 and 11 are primarily a function of the drift of the EMIT calibration graph under the conditions of the experiment, rather than cross-reactivity with other substances present in patients' sera. On the other hand, the relatively unsatisfactory performance of the TDX assay of sodium valproate, compared with the performance of the other TDX assays examined in this study, may be a function of antibody cross-reactivity with derivatives of fatty acids present in patients' sera, as the fluctuations in the results of the analysis of pooled patients' sera by TDX, shown in Table 11, are purely random in character.

The two immunoassay procedures under investigation here gave comparable results for all five anticonvulsant drugs when used for the analysis of random patients' samples over a wide range of concentration. Nevertheless, recent reports of interferences in immunoassay procedures from substances present in patients' sera have given rise to concern over the specificity of commercial kits for the assay of anticonvulsant drugs.[41,42] A major problem has been the report of interference with the EMIT assay of phenytoin by a metabolite of phenytoin or its derivatives.[43,44] Problems have also been described, although to a lesser extent, with the TDX assay for phenytoin.[41,45] The increasing development and use of monoclonal antibodies will undoubtedly improve the specificity of assays for anticonvulsant drugs based on immune reactions in the future, although there may be problems still to overcome. The qualities of simplicity and speed already make immunoassays indispensable in the situation of ever-expanding need and increase in workload for the analysis of anticonvulsant drugs.

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An Iodine-125 Radioimmunoassay for the Direct Detection of Benzodiazepines in Blood and Urine

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A radioimmunoassay (RIA) for the direct detection of benzodiazepines in blood and urine is described. It is based on a commercially available antiserum and an easily synthesised radio-iodinated derivative of clonazepam that allows the use of relatively simple gamma-counting procedures. The assay can detect low therapeutic levels of all of the benzodiazepines currently available in the UK in 50-μl samples of blood and urine (1–50 ng ml^{-1}, depending on the drug); no prior sample preparation is required. It is inexpensive, rapid, simple to perform and is broadly specific for the benzodiazepine class of drugs. The assay offers a most suitable means of screening large numbers of samples of forensic interest for the presence of the benzodiazepines.

Keywords: *Radioimmunoassay; benzodiazepine detection; blood; urine*

The benzodiazepines are a group of chemically related drugs used mainly as hypnotics and sedatives. Forensic interest in these compounds is due primarily to their frequent occurrence in drugs/driving cases,[1-3] *i.e.*, those cases where a drug is suspected of having made a significant contribution to the impairment of driving ability.

A large number of drugs/driving cases are seen by forensic science laboratories each year, each case requiring an analysis for unknown drugs and metabolites in very small volumes of blood. With the benzodiazepines in particular the analyst is faced with further problems arising from the fact that there is a steadily increasing number of different compounds available (20 in the UK alone[4]) and, because commercial companies are producing more potent drugs all the time, the newer compounds are often present at very low concentrations. A rapid and simple screening assay for benzodiazepines that is sensitive and broadly specific to this group of drugs is therefore desirable.

Methods currently used to detect benzodiazepines include high-performance liquid chromatography (HPLC),[5,6] gas - liquid chromatography with electron-capture detection (GLC - ECD)[7-9] and radioimmunoassays (RIA)[10-14] and radioreceptor assays (RRA)[15,16] using tritiated labels. Use of HPLC and GLC for routine screening is relatively time consuming. In comparison, immunoassay techniques offer many advantages as methods for the efficient screening of large numbers of biological samples for the presence of chemically related drugs.

Unfortunately, of those RIAs available for the detection of benzodiazepines most are specific to particular drugs, and assays of this type are, in general, of limited use for drug screening. Radioreceptor assays and screening RIAs capable of detecting a broad range of benzodiazepines are convenient to use, but published methods are based on tritiated labels and suffer the disadvantages associated with liquid scintillation counting, *viz.*, the need for long counting times, the need for prior sample extraction, the use of expensive scintillant and problems associated with quenching when coloured biological samples are assayed. Two enzyme immunoassay kits, one for benzodiazepines in serum and one for benzodiazepine metab-

olites in urine, are commercially available [Syva (UK), Maidenhead, Berkshire], but neither has the sensitivity required to detect low therapeutic levels of all available benzodiazepines. In addition, neither can cope with the haemolysed blood samples often encountered in forensic cases.

The screening assay described in this paper is based on an iodine-125 labelled derivative of clonazepam. It is sensitive, broadly specific and allows the direct analysis of forensic samples.

Experimental

Materials and Equipment

All chemicals, unless specified otherwise, were obtained from BDH Chemicals, Poole, Dorset.

Phosphate buffer (0.1 M, pH 7.4) containing 0.2% m/V of bovine γ-globulin (Cohn fraction II from Sigma Chemical, Poole, Dorset) and 0.01% m/V of sodium azide was used throughout the assay. The antisera were obtained from Emit TOX serum benzodiazepine and Emit DAU benzodiazepine metabolite kits [Syva (UK)]. The Emit TOX antiserum was diluted 1 + 299 and the Emit DAU antiserum 1 + 999 with assay buffer immediately before each run of the assay. These dilutions give approximately 50% binding with the amount of radiolabel added to each assay tube. The maximum binding of label to each antiserum was approximately 75–80%.

The radiolabelled benzodiazepine derivative [7-^{125}I]-iodoclonazepam (specific activity: 5.6 TBq mmol^{-1}; 12.7 MBq μg^{-1}) was prepared from the 7-amino derivative of clonazepam via a diazonium intermediate as previously described[17] and stored in methanol at 4 °C. It was diluted with assay buffer to give approximately 10 000 counts min^{-1} (550 Bq; 0.099 ng) per 100 μl.

Standard solutions of diazepam were prepared in synthetic urine at concentrations of 0, 1, 2, 3, 5, 10 and 20 ng ml^{-1}. The synthetic urine was prepared by adding 22 g of urea, 1.8 g of NaH$_2$PO$_4$.2H$_2$O, 1.1 g of Na$_2$HPO$_4$, 8.25 g of NaCl, 5.2 g of KCl, 1.5 g of creatinine and 0.1 g of NaN$_3$ to distilled water and making the total volume up to 1 l (modified from reference 18).

Swine serum was obtained from Flow Laboratories Ltd., Irvine, Ayrshire.

Polyethylene glycol (PEG, M_r 8000) was obtained from Sigma Chemical and was used to prepare a 27.5% m/V solution in distilled water (550 g of PEG plus 1525 ml of water).

Disposable polypropylene microcentrifuge tubes (plastic point) were obtained from Alpha Laboratories, Eastleigh, Hampshire.

Gamma-counting was performed using an NE 1600 counter (Nuclear Enterprises, Beenham, Berkshire), which had an efficiency of approximately 50% for iodine-125.

Method

The dilution for the two commercially available antibodies was determined by plotting dilution curves for each against the purified [7-^{125}I]iodoclonazepam and measuring the dilution needed to bind approximately 50% of the total radioactivity.

Cross-reactivities of a small number of benzodiazepines (chlordiazepoxide, diazepam, flurazepam, lorazepam, nitrazepam, oxazepam, temazepam and triazolam) were obtained using both the TOX and the DAU antisera at the optimum dilution by comparing the calibration graph of each with that of diazepam. The results indicated that the TOX antiserum was more suitable for a general screening assay owing to a narrower range of cross-reactivities; all further work was performed using only this antiserum. The cross-reactivities of all the benzodiazepines currently available in the UK, together with some of their major active and inactive metabolites, were then obtained. The concentration of each drug required to give a 50% depression of binding was measured and expressed relative to the value for diazepam.

The extent of cross-reaction of several commonly encountered but structurally unrelated drugs was also measured and compared with the cross-reactivity of diazepam. The depression of binding was measured using solutions containing 100 μg ml^{-1} of the drug unless stated otherwise. The drugs included acetylsalicylic acid (400 μg ml^{-1}), amitriptyline, amphetamine, bromocriptine, butriptyline, caffeine, codeine, ephedrine, glutethimide, imipramine, isoprenaline, lysergic acid diethylamide, methadone, methylphenobarbitone, mianserin, morphine, nicotine, paracetamol (250 μg ml^{-1}), phenytoin and protriptyline.

As diazepam is one of the strongest cross-reacting benzodiazepines, and is also one of the most commonly prescribed, it was used to prepare solutions for the standard curve.

Assays were performed in duplicate using the following conditions. Into each microcentrifuge tube were pipetted 100 μl each of sample, radiolabel and antiserum. The 100 μl of sample consisted of (for urine samples and standards) urine or standard (50 μl) and swine serum (50 μl) or (for blood samples) blood (50 μl) together with synthetic urine (50 μl). This procedure ensured that each tube contained approximately the same amounts of salt, protein and water.

The tubes were then capped, vortexed and incubated at room temperature for at least 1 h. Equilibration was attained after 60 min and was stable for up to 24 h after that time. PEG solution (500 μl) was then added, and the tubes were re-capped, vortexed thoroughly and then centrifuged at 9000 g for 3.5 min. The supernatant was removed by aspiration and the tubes containing the residual bound fraction were each counted for 60 s in the gamma counter.

Whole blood samples were assayed neat and after dilution by factors of 10 and 100 with swine serum. Urine samples were assayed neat and after dilution by factors of 10, 100 and 1000 with synthetic urine.

Cut-off levels for the assay were determined by the analysis of 100 unpreserved blank urine and blood samples obtained from volunteers not taking any drugs. The conditions of the blood samples varied from fresh unhaemolysed to haemolysed/putrefied. Intra- and inter-assay coefficients of variation for the assay were determined by repeated analyses of synthetic urine samples containing 1.5 and 5 ng ml^{-1} of diazepam.

The effects of various urine and blood preservatives on the assay were studied by adding the following preservative tablets: (1) to known blank urine samples (2.5 ml), phenylmercury(II) nitrate and sodium fluoride (50 + 100 mg), sodium fluoride and sodium sulphate (300 + 300 mg) and sodium azide (50 mg); (2) to known blank blood samples (1 ml), sodium fluoride and potassium oxalate (37.5 + 18.7 mg) and sodium nitrite (25 mg). The final concentrations of the added preservatives were approximately 5 (urine) and 2.5 (blood) times greater than those recommended for forensic use.

The assay was used to measure serum levels of diazepam following oral ingestion of single therapeutic doses of the drug (10 mg) by two volunteers. Blood samples were taken before drug ingestion and then at intervals over a period of 36 h afterwards.

The Northern Ireland Forensic Science Laboratory (NIFSL) conducted a trial to compare the results obtained using the described RIA with those obtained by conventional methods. The NIFSL routinely uses GLC with ECD, GLC with NPD and HPLC methods for the detection and quantification of benzodiazepines in case samples. The trial involved using chromatographic techniques in parallel with the RIA method on each of 80 samples (including clinical, driving and post-mortem cases) where benzodiazepines were suspected. The levels of both parent drug and any major active metabolites were obtained by the chromatographic methods, and these were compared with the total benzodiazepine level as measured by RIA.

Results and Discussion

The optimum antibody dilutions (*i.e.*, those giving 50% binding of the added radiolabel) were measured as 1 + 299 for the Emit TOX antiserum and 1 + 999 for the Emit DAU, and these dilutions were used in all further work.

Table 1. Cross-reactivities of eight common benzodiazepines relative to diazepam. Determined at 50% depression of binding (equivalent to 2.5 ng ml^{-1} of diazepam)

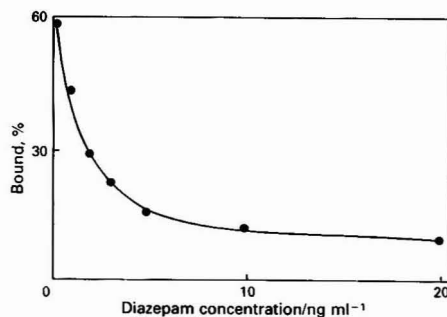| | Relative reactivity | |
Benzodiazepine	Emit TOX	Emit DAU
Diazepam	1.0	1.0
Triazolam	1.4	3.3
Flurazepam	2.8	2.5
Temazepam	4.8	12
Nitrazepam	6.4	8.8
Oxazepam	11	38
Lorazepam	36	107
Chlordiazepoxide	52	115

Fig. 1. Typical calibration graph for diazepam obtained using radioimmunoassay

Table 2. Cross-reactivities of benzodiazepines and their metabolites with the Emit TOX antiserum

Compound						Relative activity*	Concentration required for 50% depression of binding/ng ml⁻¹	Therapeutic range/ ng ml⁻¹†
Prazepam	..	..	..	..	..	0.60	1.5	8–40
Diazepam	..	..	..	..	..	1.00	2.5	50–2000
Pinazepam	..	..	..	..	..	1.20	3.0	—
Alprazolam	..	..	..	..	..	1.20	3.0	—
Triazolam	..	..	..	..	..	1.40	3.5	5–25
N-Desmethyldiazepam		..	..	..	..	2.40	6.0	100–1500
Flurazepam	..	..	..	..	..	2.80	7.0	1–5
Midazolam	..	..	..	..	..	3.20	8.0	—
Temazepam	..	..	..	..	..	4.80	12	360–850
Tetrazepam	..	..	..	..	..	5.6	14	—
Clorazepate	..	..	..	..	..	6.0	15	300–1500
Nitrazepam	..	..	..	..	..	6.4	16	20–150
Clobazam	..	..	..	..	..	10.8	27	300–900
Flunitrazepam	..	..	..	..	..	10.8	27	5–20
Medazepam	..	..	..	..	..	10.8	27	10–160
Oxazepam	..	..	..	..	..	11.2	28	500–2000
Lormetazepam	..	..	..	..	..	11.2	28	—
Ketazolam	..	..	..	..	..	14.8	37	—
Loprazolam	..	..	..	..	..	21.6	54	—
Clonazepam	..	..	..	..	..	32	80	10–100
Lorazepam	..	..	..	..	..	36	90	50–240
Desalkylflurazepam	..	..	..	..	..	44	110	30–90
Bromazepam	..	..	..	..	..	52	130	80–150
Chlordiazepoxide	..	..	..	..	..	52	130	1000–3000
Desmethylclobazam	..	..	..	..	..	52	130	2500–3500
Demoxepam	..	..	..	..	..	>100	>250	—
Desmethylchlordiazepoxide		..	..	..	..	>100	>250	—
Prazepam benzophenone	..	..	..	..	..	>100	>250	—
Diazepam benzophenone	..	..	..	..	..	>100	>250	—
7-Acetylaminonitrazepam	..	..	..	..	..	≫100	≫250	—
Clozapine	..	..	..	..	..	≫100	≫250	—

* Determined at 50% depression of binding (equivalent to 2.5 ng ml⁻¹ of diazepam).
† Approximate range of blood concentrations at the steady state wherever possible; data from references 19 and 20.
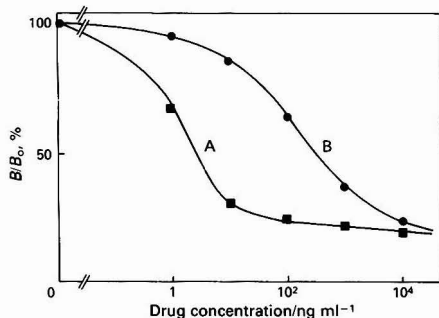

Fig. 2. Full range of cross-reactivities for benzodiazepines. B = bound activity; B_o = bound activity when no unlabelled drug is present. A, Prazepam: B, bromazepam

The relative cross-reactivities of eight common benzodiazepines were measured for both the Emit TOX and the Emit DAU antisera. The results (Table 1) indicate that the two antisera are very similar, but that the spread of cross-reactivity values is less with the Emit TOX antiserum. The most important feature of any screening assay is that the sensitivity towards the least cross-reacting drugs is sufficient to detect them at the required levels. The Emit TOX antiserum is more sensitive towards drugs of lower cross-reactivity and consequently it was selected for the development of the general screening assay; this antiserum was used in all further work. A typical assay calibration graph for diazepam is shown in Fig. 1.

The relative cross-reactivities of all available benzodiazepines, together with a number of their active and inactive metabolites, were measured using the Emit TOX antiserum. The results are given in Table 2. The full range of cross-reactivities of the 20 benzodiazepines available in the UK,[4] from prazepam (the strongest) to bromazepam (the weakest), is shown in Fig. 2. The range of cross-reactivities demonstrated by these results shows that all of the parent benzodiazepines bind relatively strongly to the antiserum, their desalkyl metabolites less so, and compounds in which the diazepine ring structure is broken (the substituted benzophenones) exhibit greatly decreased binding. Further, the range suggests that the assay is sufficiently sensitive to detect all available benzodiazepines. Even low-dose drugs such as alprazolam, prazepam and triazolam should be easily detected at therapeutic levels because their cross-reactivities are approximately the same as that of diazepam. Ironically, because of its poor cross-reactivity, one of the drugs that may be most difficult to detect at low therapeutic levels is clonazepam, the drug from which the radiolabel is derived. Other drugs that might prove difficult to detect in similar circumstances include flunitrazepam and flurazepam. However, even with these compounds, therapeutic levels will usually be above the assay cut-off values and therefore will be detectable.

Of the 20 common non-benzodiazepine drugs that were tested for their cross-reactivity in the assay, only morphine at a concentration of 100 μg ml⁻¹ caused a depression in binding greater than the positive/negative cut-off value of the assay (see below). This concentration is much higher than normal fatal levels of the drug.

The analysis of 100 unpreserved blank blood samples gave a mean level of background cross-reactivity of 0.28 ± 0.18 (S.D.) ng ml⁻¹. Similar analyses of 100 blank urine samples gave a mean value of −0.02 ± 0.16 ng ml⁻¹. The mean plus

Table 3. Comparison of RIA and GLC/HPLC analyses of blood and urine samples from clinical, driving and post-mortem cases involving benzodiazepines

| | | GLC or HPLC result/ng ml^{-1} | | | |
Drug	Sample [blood (b), urine (u)]	Parent	Metabolite*	Diazepam equivalent (total)†	RIA result/ ng ml^{-1}
Diazepam	b	30	–	30	98
	u	70	–	70	147
	b	–	340	142	210
	b	200	130	254	250
	b	420	–	420	270
	b	260	90	298	350
	b	460	–	460	570
	b	460	670	739	630
	b	1250	–	1250	820
	b	1000	–	1000	850
	b	70	–	70	1000
	b	1820	–	1820	1230
Lorazepam	b	130		3.6	2.3
	b	160		4.4	8.0
	b	320		8.9	10.4
	b	100		2.8	23
	u	1400		38.9	41
Flurazepam	b	450	+	>161	1630
	b	310	390	120	2300
Temazepam	b	750		156	120
	b	340		71	240
	b	2180		454	520
Lormetazepam	b	40		3.6	3.8
Chlordiazepoxide	b	240		4.6	27
Bromazepam	b	2600		50	32
Desmethyldiazepam/flurazepam	b	400/1000		524	430
Diazepam/desmethyldiazepam/ temazepam	b	300/440/310		548	540

* Metabolite levels were not determined unless stated otherwise; –, no or only trace amounts of parent or metabolite detected; +, metabolite detected but not quantified.

† Corrected for cross-reaction of parent drug and/or its metabolite.

three standard deviations is 0.82 ng ml^{-1} for blood and 0.46 ng ml^{-1} for urine. A single positive/negative cut-off level of 1.0 ng ml^{-1} was set for both blood and urine samples for simplicity, ensuring a >99.7% probability of obtaining a true positive result. Coefficients of variation for 1.5 and 5 ng ml^{-1} of diazepam in synthetic urine were 6.7 and 7.9% intra-assay ($n = 20$) and 6.4 and 11.4% inter-assay ($n = 20$), respectively.

Of the blood and urine preservatives tested, the only one to cause a measurable depression in binding of the radiolabel to the antiserum was the sodium fluoride - sodium sulphate (300 + 300 mg) preservative when added to urine at concentrations five times the normal. However, as urinary benzodiazepine concentrations are generally much higher than those seen in blood, and as the measured background levels in urine caused by this preservative are only equivalent to approximately 24 ng ml^{-1} of diazepam, it is unlikely that the interference will create any serious analytical problem. Despite this, urinary levels of less than 30 ng ml^{-1} should be treated with caution unless a complementary blood level is available.

The results of RIA analysis of blood samples obtained from two volunteers given a single oral dose of diazepam are shown in Fig. 3; peak levels of diazepam in both subjects are over 300 ng ml^{-1}. Most points shown in Fig. 3 were obtained following dilution of samples by a factor of 10 or 100 (shortly after

ingestion), demonstrating that the assay is capable of measuring therapeutic levels of diazepam in blood with at least an order of magnitude to spare. Because of this, assay results must be interpreted with great care as a positive result might easily be obtained in a sample taken long after any pharmacological effects have ceased. In practice, it is recommended that the assay is performed both on neat samples of blood and after dilution of samples by factors of 10 and 100, and the higher drug levels found in urine may require sample dilution by factors of 10, 100 and 1000 in order to obtain results that can be measured accurately from the calibration graph.

Of the 80 samples tested by the NIFSL, 55 were positive for benzodiazepines by RIA; 19 gave results (mean 28 ng ml^{-1} diazepam equivalents; range 1.3–180 ng ml^{-1}) that could not be confirmed independently by GLC or HPLC. The remaining 36 samples were positive both by GLC or HPLC and by RIA. Nine of these 36 results were rejected for the comparison study either owing to lack of data on metabolite concentrations or because the parent drug was only detected in trace amounts by GC or HPLC. Table 3 summarises the data obtained from the remaining 27 cases. The range of drugs identified in the case samples is fairly typical of that occurring in routine toxicology. Parent drug and/or metabolite levels as measured by GLC or HPLC are presented. These results are
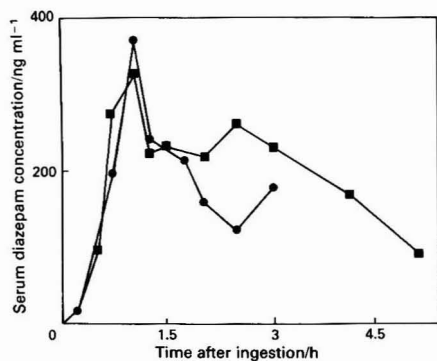
Fig. 3. Serum concentrations of diazepam in two volunteers given a single 10-mg oral dose of the drug

also expressed as total diazepam equivalents, calculated from the cross-reactivities of the respective compounds, to allow direct comparison with the RIA results.

A statistical comparison of the RIA and GLC or HPLC methods was performed using diazepam and lorazepam as examples. Diazepam provides an example typical of those benzodiazepines that undergo complex metabolism giving rise to one or more active metabolites; its major metabolite in blood is desmethyldiazepam. Lorazepam provides an example of a benzodiazepine that is not extensively metabolised and for which active metabolites have not been identified. Good correlations exist between results from the two methods if the parent drug and its major active metabolite in blood are measured, as with diazepam ($r = 0.76; p < 0.005, y = 0.506x + 259, n = 12$). Similarly, where the drug has no active metabolites and only the parent compound is measured, as with lorazepam, there is again a relatively high degree of correlation ($r = 0.85, p < 0.02, y = 0.0849x + 6.99, n = 5$). In both instances drug levels measured by RIA are generally higher than those obtained by the chromatographic methods. This is not unexpected, as it is likely that the RIA will detect those chemically related metabolites of the benzodiazepines that the more specific GLC and HPLC techniques will not.

The only results to show large differences between the two methods were observed with flurazepam. RIA results were much higher than chromatographic results, even after allowing for the major metabolite desalkylflurazepam. It is possible that other metabolites that cross-react well are present in sufficient amounts to raise the level obtained by RIA. Metabolites such as desethylflurazepam, didesethylflurazepam and N-1-hydroxyethylflurazepam were not measured by chromatography but should cross-react and may be present in significant concentrations.

The combination of the Emit TOX antiserum and the [7-^{125}I]iodoclonazepam label provides an assay that is specific for the benzodiazepines as a group, making it ideal as a general screen for this class of drugs. The examination of case samples has shown that, by comparison with the less sensitive chromatographic techniques used, the RIA gives no false negative results. There is, overall, good agreement between the results obtained by RIA and those obtained using these chromatographic methods. However, the broad range of cross-reactivities and the interaction of active metabolites with the assay means that quantification based on RIA is of little value unless the identity of the drug is known; even low levels detected in screening may represent significant amounts of

drugs such as lorazepam and bromazepam. A positive result can only be a semi-quantitative indication of the presence of a drug and, even if a single compound is known to be present, an accurate quantification of concentrations should not be attempted by this method unless a calibration graph for the specific drug is prepared. All positive results should be confirmed and the drug identified by more specific procedures such as GLC, HPLC or mass spectrometry.

Conclusions

In conclusion, the assay is easy to perform, quick to set up and run, inexpensive and very reliable. Intra- and inter-assay results confirm its reproducibility. The sensitivity and group specificity of the assay allow low therapeutic levels of all of the benzodiazepines currently available in the UK to be detected in very small volumes (50 µl) of sample without the need for any prior preparation procedures. The antiserum used is commercially available. The assay has been used successfully for testing for the presence of benzodiazepines in a large number of cases submitted for forensic analysis and should prove extremely useful to both the clinical and the forensic toxicologist.

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Indirect Micro-scale Method for the Determination of Desferrioxamine and its Aluminium and Iron Chelated Forms in Biological Samples by Atomic Absorption Spectrometry with Electrothermal Atomisation

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An indirect micro-scale method is described for the determination of desferrioxamine (DFA) itself and in its iron and aluminium complexed forms (FeA and AlA) in blood plasma and urine. AlA, FeA and DFA, after its conversion into FeA with an excess of iron, are selectively extracted with benzyl alcohol, and iron and aluminium are determined in the benzyl alcohol extract by electrothermal atomic absorption spectrometry using partition pyrolytically coated graphite tubes with a cuvette to prevent benzyl alcohol from spreading to the tube extremities.

The specificity of the method was assessed and the sensitivity is sufficient for the determination of DFA, FeA (44 μg l⁻¹) and AlA in blood plasma and urine after the administration of desferrioxamine as Desferal. However, because of the wide range of concentrations observed in biological samples (from 1 to 300 mg l⁻¹ of DFA) the use of two calibration graphs and sometimes a preliminary dilution of the high-concentration samples are required.

The use of this indirect micro-scale method for more than 1000 assays without particular problems has allowed the study of DFA pharmacokinetics.

Keywords: *Desferrioxamine determination; aluminium and iron complexes; atomic absorption spectrometry; electrothermal atomisation; biological samples*

Desferrioxamine (DFA), a well known iron chelator, has been used as an aluminium chelator in dialysed patients following the work of Ackrill *et al.*[1] Many papers have reported the efficiency of DFA for Fe and Al removal in patients, but the pharmacokinetics of DFA itself and of its Fe and Al complexed forms, which are referred to as ferrioxamine (FeA) and aluminoxamine (AlA), respectively, are not yet well known and there is as yet no suitable method for their determination. The spectrophotometric method of Meyer-Brunot and Keberle[2], which has a detection limit of 5 mg l⁻¹, lacks sensitivity and cannot be adapted to the determination of AlA. High-performance liquid chromatography is a promising method, but the results obtained by Cramer *et al.*[3] do not allow the determination of DFA, AlA and FeA in biological samples, although an application of this method to biological samples has recently been described.[4]

In this paper, a micro-scale method consisting in the specific extraction of DFA in complexed forms, *i.e.*, FeA and AlA with benzyl alcohol (as used by Meyer-Brunot and Keberle[2]) is described but, instead of using spectrophotometric detection for FeA, the concentration of Fe or Al is measured by electrothermal atomic absorption spectrometry. This indirect method has been adapted to the determination of DFA, AlA and FeA at therapeutic concentrations in blood plasma or urine.

Experimental

The instrument used was a Varian Model 975 atomic absorption spectrometer with a GTA 95 graphite furnace and an autosampler. Partition pyrolytically coated graphite tubes with a cuvette (Varian 63.100008.00) were always used to prevent the spreading of benzyl alcohol to the tube extremities. To improve the benzyl alcohol delivery in the graphite tube, 5 μl of ethanol, taken from the modifier beaker of the programmable sample dispenser, were automatically dispensed with the blank, standards and samples. The main instrument settings are indicated in Table 1. Background correction by a deuterium lamp was always used.

Reagents

For the conversion of DFA into FeA and AlA, iron(III) chloride solution (0.01 M) and aluminium chloride solution (0.02 M), respectively, were used. Instead of using solid sodium chloride as used by Meyer-Brunot and Keberle,[2] a saturated solution of ammonium nitrate (Prolabo) dissolved in Merck buffer (pH 8), giving a solution with a final pH of about 7, was used.

DFA as the methanesulphate, $C_{26}H_{52}N_6O_{11}S$ (relative molecular mass 656), was obtained from Ciba Laboratories.

Table 1. Main instrument settings for the determination of FeA and AlA

	Fe (FeA) determination at		Al (AlA) determination at	
Parameter	Low levels	High levels	Low levels	High levels
Wavelength/nm	248.3	248.3	309.3	309.3
Furnace temperature/°C:				
Charring	700	700	1000	1000
Atomisation	2400	2400	2500	2500
Injected volume of				
benzyl alcohol/μl	10	5	30	10
Argon purge gas flow-rate/				
l min⁻¹	0	0.5	0	0

Eppendorf polypropylene microtubes of 1.5 ml were used for the extraction and polypropylene solvent-resistant cups for the autosampler.

Procedure

For each sample of plasma or urine to be analysed, the following two methods were used.

Determination of AlA and FeA already present in biological samples

To 100 µl of blood plasma or urine in an Eppendorf microtube, 200 µl of saturated NH_4NO_3 (pH 7) and 750 µl of benzyl alcohol were added. The tube was shaken for 60 s on a Thermolyne apparatus and centrifuged at 10 000 g for 5 min. Then, 500 µl of benzyl alcohol were transferred into a cup of the autosampler for the determination of Al and Fe by graphite furnace atomic absorption spectrometry.

Determination of FeA and DFA

The same procedure was used except that 25 µl of 0.01 M iron(III) chloride were added to convert DFA into FeA [100 µl of plasma, 25 µl of 0.01 M iron(III) chloride, 200 µl of NH_4NO_3 and 750 µl of benzyl alcohol]. Standards for the preparation of calibration graphs were prepared by adding to normal blood plasma or urine known amounts of DFA (1.25, 2.5, 5 and 10 mg l^{-1}, low concentrations graph, and 12.5, 25 and 50 mg l^{-1}, high concentrations graph). DFA was then converted into FeA by the addition of 25 µl of 0.01 M iron(III) chloride solution or into AlA by the addition of 25 µl of 0.02 M aluminium chloride solution. The extraction procedure was used as described.

The concentrations of FeA and AlA already present in biological samples were measured directly but the concentration of DFA was calculated by taking the difference between the FeA + DFA concentration (after FeCl$_3$ addition) and the FeA concentration.

Results

Fig. 1 shows the absorbance signals obtained for DFA after its conversion into FeA or AlA by increasing the amounts of FeCl$_3$ or AlCl$_3$ added in a constant volume of 25 µl to a 100-µl blood plasma sample containing 100 mg l^{-1} (152.4 µmol l^{-1}) of DFA. Concentrations of 0.01 M of Fe and 0.02 M of Al, sufficient to obtain maximum signals, were used for the analysis.
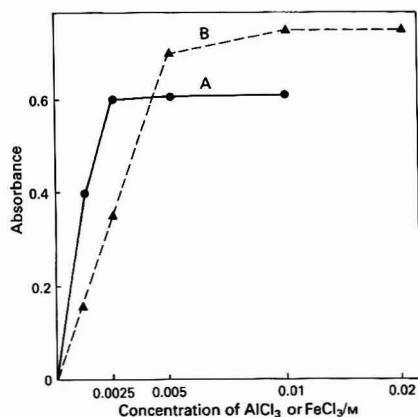
Fig. 2 shows typical calibration graphs obtained after the addition of DFA to normal blood plasma at low concentrations (2.5, 5 and 10 mg l^{-1}) and high concentrations (2.5, 5, 50 and 100 mg l^{-1}) and its conversion into FeA or AlA subsequently extracted in benzyl alcohol. The absorbances of low and high concentrations of FeA are approximately the same (Fig. 2) because a lower volume of benzyl alcohol was injected and a flow of argon gas was used to reduce the sensitivity for high concentrations (Table 1). For FeA measurements, a curvature of the two calibration graphs is observed above 25 and 2.5 mg l^{-1}, respectively, whereas for AlA measurements the response is almost linear up to 10 mg l^{-1}. We tried to cover the largest scale of DFA and FeA concentrations found in biological samples using the same extraction procedure but under two different sets of conditions (Table 1). However, when the DFA or FeA concentrations were higher than 25 mg l^{-1}, a preliminary dilution (1 + 2 to 1 + 10) of the biological sample was necessary.

The detection limit, calculated as equivalent in concentration to twice the standard deviation of the absorbance signal of a sample of blood plasma without DFA, was equivalent to 95 µg l^{-1} of DFA. The sensitivity, defined as the concentration that produces a 0.0044 absorbance for iron, was equivalent to 44 µg l^{-1} or 0.07 µmol l^{-1} of DFA. The reproducibility of the method, tested by ten successive assays of the same sample, was 2.5 and 8% for DFA concentrations of 12.5 and 1.25 mg l^{-1}, respectively. The recovery, studied by adding 1.25, 12.5 and 25 mg l^{-1} of DFA to different biological samples and measuring their concentrations with respect to the calibration graphs, gave an average very close to 100%. The DFA concentrations measured in the blood plasma of patients extended from 1 to 300 mg l^{-1} (1.5 to 457 µmol l^{-1}), depending on the doses given (10–80 mg kg^{-1} body mass), the route of administration (intravenous or intramuscular) and the time of blood sampling.

Discussion

DFA is more soluble in water than in benzyl alcohol, but FeA and AlA are more soluble in benzyl alcohol than in water. This indirect micro-scale method, based on the selective extraction of FeA and AlA with benzyl alcohol and the determination of the metal extracted by electrothermal atomic absorption spectrometry, is applicable only if Fe^{3+} and Al^{3+} added to the sample are not found in the benzyl alcohol phase, in the absence of DFA.

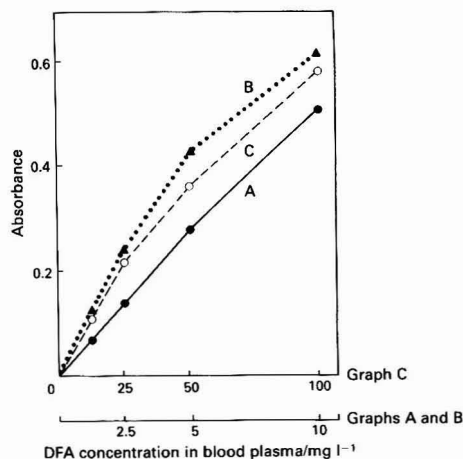After addition of 25 µl of 0.01 M FeCl$_3$ or 0.02 M AlCl$_3$

Fig. 1. Effect of 25 µl of different concentrations of AlCl$_3$ and FeCl$_3$ on the conversion of 100 mg l^{-1} (152 µmol l^{-1}) of DFA into A, FeA and B, AlA, under the conditions described in the text

Fig. 2. Calibration graphs for A, AlA; and B and C, FeA

solutions to 100 µl of blood plasma or urine without DFA, FeA and AlA, we found, at an acidic pH of about 2, very small amounts of Fe and Al in the benzyl alcohol phase but not at pH 5 or higher. Therefore, to guarantee optimum conditions of pH, 200 µl of a buffered solution of NH_4NO_3 at pH 7 were added. Under these conditions, all the blanks (plasma, urine without DFA, AlA and FeA) gave similar absorbance values to those obtained with benzyl alcohol. Moreover, we checked that substances such as citrate, ascorbate and $EDTANa_2$, added to the sample in high concentrations (250 mg l^{-1}), did not modify the blank value. Hence, to our knowledge, DFA is the only molecule present in blood plasma and urine able to transfer Fe and Al into the benzyl alcohol phase and the method described can be considered as specific. In addition to Fe and Al, DFA can form a soluble complex in benzyl alcohol with vanadium. However, when present at only very low levels in biological samples, vanadium does not interfere in the determination of DFA. However, if necessary, it is also possible to measure indirectly the concentration of the vanadium DFA complex. It is interesting that human serum transferrin also binds vanadium.[5]

This indirect micro-scale method with a sensitivity of 44 µg l^{-1} for DFA, is about 100 times more sensitive than the spectrophotometric macro-scale method of Meyer-Brunot and Keberle[2], which is based on an FeA molecular determination at 430 nm in which the smallest amount that can be determined with certainty is 5000 µg l^{-1}. The sensitivity of this indirect micro-scale method could be improved by increasing the volume of benzyl alcohol injected into the graphite furnace, or by automatic multiple injections. In contrast, the sensitivity could be decreased by reducing the amount of biological samples by a preliminary dilution, by increasing the volume of benzyl alcohol used for extraction and by increasing the argon gas flow-rate during atomisation. Therefore, the proposed analytical conditions are a compromise trying to cover the large range of DFA and FeA concentrations found in biological samples after DFA (Desferal) administration.

It is necessary to emphasise that the use of graphite tubes with a cuvette is essential to prevent benzyl alcohol from spreading to the tube extremities, giving very high background signals and non-reproducible specific signals, a problem encountered with other organic solvents.[6] We have been using this indirect method for 1 year and more than 1000 biological samples have been analysed without particular problems.

The results of the study of desferrioxamine pharmacokinetics in healthy subjects and patients with renal failure on haemodialysis and with haemochromatosis will be published elsewhere.

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Quenching Behaviour of Lanthanides on the Ultraviolet Fluorescence of Uranium(VI)

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All of the lanthanides can act as quenchers of the UV fluorescence of uranyl ions in an NaF - LiF flux. Half-concentrations, derived from the semi-logarithmic dependence of the relative fluorescence intensity on the quencher concentration, were found to be constant for Sm, Eu, Gd, Dy, Ho, Er, Tm, Yb and Lu ($C_{1/2}$ *ca.* 1100 p.p.m.). However, Ce, Pr, Nd and Tb showed different quenching behaviour with half-concentrations lower than 300 p.p.m. This peculiar trend exhibited by these elements may be correlated with their chemical behaviour during fusion in an ambient atmosphere yielding coloured flux pellets owing to an at least partial formation of a higher oxidation state of the corresponding element.

Keywords: *Lanthanide quenching behaviour; uranium(VI) fluorescence*

Uranium ores may contain members of the rare-earth group in various concentrations. In the course of the extraction of uranium from mineral leach solutions, some of these elements may accompany it, thus interfering with its fluorimetric determination. Consequently, the quenching power[1] of rare earths has been investigated using the well known NaF = LiF flux mixture.

Although the 14 lanthanides are known to exhibit similar physico-chemical properties, a discontinuity in the quenching power of the series was observed by Veselsky,[2] close to the middle of the series in which light lanthanides were found to be medium quenchers, whereas heavy lanthanides showed a less pronounced effect.

Up to a certain limit, the quenching effect of an element obeys a semi-logarithmic function for the dependence of the relative fluorescence intensity (I with quencher/I_0 without quencher) on the quencher concentration in the pellet.[1,3,4] This behaviour results from the validity of Lambert's law for the absorption of UV and/or fluorescence light of the uranyl ion in the flux pellet.

Lanthanides are commonly trivalent; however, in certain media they may exhibit higher valency states. Molten fluorides support the formation of anomalously high oxidation states. These molten salts may, for example, shift the Ln(III) - Ln(IV) equilibrium in an oxidising atmosphere towards the higher step. Nugent *et al.*[5] studied the prediction of the M(III) - M(IV) standard potential for actinides and lanthanides in aqueous media. These studies were extended by Degueldre[6] to other (organic) media while Duyckaerts and Gilbert[7] extrapolated Nugent *et al.* standard potential predictions to other molecular and ionic solvents of a very different nature (*e.g.,* molten salts). After formation of the higher valency form in a molten flux, the equilibrium is usually frozen during solidification of the fused matrix substance. Hence, the presence of an interfering element at a given valency state in the original sample will yield a quenching effect, the degree of which is closely linked to the oxidation state of that element in the final flux.

In this work we have studied the quenching power of nine lanthanides and compared their half-concentrations with those found previously.[2]

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Experimental

All experiments were carried out with pellets containing 400 mg of NaF - LiF flux (98 + 2). The flux mixture was fused in small platinum dishes at a temperature of 1000 °C in a model 3A Simon-Mueller furnace. Standard amounts of uranium (1 µg) were added prior to fusion, together with variable amounts of the quenching material.[1]

The platinum tray used in our laboratory holds ten dishes. For every experiment one standard (1 µg of U), one blank and eight sample pellets containing the same amount of uranium but at four different quencher concentrations were prepared in duplicate. In another series of experiments the lanthanide and uranium concentrations were kept constant but different quenchers were used.

The quencher solutions were prepared by dissolution of very pure rare earth oxides supplied by Koch-Light (UK) in approximately 6 M hydrochloric acid; the acidity of the solution was kept to a minimum. The uranium standards were prepared by suitable dilution of a uranium stock solution (1000 µg ml^{-1}).

A Galvanek-Morrison fluorimeter was used throughout.

The data obtained were submitted to linear regression analysis.

Results and Discussion

Verification of the Quenching Function

The reduction of the UV fluorescence of U(VI) caused by a quencher is given by a semi-logarithmic function of the quencher concentration (C) in the pellet:

$$\ln (I/I_0) = -kC \quad \text{and} \quad C_{1/2} = \frac{\ln 2}{k}$$

where I is the fluorescence intensity at a quencher concentration C; I_0, the fluorescence intensity without quencher; k, absorption constant; and $C_{1/2}$, the half-concentration at $I = I_0/2$.

At first, a verification of this concept (theoretically derived by Price *et al.*[3] and confirmed by Veselsky[1]) was attempted for nine lanthanides. Figs. 1, 2 and 3 show semi-logarithmic graphs of the relative fluorescence intensities observed experimentally for various concentrations of quencher. All the pellets prepared represented uniform fluxes that did not require any homogenisation by a second fusion. Linear regression analysis was required for the half-concentration determinations because Sm, Eu, Dy, Ho, Er, Tm and Lu exhibited

Table 1. Dependence of relative fluorescence intensity on the lanthanide concentration

Element				N^*	$C_{1/2}$,† p.p.m.	$(I/I_0)_{C=0}$,‡ %
Pr	..	..	..	12	194 ± 18	104.3 ± 1.03
Sm	..	..	..	14	1163 ± 132	93.1 ± 1.03
Eu	..	..	..	14	1201 ± 160	91.6 ± 1.04
Tb	..	..	..	09	141 ± 21	91.5 ± 1.08
Dy	..	..	..	14	1095 ± 107	102.4 ± 1.03
Ho	..	..	..	13	1060 ± 89	104.8 ± 1.03
Er	..	..	..	14	1026 ± 80	99.4 ± 1.02
Tm	..	..	..	13	1045 ± 86	103.3 ± 1.03
Lu	..	..	..	22	1189 ± 117	107.0 ± 1.04

* N, number of samples.

† $C_{1/2}$, half-concentration and its standard deviation.

‡ $(I/I_0)_{C=0}$, intercept and its standard deviation
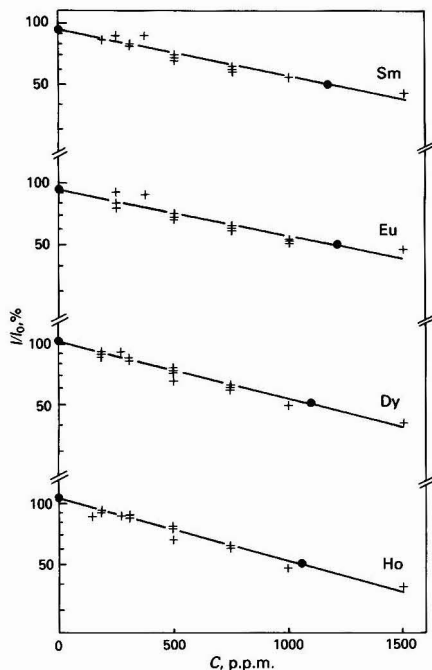
Fig. 1. Semi-logarithmic dependence of uranyl fluorescence intensity on quencher concentration for Sm, Eu, Dy and Ho

Fig. 2. Semi-logarithmic dependence of uranyl fluorescence intensity on quencher concentration for Er, Tm and Lu

similar (weak) quenching power whereas Pr and Tb behaved as medium quenchers. Table 1 shows the calculated concentrations for which the relative standard deviation was around 10%. The theoretical intercept value ($I/I_0 = 100\%$ for $C = 0$) was not introduced in the calculation treatment as an observed value but it was calculated by means of the experimental values. In all instances the average of the intercept was around 100% and although their standard deviations were small (1.0–1.1% for each element) a relative standard deviation of 10% was also assumed here. According to Figs. 1–3 the semi-logarithmic dependence, up to a certain limit (30% for Tb in Fig. 3) of the relative fluorescence intensity on the quencher, was assumed to be valid for the nine lanthanides investigated.

Comparison of the Quenching Power of the Lanthanides

The half-concentration plotted in Fig. 4 for lanthanum and the lanthanides are taken from measurements on fluxes obtained

by addition of U and quencher (trivalent lanthanide) before fusion. From Table 2 it can be seen that the quenching power is constant for most of these elements (*i.e.*, 1100 ± 300 p.p.m. for Sm, Eu, Gd, Dy, Ho, Er, Tm, Yb and Lu). Pellets containing any of these elements at a concentration equal to the half-concentration were white or near-white, and because of their high $C_{1/2}$ values, were categorised as weak quenchers. Ce, Pr, Nd and Tb act as medium quenchers (with $C_{1/2} < 300$ p.p.m.) but although pellets containing Pr or Tb were strongly coloured, those containing Ce or Nd were very pale (almost white).

As some lanthanides (Ce, Pr, Nd, Tb, Dy and Tm) are reported to yield tetravalent compounds,[8] our attention was drawn to those rare earths that may be oxidised during the fusion.

Some lanthanide sesquioxides (Ln_2O_3) or lanthanide compounds prepared from volatile acids give dioxides (LnO_2) or at least oxides (LnO_x with $1.5 < x < 2$) on heating in air (*e.g.*, CeO_2, white or pale yellow[9]; Pr_6O_{11} or PrO_2, black[10,11]; TbO_2, chocolate[12]; whereas Nd_4O_7, proposed by Brauner, is doubtful). Hence, after fusion, a certain amount of Ln(IV) can subsist or be formed in the molten fluorides where the presence of oxygen acts as an oxidising agent, while oxide anions present in the molten flux stabilise the higher valency states. During the pellet solidification, the Ln(III) - Ln(IV) equilibrium is frozen. In the final flux, covalent Ln(IV) compounds show different quenching powers to the more ionic Ln(III) forms.

Table 2. Comparison of the quenching power of lanthanides in the NaF - LiF flux and their tendency to yield the tetravalent state

Element				$C_{1/2}$, p.p.m.	Colour of pellet*	$E°$ †/V vs. Ce	Compounds of tetravalent element‡
Ce	..	..	..	300§	Slightly grey	0.00	CeO_2, CeF_4, Cs_3CeF
Pr	..	..	..	194¶	Black	1.45	PrO_2, PrF_4, Cs_3PrF_7
Nd	..	..	..	250§	Slightly grey	2.65	Nd_4O_7 ? Cs_3NdF_7
Pm	..	..	..	—	—	2.95	
Sm	..	..	..	1163¶	Hardly brown	3.35	
Eu	..	..	..	1201¶	Hardly ochre	4.55	
Gd	..	..	..	1487§	Hardly ochre	6.15	
Tb	..	..	..	141¶	Strong chocolate	1.35	TbO_2, TbF_4, Cs_3TbF
Dy	..	..	..	1095§	Slightly yellow-pink	3.15	Cs_3DyF_7
Ho	..	..	..	1060¶	Slightly ochre-pink	4.25	
Er	..	..	..	1026¶	Pink	4.35	
Tm	..	..	..	1045¶	Slightly yellow	4.35	Cs_3TmF_7
Yb	..	..	..	800§	Slightly ochre	5.55	
Lu	..	..	..	1189¶	Slightly yellow	7.35	

* Pellet colour for C = 4000 p.p.m.

† $E°$ from Nugent et al.,[5] extrapolated to other media,[7] reported vs. $E_{Ce(III) - Ce(IV)}$.

‡ Ln(IV) compounds from references 5 and 8–12.

§ $C_{1/2}$ values from reference 2.

¶ $C_{1/2}$ values from this work.
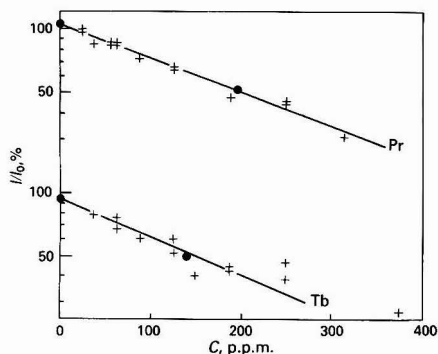
Fig. 3. Semi-logarithmic dependence of uranyl fluorescence intensity on quencher concentration for Pr and Tb
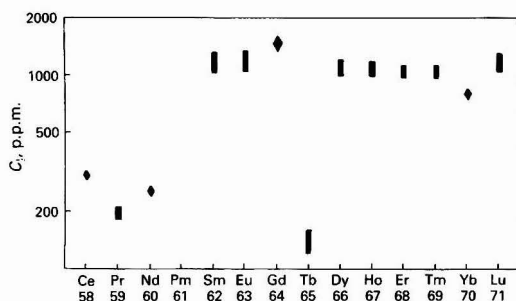
Fig. 4. Dependence of $C_{1/2}$ on the atomic number of lanthanides: ■, this work; ◆, from reference 2

Tests to prove the presence of tetravalent lanthanides were carried out with cerium. Fluxes containing no uranium were spiked with a Ce(III) standard solution (up to 2000 p.p.m.). After fusion, the pellets containing cerium were not completely soluble in water, but they were completely dissolved in concentrated sulphuric acid (70 °C). The flux leaching solution exhibited the yellow colour of Ce(IV). This test shows the presence of tetravalent cerium in the pellets investigated. Another significant observation, which proved the valency

change during the fusion - solidification process, was that the Ce(III) half-concentration (300 p.p.m.) reported earlier[2] was lower than the average $C_{1/2}$ for the weaker quenching lanthanides ($C_{1/2}$, 1100 p.p.m.) whereas the half-concentration from pellets obtained by spiking Ce(IV) standard solutions was 138 p.p.m.[4] This result can be explained by a partial oxidation of the initial trivalent cerium during the pellet preparation.

For praseodymium, terbium and neodymium, the low half-concentrations could also be explained on the basis of a modification of their initial valency state. As shown in Table 2, the colours of the pellets spiked with Pr and Tb corresponded to the colour of their dioxides. Pellets containing Nd were pale grey, but information on the colour of neodymium dioxide was not available from the literature. Hence, the presence of Nd(IV) and Ce(IV) (pale, covalent compounds), which would absorb in the wavelength range of the UV absorption of the uranyl ion, were assumed to explain their medium quenching power. However, this behaviour can be explained in the same way for Tb and Pr and by considering also the absorption of the emitted fluorescent light on addition of coloured terbium or praseodymium tetravalent compounds.

Comparison of the quenching half-concentration of the rare earths and their ability to form tetravalent compounds was made on the basis of the Ln(III) - Ln(IV) potential calculated by Nugent et al.[5] and reported (Table 2) versus the potential of cerium. This classification of potential can be extrapolated to other media[7] and thus to the molten flux where Ce(IV), Pr(IV), Tb(IV) and Nd(IV) (to a lesser extent) can be formed or maintained at their valency state because their potentials were near to the Ce(III) - Ce(IV) potential. The other lanthanides, however, are much more difficult to oxidise in the flux where they behave as weak quenchers in the trivalent state.

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Quantitative Determination of Thiourea in Aqueous Solution in the Presence of Sulphur Dioxide by Raman Spectroscopy

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A method has been developed for the quantitative determination of thiourea in aqueous solutions acidified with sulphuric acid in the presence of sulphur dioxide. The method uses laser Raman spectroscopy and is valid in the presence of high concentrations of sulphur dioxide and inorganic salts. This is particularly important with respect to the use of such solutions for the leaching of gold from ores and concentrates. The method is simple, rapid and accurate to within ±1.2% and has been applied to the study of solutions used in leaching experiments.

Keywords: *Thiourea determination; sulphur dioxide; Raman spectroscopy; gold ores*

The use of an aqueous solution of thiourea as a leaching agent for gold has been widely reported in the literature.[1-3] It offers several advantages over sodium cyanide in that it operates in acidic media, forming a cationic gold complex, and has faster dissolution kinetics. However, a major disadvantage is its tendency to oxidise, eventually forming elemental sulphur and several other decomposition products. This leads to an excessive consumption of the thiourea, which generally makes its use uneconomical in the treatment of gold-bearing ores and concentrates.

The addition of sulphur dioxide during the leaching process has been found to decrease the thiourea consumption considerably by, it is thought, preventing the oxidation process.[4-6] In order to evaluate the economic feasibility of a gold leaching operation based on thiourea, it is necessary to have a reliable method for determining its concentration in acidified aqueous solutions containing sulphur dioxide and a range of metal salts. The standard methods for quantitatively determining thiourea in water are: (1) titration with *N*-bromosuccinimide[7]; (2) titration with mercury(III) nitrate[8]; and (3) potentiometric titration with potassium iodate.[9]

It was found that iron(II) ions, present in the ores, interfere with method 1 and sulphur dioxide interferes with methods 2 and 3. An alternative method is therefore required for this type of study, and the technique of Raman spectroscopy offers some distinct advantages for this purpose.

Experimental

Apparatus

Potentiometric titration was carried out using a Metrohm titroprocessor. The titration curve and titre volume were plotted automatically using a Knauer dual-pen recorder. All Raman spectra were recorded using an Anaspec Model 36 laser Raman spectrometer fitted with a Tracor Northern Reticon Type S intensified diode array detector.

Reagents

All of the reagents used were of analytical-reagent grade or equivalent. Solutions of thiourea were made up in sulphuric acid (0.1 M) in various concentrations between 3 and 20 g l⁻¹, the mass of the thiourea being determined to three decimal places. Solutions for analysis by Raman spectroscopy also contained an accurately weighed amount of acetic acid as an internal standard (see below). An approximate 1 : 1 molar ratio of acetic acid to thiourea was used. Sodium metabisulphite was used to dope the thiourea solutions with sulphur dioxide, assuming that 1 mol of sodium metabisulphite forms 2 mol of sulphur dioxide in acidic solution.

Procedures

Potentiometric titration of thiourea

Thiourea is oxidised by potassium iodate in acidic solution to give formamidine disulphide:

$$6SC(NH_2)_2 + KIO_3 + 6H^+ \rightarrow 3[SC(NH_2)_2]_2^{2+} + 3H_2O + KI$$
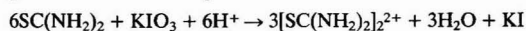
The end-point of the titration is shown by a sharp increase in potential at a platinum redox electrode. A known volume of the solution to be analysed (5–20 ml) was pipetted into 50 ml of 1 M phosphoric acid and titrated with 0.017 M potassium iodate solution using the titroprocessor. The thiourea concentration was calculated from a knowledge of the volumes of thiourea solution taken and potassium iodate solution used and the molarity of the potassium iodate solution.

Using this technique a thiourea solution, containing a weighed concentration of 7.602 g l⁻¹ in 0.1 M sulphuric acid, was analysed nine times and gave a mean of 7.57 g l⁻¹ with a standard deviation of 0.05 g l⁻¹.

Laser Raman spectroscopy

The Raman spectrum of an aqueous solution of thiourea, showing the C=S stretching mode of thiourea at 735 cm⁻¹, is shown in Fig. 1; the γ (OCO) mode of acetic acid at 880 cm⁻¹ is shown in Fig. 2. As Raman spectroscopy is not intrinsically quantitative, an internal standard must be used for analyses of this type. Because of the particular application to gold leaching, in this study the aqueous solution of thiourea would contain sulphuric acid, sulphur dioxide, various inorganic salts and oxidation products of thiourea. Hence, the best internal standard for this application is one with a strong Raman band that is well separated from any bands from other components likely to be present and stable in sulphuric acid. The compound chosen was acetic acid, which has a strong Raman band at 880 cm⁻¹ (Fig. 2) that is well separated from thiourea,
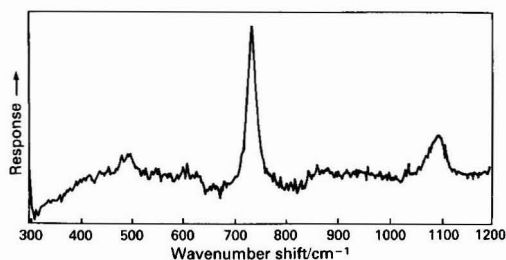

Fig. 1. Raman spectrum of aqueous thiourea

sulphuric acid, sulphur dioxide or common anion bands. The intensity of this band was found to be similar to that of the thiourea band at 735 cm^{-1} (Fig. 1) and so acetic acid was added to the solutions to be analysed at approximately the same molar concentration as the thiourea.

The concentration of thiourea is then obtained from the ratio of the integrated areas of the 735 and 880 cm^{-1} bands, and the known mass of acetic acid added to the solution. The Raman technique was calibrated using a range of standard solutions (Table 1) in order to establish the relationship between the 735 and 880 cm^{-1} bands. It was found necessary, because of differences in response of the various component diodes of the diode array with changes in laser power, to re-calibrate with a known standard before each analysis, as the ratio of the 735 to 880 cm^{-1} bands could vary by as much as 5%. It was also necessary to use the same area of the detector for each measurement to increase the accuracy of the method.

Leach tests

Samples (50 g) of pyrite concentrate (head assay = 17 p.p.m. of gold) were leached with 117 ml of 0.1 M sulphuric acid, containing 10 g l^{-1} of thiourea, at 25 °C. The concentrate was stirred at 700 rev. min^{-1} in a 500-ml flat-bottomed glass vessel with indented baffles to improve the mixing. The potential of the solution was monitored by means of a platinum redox electrode, the oxidation potential being maintained between 380 and 420 mV for the first 3 h by the addition of iron(III)
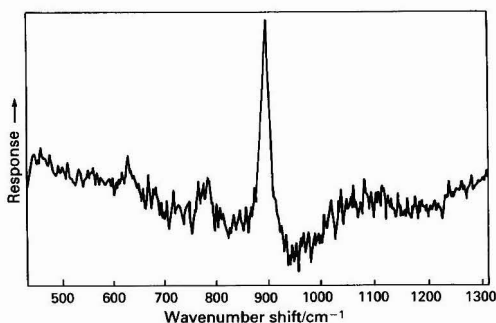
Fig. 2. Raman spectrum of aqueous acetic acid

Table 1. Determination of the thiourea concentration with no sulphur dioxide

	Thiourea concentration/g l^{-1}	
Thiourea concentration (by mass)/g l^{-1}	Potentiometric titration	Raman spectroscopy
3.087	3.1	Standard
5.022	5.1	Standard
9.868	9.9	Standard
9.960	9.9	Standard
5.02	5.0	4.96
10.00	9.9	10.02
19.98	19.9	19.83

sulphate solution. The appropriate mass of sodium metabisulphite was added in stages over these first 3 h to give the required thiourea to SO$_2$ molar ratio. During the last hour of the test no adjustment was made to the potential of the solution, which fell to between 233 mV (SO$_2$ to thiourea molar ratio of 2 : 1 m/m) and 368 mV (no SO$_2$). At the end of the leach the pulp was filtered and the residue washed with 2 × 100 ml of distilled water, the filtrate and washing being combined. The total volume of the solution was then measured.

The solution was analysed for thiourea by both potentiometric titration and laser Raman spectroscopy, the latter after the addition of the appropriate amount of acetic acid as the internal standard. The solution was also analysed for gold by atomic absorption spectrometry and the gold content of the dried residue was determined by fire assay. The percentage of the total gold extracted into solution could therefore be calculated. The mass of thiourea in the feed and product solutions was calculated and the amount of thiourea consumed in the leach test expressed as the mass of thiourea consumed (in kg) per mass of pyrite concentrate used in the test (in tonnes) $i.e.$, kg tonne^{-1}.

Two tests were carried out using the conditions described above, but in a 1-l vessel using 100 g of concentrate and 233 ml of a 50 g l^{-1} solution of thiourea in 1 M sulphuric acid.

Results and Discussion

Determination of the Thiourea Concentration in the Absence of Sulphur Dioxide

The concentration of thiourea in five solutions containing 5.02, 10.00 and 19.98 g l^{-1} of thiourea (by mass) was determined both by potentiometric titration and laser Raman spectroscopy. The results are given in Table 1. Generally good agreement was found between the amount of thiourea added and the amount found using the two analytical techniques, the maximum error being 1% for the potentiometric and 1.2% for the laser Raman technique.

Determination of the Thiourea Concentration in the Presence of Sulphur Dioxide

The results are listed in Table 2. Typical traces from the potentiometric titration of a 0.1 M thiourea solution containing different amounts of sulphur dioxide are shown in Fig. 3. At an SO$_2$ to thiourea molar ratio of 1 : 2 the change in potential owing to the reaction of the sulphur dioxide with potassium iodate can be clearly seen (marked as the SO$_2$ end-point) although in practice the end-point of this reaction is difficult to determine. Comparing the trace of thiourea alone with that of a solution containing an SO$_2$ to thiourea molar ratio of 1 : 10, no obvious difference in curve shape is apparent. However, the calculated thiourea concentrations of the two solutions are 7.6 g l^{-1} (no SO$_2$) and 8.3 g l^{-1} (with SO$_2$). Thus, at low concentrations of SO$_2$ its reaction with potassium iodate cannot be detected from the shape of the potentiometric curve. The fact that a falsely high figure for the thiourea concentration is obtained is not apparent using this method at low sulphur dioxide concentrations.
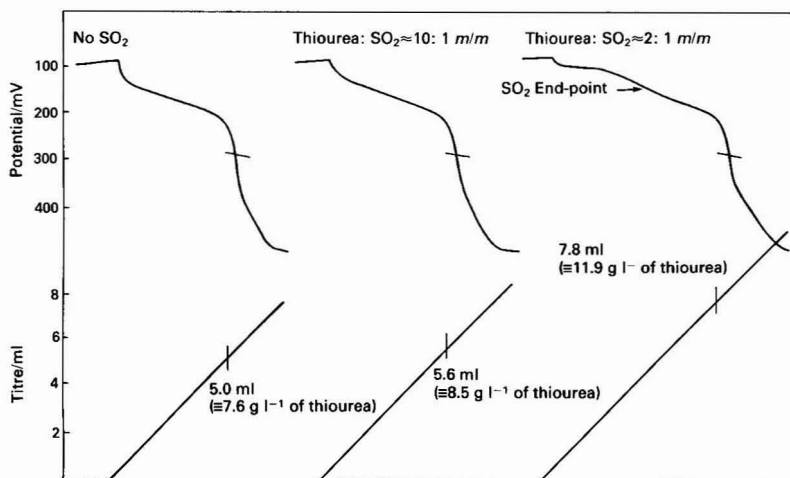
Table 2. Determination of thiourea concentration in the presence of sulphur dioxide

Thiourea concentration (by mass)/ g l^{-1}	Na$_2$S$_2$O$_5$ concentration/ g l^{-1}	SO$_2$: thiourea molar ratio	Thiourea concentration/g l^{-1}	
			Potentiometric titration	Raman spectroscopy
10.10	12.6	1 : 1	23.2, 24.1	9.95
10.10	4.6	1 : 2.7	14.6, 15.1	9.98
10.10	2.5	1 : 5	13.0, 12.5	10.11

Fig. 3. Potentiometric titration of a 7.61 g l^{-1} of thiourea solution

Table 3. Leaching of a pyritic gold ore using thiourea

Test No.	Thiourea in feed (by mass)/g l^{-1}	$Na_2S_2O_5$ added/g	SO_2: thiourea molar ratio	Gold extraction, %
1	10.12	0	—	73
2	10.12	0.75	1:2	61
3	10.00	1.4	1:1	62
4	10.00	2.8	2:1	59
5	50.03	0	—	70
6	50.03	8.0	1:2	67

Table 4. Analysis of leach liquors

Test No.	SO_2: thiourea molar ratio	Thiourea in feed by potentiometric titration/g l^{-1}	Thiourea in leach liquor by potentiometric titration/g l^{-1}	Thiourea in leach liquor by Raman spectroscopy/g l^{-1}	Thiourea consumption*/ kg tonne^{-1} Potentiometric titration	Raman spectroscopy
1	—	10.0	2.3	2.27	10.2	9.8
2	1:2	10.0	3.1	3.35	5.4	4.0
3	1:1	9.9	4.1	3.84	(−0.4)	1.0
4	2:1	9.9	7.1	3.34	(−20.1)	2.8
5	—	49.8	22.5	22.55	25.1	24.9
6	1:2	49.8	23.0	22.05	(−0.5)	4.3

* Parentheses indicate that the thiourea concentration determined is apparently greater than the initial concentration of thiourea.

A 0.1 M sulphuric acid solution containing 10.10 g l^{-1} of thiourea was doped with different amounts of sodium metabisulphite to give calculated SO_2 to thiourea molar ratios of 1:1, 1:2.7 and 1:5. The thiourea concentration of the solutions was then determined by laser Raman spectroscopy and potentiometric titration. Based on the total volume of potassium iodate used to titrate the samples, thiourea concentrations of 24, 15 and 13 g l^{-1}, respectively, were obtained by potentiometric titration. In each instance the presence of SO_2 in the solution was obvious from the shape of the potentiometric curve. The thiourea concentrations obtained for the same solutions by Raman spectroscopy were 9.95, 9.98 and 10.11 g l^{-1}, respectively, showing that the presence of SO_2 did not affect this technique.

Results of the Leaching Studies

The results of the leaching studies are given in Tables 3 and 4.

As the amount of sodium metabisulphite added during the leaching of the pyrite concentrate was increased, the difference between the thiourea concentration of the leach liquors, as determined by the two analytical methods, also increased. With no sulphur dioxide present both methods gave similar thiourea concentrations in the leach liquor, *i.e.*, 2.27 g l^{-1} by Raman spectroscopy and 2.3 g l^{-1} by potassium iodate titration. The amount of thiourea consumed was, therefore, similar, 9.8 and 10.2 kg tonne^{-1}, respectively. At an SO_2 to thiourea molar ratio of 1:2 the thiourea concentration in the leach liquor was 3.35 g l^{-1} by Raman spectroscopy and 3.1 g l^{-1} by potassium iodate titration, giving thiourea consumptions of 4.0 and 5.4 kg tonne^{-1}, respectively.

Increasing the amount of SO_2 in the leach to a 1:1 SO_2 to thiourea molar ratio and above obviously left some free SO_2 in the leach liquor as the analysis by potentiometric titration gave a higher thiourea concentration in this solution than the feed concentration. Analysis using Raman spectroscopy gave

thiourea consumptions of 1.0 kg tonne^{-1} (SO_2 to thiourea, 1:1) and 2.8 kg tonne^{-1} (SO_2 to thiourea, 2:1).

Leach tests using 50 g l^{-1} of thiourea in 1 M sulphuric acid gave a greater decrease in the thiourea consumption when sulphur dioxide was present than the tests that used 0.1 M sulphuric acid. With no sulphur dioxide present, the Raman and potentiometric methods again gave similar thiourea consumption figures, 24.9 and 25.1 kg tonne^{-1}, respectively. With an SO_2 to thiourea molar ratio of 1:2 in the leach, the calculated value for thiourea consumed fell to 4.3 kg tonne^{-1} by Raman spectroscopy, whereas the potentiometric result indicated the production of 0.5 kg tonne^{-1}, obviously an unacceptable result.

The presence of sulphur dioxide in the leach tests using 0.1 M sulphuric acid appeared to decrease the gold extraction. At present, the reason for this is not apparent, but it may relate to the redox potential being allowed to fall over the final hour of the leach in order to minimise the amount of sulphur dioxide left in solution.

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Simultaneous Collection of Sulphur Dioxide and Sulphate by a Selective Sampler and their Analysis at Background Levels

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Sulphate and sulphur dioxide (SO_2) were collected separately from the atmosphere, with high efficiency and selectivity, in a dual filter apparatus. The first filter, impregnated with perchloric acid, was used to collect the aerosol sulphate and the second filter, impregnated with sodium hydroxide solution, collected the gaseous SO_2. The analysis of these species was carried out by ion chromatography (sulphate) and voltammetry (SO_2) in the filter washings. Operating with sampling times of 30 min, at a filter face velocity of *ca.* 18 cm s^{-1}, the detection limits were estimated to be 0.8 µg m^{-3} for sulphate and 0.3 µg m^{-3} for SO_2. The possible oxidation of SO_2 by NO_x is discussed and the operating conditions required to make this reaction negligible are given.

Keywords: *Air pollution; sulphur oxides sampling; sulphur oxides analysis; aerosols*

The measurement of sulphur dioxide (SO_2) and of all types of sulphates in the air is important for studies of SO_2 oxidation processes and of the generation, transport and removal of sulphate in the atmosphere. These studies require SO_x measurements far from the emission point, where the SO_x concentration may be close to the background levels (0.2–2 µg m^{-3}). However, the sampling and the SO_x measurements, particularly at low concentration levels, leave some questions to be answered. In fact, several workers have pointed out that the collection of atmospheric aerosols on filters leads to an artifact sulphate formation as a consequence of the adsorption and oxidation of SO_2 on the filter.[1-6] Further, it should be noted that, in the presence of NO_x, which is a normal component of the atmosphere, the collection and measurement of SO_2 may be affected by the reaction between NO_x and SO_2, which occurs at a significant rate at neutral or acidic pH.[7,8] The main objective of this work was to demonstrate the possibility of simultaneous, but separate, collection from the air of SO_2 and of all types of sulphate, in such a way as to avoid sampling errors inherent to SO_2 oxidation and/or adsorption. In the proposed method, sulphate and SO_2 were collected and measured by a simple laboratory-constructed ion chromatograph and by a voltammetric method, respectively,[9] at concentrations as low as 0.8 g m^{-3}.

Experimental

Materials and Apparatus

All solutions were prepared from analytical-reagent grade chemicals and triply distilled water. High purity nitrogen - oxygen mixtures were supplied by SIO (Milan). All tubing in contact with SO_2 and air was made of PTFE to avoid adsorption phenomena. Microsorban (Delbag) and Microquartz (Gelman) filters, 47 mm diameter, were utilised to collect SO_x. The anion separating resin was prepared according to Small *et al.*,[10] and the stripper resin was a strong cation exchanger (Dowex 5W-X8) containing 5 mequiv. g^{-1} of SO_3H groups. H_2SO_4 and $(NH_4)_2SO_4$ aerosols, particle size range 0.1–1.0 µm, were produced by an aerosol generator according to Niessner and Klockow.[11]

Voltammetric analyses of SO_2 were carried out with an Amel 472 multipolarograph unit according to Rigo *et al.*[9] A laboratory-constructed ion chromatograph apparatus was utilised to measure sulphate. The volume of samples injected into the chromatograph varied from 0.1 to 2 ml and a conductivity cell, having a volume of 5 µl and a cell constant of 0.07 cm^{-1}, was used. Sodium hydroxide solution (2×10^{-3} M) was utilised as the eluent at flow-rates in the range 1–2.5 ml min^{-1}. Standard SO_2 atmospheres were obtained by passing purified air over a calibrated SO_2 permeation tube and NO_x atmospheres were prepared according to Hashimoto and Tanaka.[12] NO_x concentrations were measured by the Saltzman method.[13]

Sampling Procedure

The sulphate-collecting filters were usually impregnated with 10^{-3} M $HClO_4$ containing 10^{-4} M EDTA (acid filter) and 10^{-1} M NaOH solution was utilised for impregnating the SO_2-adsorbing filters. The impregnation was carried out by pouring the solution directly on to the filter placed on the filter holder (PVC and PTFE were utilised for the sulphate and SO_2 filter holders, respectively) and the excess of solution was removed with a stream of nitrogen.

The air was drawn through the acid and/or the basic filter at known filter face velocities. Face velocities in the range 1.2–24 cm s^{-1} (STP), which correspond to flow-rates in the range 1.25–25 l min^{-1}, were obtained with an Edwards SD 100 pump. After sampling, the filters were rinsed five times with the washing solution under a positive nitrogen pressure, to give a final volume of 5 ml. The washing solutions for the basic and acid filters were usually 10^{-1} and 10^{-3} M NaOH solution, respectively.

Results and Discussion

Collection Efficiency of Sulphates on an Acid-impregnated Filter

Preliminary laboratory experiments were carried out with two types of filters (Microsorban and Microquartz) to test their capacity to collect sulphate aerosols generated by the apparatus described by Niessner and Klockow.[11] Both dry and acid-impregnated filters were utilised. Aqueous solutions of

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Table 1. Collection efficiency of sulphate aerosols. The aerosols were generated according to Niessner and Klockow.[11] The collection efficiency using a dry or a 10^{-3} M $HClO_4$-impregnated filter was evaluated by measuring the sulphate collected on a Microsorban filter impreganted with 0.1 M NaOH solution, in series with the tested filter. The sampling time was 30 min and the filter diameter was 47 mm

Filter					Aerosol		
Type*			Conditions	Face velocity/ cm s^{-1}	Type	Concentration/ mg m^{-3}	Capture efficiency, %
MQ	..	.. ..	Dry	1	H_2SO_4	5.25	87
MQ	..	.. ..	$HClO_4$	1	H_2SO_4	5.25	78
MS	..	.. ..	Dry	1	H_2SO_4	5.25	>99.5
MS	..	.. ..	Dry	4.8	H_2SO_4	1.05	95
MS	..	.. ..	$HClO_4$	1	H_2SO_4	0.31	>99.5
MS	..	.. ..	$HClO_4$	5	H_2SO_4	0.31	>99.5
MS	..	.. ..	$HClO_4$	18	H_2SO_4	0.31	>99.5
MS	..	.. ..	$HClO_4$	18	$(NH_4)_2SO_4$	0.41	>99.5

* MQ, Microquarz (Gelman); MS, Microsorban (Delbag).
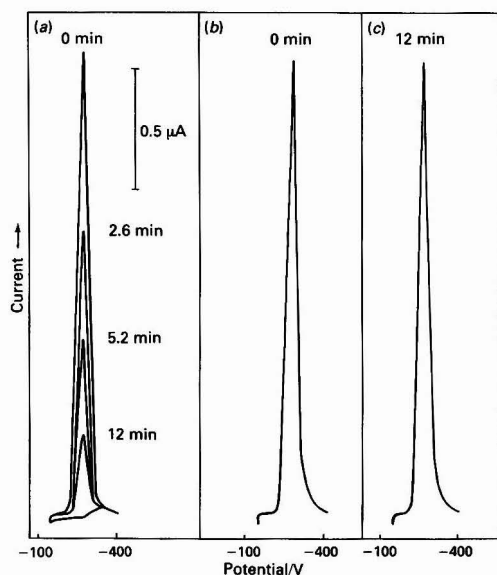
Fig. 1. Time dependence of differential-pulse voltammograms of SO_2 in the presence of nitrite. (a) The voltammograms were obtained after addition of 1 ml of 1.6 M HCl to 4 ml of a basic solution containing 1×10^{-5} M SO_3^{2-} and 2.4×10^{-5} M NO_2^-. (b) As in (a), but in this instance the HCl solution also contained 1×10^{-2} M sulphanilic acid and 0.5 M acetic acid. Other experimental conditions: pulse height, 80 mV; sweep rate, 10 mV s^{-1}; and delay time, 16 s

different acids were used to impregnate the filters in order to minimise the SO_2 adsorption. $HClO_4$ was found to be the most suitable acid as it is non-volatile and does not interfere in the ion chromatographic analysis. Usually, 10^{-4} M EDTA was added to the acid solution to chelate Pb and Ca ions, etc., which may be found in the atmospheric aerosols and give insoluble sulphates. The collection efficiency was evaluated by measuring the sulphate both on the tested acid filter and on a Microsorban filter, impregnated with 10^{-1} M NaOH, placed in series after the acid filter. As it was possible to detect 250 ng of sulphate on the basic filter, the collection efficiency of the acid filter was measured accurately. As shown in Table 1, the results were dependent on the type of filter. Under the same experimental conditions, both dry and impregnated Microquartz filters showed a lower collection efficiency than Microsorban filters. The latter, when impregnated with 10^{-3} M $HClO_4$, showed a recovery efficiency above 99.5% up to face velocities of 18 cm s^{-1}.

The washing solution usually utilised was 10^{-3} M NaOH solution, but no difference in the sulphate recovery was found when water was used instead. Finally, it was found that the Microsorban filters could be utilised for tens of measurements whereas the Microquartz filters, when impregnated with acid solutions, lost their consistency after one sampling. As a consequence, subsequent experiments were carried out with Microsorban filters.

SO_2 Collection on Basic Filters and its Interaction with the Acid Filter

As reported in a previous paper,[9] Microsorban filters, impregnated with 10^{-1} M NaOH solution, can be utilised to sample SO_2 very efficiently at concentrations as low as 0.4 µg m^{-3}. Further, the presence of an acid filter before the basic filter should permit the separation of sulphate from SO_2. In fact, whereas sulphate is collected on the $HClO_4$-impregnated filter having a pH of ca. 3, SO_2 is not retained as at this pH SO_2 exists both as HSO_3^- and $SO_2.nH_2O$, which is continuously swept out by the air stream. This minimises the residence time of SO_2 on the filter and, as a consequence, its possible oxidation to sulphate. In order to verify this hypothesis, about 425 l of N_2 - O_2 mixture (21% of oxygen) containing 75 µg m^{-3} of SO_2, which corresponds to a total amount of 500 nmol of SO_2, were drawn through to the acid-soaked filter at face velocities in the range 2–18 cm s^{-1}. The filter was rinsed with 10^{-3} M NaOH solution and the washings tested for sulphate and SO_2. In this experiment both species were found to be absent, within the detection limits of the respective methods, which are below 1% of the SO_2 present in the N_2 - O_2 mixture. This indicates that no SO_2 adsorption or oxidation occurred on the $HClO_4$-impregnated filter.

NO_x - SO_2 Reactions

Notwithstanding the low solubility of NO_x in aqueous solutions, a fraction of it is adsorbed on the NaOH-impregnated filter, where they are hydrolysed with the formation of NO_2^-. This species may react with SO_2, the reaction rate being strongly dependent on pH. As this reaction is negligible at alkaline pH,[8] during the collection on the NaOH-impregnated filter SO_2 is insensitive to the presence of NO_x. However, SO_2 could be oxidised by NO_x on the acid filter and also during the voltammetric measurements of SO_2 carried out on the washings from the NaOH-impregnated filter. With reference to the last point, it must be mentioned that, in order to obtain high sensitivities, the voltammetric measurements must be carried out at low pH,[9] where the reaction between SO_2 and NO_2^- occurs at a high rate. In fact, if NO_2^- is present in the washings from the basic filter, there is a continuous decrease in the SO_2 wave after the addition of the concentrated HCl, as can be seen in Fig. 1(a). However, we

Table 2. Effect of the face velocity on the reaction between SO_2 and NO_x occurring on the acid filter during field experiments. The NO_x concentration was increased by emission in the atmosphere of controlled amounts of NO_x. SO_2 was simultaneously collected on two Microsorban filters, impregnated with 0.1 M NaOH solution, one of them being placed in series after a $HClO_4$ treated filter

Face velocity/ cm s^{-1}	NO_x* concentration/ $\mu g\,m^{-3}$	$(SO_2)_B$† concentration/ $\mu g\,m^{-3}$	$(SO_2)_{A+B}$‡ concentration/ $\mu g\,m^{-3}$
3	965	45.5	11.1
9.5	1006	37.2	22.5
13	86	36.0	29.4
18	109	31.7	31.5
21	109	31.2	31.5
19	199	2.9	3.1
21	78	3.7	4.0

* As NO_2.

† $(SO_2)_B$: SO_2 collected on the basic filter.

‡ $(SO_2)_{A+B}$: SO_2 collected on the basic filter placed after the acid filter.

Table 3. SO_2 and sulphate concentrations in Venice in winter

		Concentration/$\mu g\,m^{-3}$		
		SO_2	$SO_4{}^{2-}$	$[SO_2]/[SO_4{}^{2-}]$
Minimum	..	4.6	18.8	0.13
Maximum	..	329	235	3.7
Average ..	..	77	68	1.2

found that the addition of concentrated HCl containing sulphanilic and acetic acid prevents the oxidation of SO_2 by NO_x, which takes place in the polarographic cell [see Fig. 1(b)]. A similar result, i.e., a very stable SO_2 voltammogram, was obtained even in the presence in the polarographic solution of $NO_2{}^-$ concentrations ten times higher than the SO_2 concentration. As regards the oxidation of SO_2 on the acid filter, we carried out laboratory and field experiments to measure the degree of SO_2 oxidation by NO_x. Laboratory experiments, performed at different face velocities and at an $[NO_x]$ to $[SO_2]$ ratio of about 3, indicated that SO_2 oxidation decreases almost linearly when the face velocity is increased from 3 to 12 cm s^{-1}, and is negligible at face velocities equal to or higher than 15 cm s^{-1}. This result was supported by field experiments, which showed no significant difference between the amount of SO_2 sampled on the basic filter, in the presence or absence of the acid filter, when the face velocity was higher than 15 cm s^{-1} (see Table 2). In these experiments the atmospheric concentration of NO_x was increased by a controlled flow of NO_x. Under these conditions, the absence of SO_2 oxidation on the acid filter may be due to the low contact time between the two gases in the aqueous phase, and to the continuous sweeping out of the two reagents by the air stream.

Application of the Method

These SO_x sampling and measurement methods were applied in the city of Venice in the winter. The sampler was operated at a face velocity of 18 cm s^{-1}, five times a day, for 30-min periods. The minimum, maximum and average values of the SO_2 and sulphate concentrations and the $[SO_2]$ to $[SO_4{}^{2-}]$ ratio over ten days of sampling are reported in Table 3.

The mean SO_2 concentration measured agreed fairly well with the mean value of 30 $\mu g\,m^{-3}$ reported, for the same period, by the SO_2 monitoring network operated in Venice by the Ente Zona Industriale of Porto Marghera. It should be noted that the minimum SO_x values are well above the background levels, although about 20% of the samplings were carried out in the presence of a strong wind from the open sea. Further, the low degree of SO_2 oxidation seems to indicate that the pollution sources in Venice are mainly local. Systematic measurements of the SO_x levels in Venice are certainly worthy of further investigation.

In conclusion, the simple collection of sulphate on an acid-impregnated filter and elimination of the SO_2 oxidation caused by NO_x are the salient features of the method reported here, which permits the collection and determination of very low levels of SO_x without significant errors due to artifact sulphate formation.

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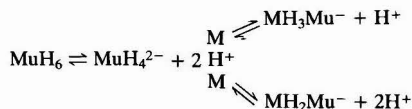# Spectrophotometric Determination of Bismuth in Pharmaceutical Preparations Using Mucic Acid*

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The formation of a 1:1 complex with mucic acid (MuH_6) has been used for the determination of concentrations of 4.5–25.5 p.p.m. of bismuth(III) at pH 4.0–5.0. The complex exhibits maximum absorption at 245 nm, with a molar absorptivity of 6.5×10^3 l mol^{-1} cm^{-1}. The method is specific and was applied to the determination of bismuth in pharmaceutical preparations.

Keywords: *Spectrophotometry; bismuth; pharmaceutical preparations; mucic acid*

Bismuth and its compounds are employed in medicine in desiccants, local astringents, antiseptics, antiacids and as radio-opaque media for X-rays. Numerous procedures have been employed for the determination of bismuth: classical gravimetric methods; titrimetric methods as recommended by the US and British Pharmacopoeias, which use ethylenediaminetetraacetic acid (EDTA) as a reagent and either xylenol orange[1] or pyrocatechol violet[2] as indicator; and spectrophotometric techniques making use of a variety of auxiliary reagents. One of the many polycarboxylic acids with which bismuth forms complexes is mucic acid (MuH_6), of which the spectrophotometric characteristics of complexes with iron, vanadium, copper and cobalt have been reported[3–5]; its potential as an analytical reagent has also been discussed.[6] The equilibria can be represented as follows:

$$MuH_6 \rightleftharpoons MuH_4{}^{2-} + 2\,H^+ \begin{array}{c} M \nearrow MH_3Mu^- + H^+ \\ M \searrow MH_2Mu^- + 2H^+ \end{array}$$

where M = metal.

This paper describes the spectrophotometric determination of bismuth using mucic acid and its application to pharmaceutical preparations.

Experimental

Apparatus

A Varian Techtron 635 spectrophotometer with quartz cells of 10 mm light path was used, together with a Beckman Electromate pH meter equipped with glass and calomel electrodes and with a sensitivity of ±0.02 pH unit.

Reagents

All solutions were prepared with analytical-reagent grade chemicals and distilled water.

Bismuth(III) standard solution. Bismuth oxide was dissolved in hydrochloric acid. The resulting solution was diluted to volume and its concentration determined titrimetrically.[7]

Mucic acid solution, 0.05%. A weighed amount of mucic acid was dissolved in the least possible amount of 0.1 M sodium hydroxide solution and the resulting solution made up with distilled water.

Mucic acid standard solution. Prepared as above, its concentration being determined titrimetrically.[8]

Acetic acid - acetate buffer, pH 4.5. Prepared by mixing the appropriate volumes of 0.2 M sodium acetate solution and 0.2 M acetic acid.

Procedure

A sample of the solution to be assayed containing between 4.5 and 25.5 µg of Bi(III) is transferred into a 25-ml calibrated flask to which 5 ml of sodium acetate - acetic acid buffer and 2 ml of 0.05% mucic acid solution are added. The solution is diluted to volume with distilled water and its absorbance is measured at 245 nm against a reagent blank prepared at the same time.

Results

Absorption Spectra

The UV absorption spectra of the Bi(III) complex of MuH_6 at various pH values are shown in Fig. 1. The absorption maximum occurred in all instances at 245 nm, which was the wavelength employed in subsequent measurements.
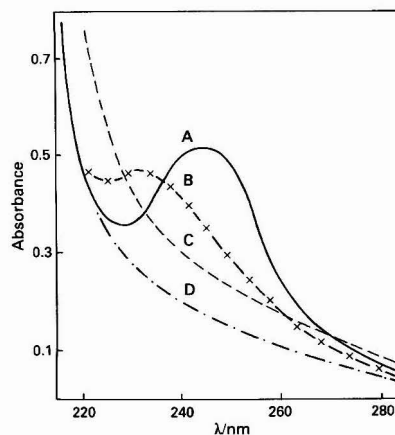
Fig. 1. Absorption spectra of Bi(III) - MuH_6 complex with 9.0 µg ml^{-1} of Bi(III) at: A, pH 4.5; B, pH 7.1; C, pH 9.5; and D, pH 1.5

Effects of pH, Time and Temperature

The greatest absorption occurred in the pH range 4.0–5.0 and was constant throughout this range, and all subsequent measurements were accordingly carried out at an acidity of pH 4.5 adjusted with sodium acetate - acetic acid buffer. The absorbance of the complex was not affected by standing for up to 24 h or by heating to 80 °C.

Effects of MuH_6 Concentration and Order of Addition of the Reagents

The results of measuring the absorbance of a series of solutions containing 9.0 µg of bismuth and different amounts of 0.05% MuH_6 solution showed that 1 ml of 0.05% MuH_6 solution sufficed to complex the 9 µg of bismuth. There was no appreciable change in the absorbance of the complex if the order of addition of the reagents was varied.

Beer's Law, Molar Absorptivity, Sensitivity and Reproducibility

Beer's law was obeyed over the range 4.5–25.5 µg ml^{-1} of Bi(III) with the Ringbom optimum interval between about 6.5 and 18.5 µg ml^{-1} of Bi(III). Under the conditions recommended the molar absorptivity was 6.5×10^3 l mol^{-1} cm^{-1} and Sandell's sensitivity was 0.020 µg cm^{-2}. The reproducibility was good, the coefficient of variation (C.V.) of 20 determinations of solutions containing 9.0 µg of bismuth being 0.80%.

Effects of Foreign Ions

Solutions containing known amounts of bismuth and various other ions were prepared and assayed for bismuth by the standard procedure. No interference was observed from concentrations of Al(III), Cu(I), Cu(II), Mn(II), Cd(II), U(VI), phosphate, sulphate, nitrate, chloride or acetate 100 times greater than that of bismuth, or by concentrations of Fe(III), Pb(II), Ti(IV), Zn(II), Co(II), Co(III), V(IV), tartrate, molybdate or nitrite 10 times greater.

Stoicheiometry of the Complex

The molar ratio[9] and continuous variation[10] methods (Figs. 2 and 3) showed that MuH_6 and bismuth formed a 1 : 1 complex whose structure is presumably[11]

$$HOOC - (CHOH)_2 - CH - CH - CO$$
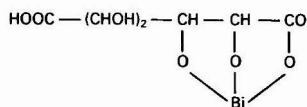
A spectrophotometric study of the reaction together with its stoicheiometry allowed the stability constant of the complex to be calculated as 1.5×10^6.
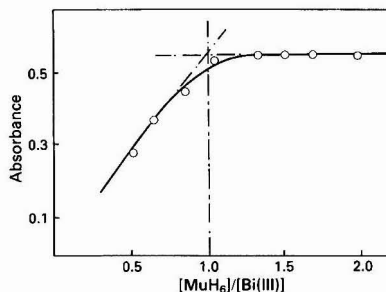
Fig. 2. Determination of the composition of the Bi(III) - MuH_6 complex by the Yoe and Jones method.[9] pH = 4.5
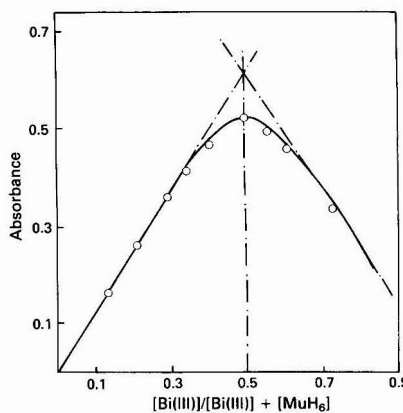
Fig. 3. Determination of the composition of the Bi(III) - MuH_6 complex by Job's method.[10] pH = 4.5

Applications

The mucic acid method described above was applied to the determination of bismuth Sualyn digestive regulators (Vita Laboratories, Barcelona, Spain), which are marketed either as colloidal suspensions of particle size <1 µm containing bismuth subnitrate mixed with metochlopramide, magnesium carbonate and sorbitol, or as 1.5-g tablets containing 0.125 g of bismuth polyuronate (equivalent to 60.5 mg of bismuth oxide) together with metochlopramide, magnesium oxide and excipients.

Table 1. Determination of bismuth in pharmaceutical preparations

Sample		Standard titrimetric method[2]		ADA method[12]†		Proposed method	
		Concentration/ µg ml^{-1}	C.V., %	Concentration/ µg ml^{-1}	C.V., %	Concentration/ µg ml^{-1}	C.V., %
Colloidal suspension	..	10.5 ± 0.08	0.76	10.8 ± 0.15	1.40	10.6 ± 0.08	0.75
Tablets	..	12.6 ± 0.10	0.80	12.5 ± 0.16	1.28	12.8 ± 0.09	0.70

* Each value represents the mean of three analyses; each determination was carried out five times and the results averaged.
† N-(2-Acetamido)iminodiacetic acid as reagent.

Table 2. Sensitivities of different methods for the determination of bismuth

Method	Sensitivity/ $\mu g\ cm^{-2}$	Range/ $\mu g\ ml^{-1}$	Reference
Standard* ..	—	200–300	2
ADA†	0.023	3.8–17.9	12
Proposed	0.020	4.5–25.5	This work

* Titrimetric with EDTA and pyrocatechol violet.
† N-(2-Acetamido)iminodiacetic acid as reagent.

Procedure

For each determination by the mucic acid method a 0.2-g powdered tablet sample or 0.2 ml of colloidal suspension was placed in a Kjeldahl flask and drops of hydrogen peroxide and perchloric acid added until the transparency of the solution showed oxidation to be complete. After cooling to room temperature, the solution was transferred into a calibrated flask and diluted to volume with distilled water. A sample of this dilute solution was then transferred into a 25-ml calibrated flask such that on making the volume up to 25 ml the final concentration of Bi(III) would lie in the range 6.5–18.5 $\mu g\ ml^{-1}$. After making up the volume accordingly, the assay procedure described under Experimental was followed.

The bismuth contents of the pharmaceutical samples were also determined by a standard titrimetric method[2] and by another spectrophotometric method.[12] The results are compared in Table 1.

The sensitivities of this and other methods for the determination of bismuth are shown in Table 2.

Conclusions

The method proposed in this paper for the determination of bismuth in pharmaceutical preparations is simpler and quicker than other methods. The high molar absorptivity of the MuH_6-Bi(III) complex confers on the proposed method an accuracy, reproducibility and specificity that compare favourably with those of other sensitive methods.[12–16]

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Spectrophotometric and High-order Derivative Spectrophotometric Study of the Indium - 1-(2-Pyridylazo)-2-naphthol Complex in the Aqueous Phase

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A spectrophotometric and derivative spectrophotometric study of the indium - 1-(2-pyridylazo)-2-naphthol complex in the aqueous phase in the presence of cetyltrimethylammonium bromide, a surfactant, is reported. The molar absorptivity, specific absorptivity and Sandell's sensitivity of the complex at its $\lambda_{max.}$ (550 nm) were found to be 1.91×10^4 l mol^{-1} cm^{-1}, 166 l g^{-1} cm^{-1} and 6.02 ng cm^{-2}, respectively. Beer's law is valid for the range 5.0×10^{-6} to 1.0×10^{-4} M In^{3+}.

Keywords: *1-(2-Pyridylazo)-2-naphthol; indium complex; derivative spectrophotometry; spectrophotometry*

Indium forms a red complex with 1-(2-pyridylazo)-2-naphthol (PAN), which has been the basis of a number of titrimetric[1-3] and spectrophotometric[4-16] methods for determining microgram amounts of indium(III). Although the reaction is sensitive at the parts per million level, it lacks selectivity. Also, the insolubility of both the reagent and the resulting complex in water necessitates the use of an organic solvent or a mixture of solvents. These limitations have now been overcome by using a suitable surfactant and carrying out the absorption studies in both the derivative and normal modes.

Experimental

Reagents

All chemicals used were of analytical-reagent grade. Aqueous solutions of indium(III) (1×10^{-3} M), surfactant (1×10^{-2} M) and buffer (10% m/V) were prepared by dissolving appropriate amounts of In$_2$(SO$_4$)$_3$, cetyltrimethylammonium bromide (CTMAB) and ammonium acetate, respectively, in water. A 5.0×10^{-4} M solution of PAN was prepared by diluting its solution in concentrated HCl with water (final concentration of HCl $\approx$ 1.5 M).

Instruments

A Shimadzu 260 UV - visible recording spectrophotometer and an EC digital pH meter were used.

Preparation of Working Solutions

To study the effect of pH on complex formation, two sets of solutions, one 2.5×10^{-5} M with respect to the metal ion and the other without metal ion, each in the pH range 2.0–9.5 and containing PAN (1.25×10^{-4} M), CTMAB (1.0×10^{-3} M) and ammonium acetate (6.48×10^{-2} M), were prepared.

To study the effect of CTMAB concentration on the absorbance of the In - PAN system, a set of solutions containing increasing amounts of CTMAB, 2.0×10^{-5} M In^{3+}, 2.5×10^{-4} M PAN and 6.48×10^{-2} M ammonium acetate at the pH of maximum complex formation, *i.e.*, 4.2 (established as described above) was prepared.

To study Beer's law, a set of 10-ml solutions, under optimum conditions of maximum complex formation and containing increasing amounts of the metal ion, 2.5×10^{-4} M PAN, 2.0×10^{-3} M CTMAB and 6.48×10^{-2} M ammonium acetate were prepared.

To study the effect of PAN concentration and the presence of interfering ions, solutions were prepared as described for Beer's law, maintaining the required reagent concentrations.

Results and Discussion

Absorption Spectrum and Effect of pH on the Absorbance of the System

The complex shows an absorption maximum at 550 nm. As the ligand also absorbs at this wavelength, the relative increase in absorbance of the complex was calculated and plotted against the pH of the solution. As the plot has its highest point at pH 4.2, this is taken as the pH of maximum complex formation.

Effect of Surfactant Concentration on the Absorbance of the System

The absorbance at 550 nm of solutions containing increasing amounts of the surfactant was found to be proportional to CTMAB concentration up to 1.2×10^{-3} M. A small decrease was observed above 1.3×10^{-3} M, which is considered to be due to a local increase in the concentration of the complex forming anions in the vicinity of the micelle that dissolves the complex and the ligand. In all subsequent studies, therefore, the surfactant concentration was maintained above 1.3×10^{-3} M, the critical micellar concentration of the surfactant.[17]

Effect of PAN Concentration on the Absorbance of the System

To investigate the effect of increasing PAN concentration, a set of solutions containing increasing amounts of PAN solution were prepared. The absorbance of the solutions at 550 nm increased with increase in PAN concentration and was highest at a 10-fold excess of PAN. However, it lay within ±4% of the maximum value when the excess PAN concentration was varied from 5- to 20-fold relative to that of the metal.

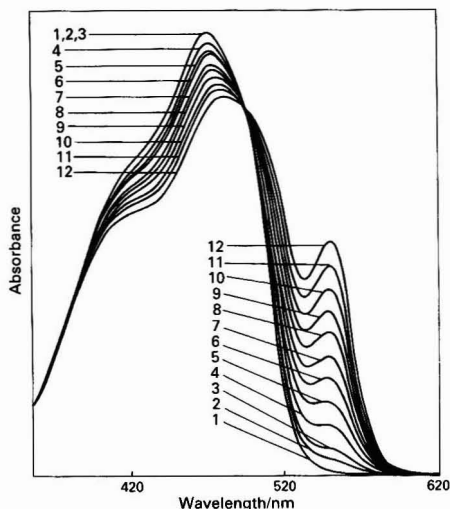* To whom correspondence should be addressed.

Fig. 1. Absorption spectra of the In - PAN - CTMAB system. No. 1 contains no In; Nos. 2–12 contain increasing In concentrations from 5.0×10^{-6} to 1.0×10^{-4} M
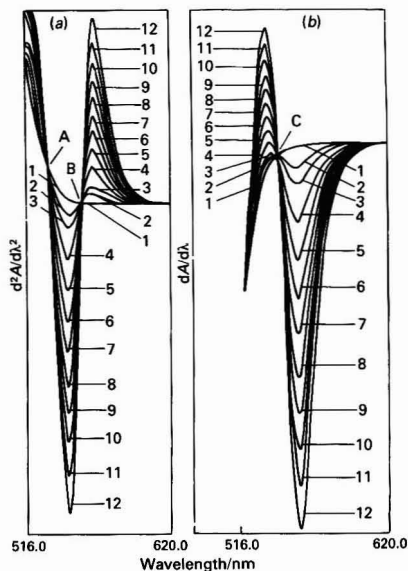

Fig. 2. Derivative spectra of the spectra in Fig. 1. (*a*) Second order; (*b*) first order. A, B and C, cross-over points

Effect of Metal Ion Concentration on the Absorbance of the System

The absorbance at 550 nm of solutions containing 5.0×10^{-6} to 1.0×10^{-4} M of In^{3+} shows an excellent linear fit with a correlation coefficient of 0.9998 (Fig. 1). The equation governing the concentration - absorbance relationship is

$$C = 10.493A - 0.2169$$

where C is the concentration (in mol $l^{-1} \times 10^5$) and A the absorbance. The negative intercept is due to the absorption of the ligand at the $\lambda_{max.}$ of the complex as the spectra were recorded against a water blank. This option facilitates the

study of the absorption characteristics of the system in the visible region of the spectrum using both the derivative and normal modes. It also enables proper correction(s) to be made to the absorbance values of the solutions, which otherwise is not possible. In the set under consideration two corrections, giving corrected absorbances A_I and A_{II}, due to the total and proportional amounts of ligand left uncomplexed, respectively, were made in the absorbance of the system:

$$A_I = (A)_{\text{observed 550}} - (A)_{\text{ligand blank 550}}$$
$$A_{II} = (A)_{\text{observed 550}} - (A)_{468} \left[\frac{(A)_{550}}{(A)_{468}} \right]_{\text{ligand blank}}$$

Construction of a graph of these corrected absorbance values followed by regression analysis gave the following concentration - absorbance relationships for the same concentration range of metal with the same correlation coefficient of 0.9998 as was given by the uncorrected absorbance A:

$$C_I = 10.49 A_I - 0.0012$$
$$C_{II} = 10.45 A_{II} - 0.0001$$

However, when the molar absorptivities (ε) were calculated from these three sets of absorbance values, *viz.*, A, A_I and A_{II}, those obtained from A_{II} showed the smallest coefficient of variation (0.572), compared with 2.861 and 0.649 for A and A_I, respectively. The average ε value (nine determinations) for the complex at 550 nm is 1.91×10^4 l mol^{-1} cm^{-1}, whereas the specific absorptivity (a) and Sandell's sensitivity (s) of the system (calculated with respect to the metal ion) are 166 l g^{-1} cm^{-1} and 6.02 ng cm^{-2}, respectively.

Derivative spectra of these solutions were also recorded (Fig. 2). The first derivative spectrum shows a maximum at 542 nm and a minimum at 560 nm with a cross-over point at 550 nm, corresponding to the $\lambda_{max.}$ of the complex. In the absence of the complex, *i.e.*, in the presence of ligand alone, no minimum or maximum is observed in the first-derivative spectrum at the above wavelengths. The second-derivative spectrum of the indium complex shows a maximum at 570 nm, a cross-over point at 563 nm, a minimum at 555 nm and another cross-over point at 541 nm, whereas with the ligand alone, a flat base line passing through both of the cross-over points and not showing any maximum or minimum is observed (Fig. 2).

In the first-derivative spectrum of the solutions, the depth of the minimum at 560 nm from the ligand base line and the distance between the maximum (at 560 nm) and the minimum (at 542 nm) were measured. In the second-derivative spectrum the minimum at 555 nm and the maximum at 570 nm were chosen for the measurement. These depths and distances were found to be directly proportional to the concentration of metal ion in solution as the correlation coefficients lie in the range 0.998–1.000. This permits the successful extension of this method to the determination of In^{3+} in solutions containing relatively small concentrations of In^{3+}. Dilutions of up to five times the concentration of the lowest point in the Beer's law graph can conveniently be handled by proportionately increasing the absorption path length. However, the determination of In^{3+} by absorptiometry when only one hundredth of the normally determinable amount (*i.e.*, 5.0×10^{-6} M) was present gave erratic results. In such situations, the first-derivative spectrum was found to be of great value as the depth of the minimum at 560 nm was found to be linearly proportional to In^{3+} concentration down to 0.2×10^{-6} M; linear regression showed a correlation coefficient of 0.9648. Although the second-derivative spectrum was expected to give better results it could not be made use of because of excessive background noise. The use of longer path lengths can therefore facilitate the determination of indium at the parts per billion level using absorptiometry in the first-derivative mode.

Composition of the Complex

From the plot of Job's method of continuous variations, the In^{3+} to PAN ratio in the complex was found to be 1:1. The complex can therefore be represented as $[In(PAN)]^{2+}$. As Br^-, Cl^-, CH_3COO^- and OH^-, and also small amounts of SO_4^{2-}, which are capable of complexing with In^{3+}, are also present, a more realistic expression of the complex formed under the experimental conditions would be $[In(PAN)X_n]^{2-n}$ where X is any of the above monovalent anionic ligands.

Stability Constant of the Complex

Based on stability constant data for the complexes of In^{3+} with the anions present in solution, it has been inferred that X in the above expression can only be Cl^-. Therefore, the conditional stability constant for the equilibrium

$$InCl_3 + PAN^- \rightleftharpoons [In\ PANCl_3]^-$$
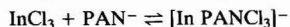
was calculated after accounting for the simultaneously existing equilibria involving other ligands present in solution. The average stability constant thus calculated was 1.77×10^{13} l mol^{-1} with a coefficient of variation of 14.9%.

Interferences Due to Foreign Ions

Interferences in the photometric determination of In^{3+} from common cations and anions were investigated. Equal amounts of Ga^{3+}, Tl^{3+}, Hg^{2+}, Cd^{2+}, Mn^{2+} and Cr^{3+} and 5- and 10-fold amounts of Pb^{2+} and Al^{3+}, respectively, did not interfere. Serious interference was caused by Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, Fe^{2+} and Fe^{3+}. The interference due to Fe^{2+} and Fe^{3+}, even in 50-fold excess, could be easily overcome by carrying out the determination in the derivative mode. VO^{2+} shows similar behaviour with a peak hump at *ca.* 570 nm; the interference due to a 10-fold excess of vanadyl ions could be avoided by using second-order derivative spectrophotometry. The interference due to the presence of Ni^{2+} and Co^{2+} could also be overcome as these have well separated maxima and minima in the derivative modes. A 20-fold excess of Co^{2+} and a 5-fold excess of Ni^{2+} could thus be tolerated in determination of

0.2×10^{-4} M In^{3+}. A 10-fold excess (with respect to the metal) of I^-, CN^-, citrate, tartrate and oxalate decreased the absorbance of the system by 4.7, 1.3, 3.9, 0.6 and 7.6%, respectively. EDTA strongly complexes indium and completely prevents its complexation with the complexone, thus causing serious interference.

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Determination of Iron in Sea Water by Densitometry after Enrichment as a Bathophenanthroline Disulphonate Complex on a Thin Layer of Anion-exchange Resin

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A rapid, simple and sensitive method for the determination of trace amounts of iron in sea water has been developed. The method is based on the enrichment of iron as a red bathophenanthroline disulphonate complex by a thin layer of anion-exchange resin followed by densitometric scanning of the thin layer. Except for the chloride, the major constituents and the trace elements of sea water, when present in concentrations found in sea water or higher, did not affect the determination of iron. Chloride ions at concentration levels found in sea water affect the fixation of the iron(II) - bathophenanthroline disulphonate complex on a thin layer of anion-exchange resin. The effect of chloride in sea water samples was reduced by dilution. The concentration of iron in sea water was found to be 2.55 µg l^{-1} with a relative standard deviation of 3.5% ($n = 11$) for 50 ml of sea water.

Keywords: *Iron determination; sea water; densitometry; bathophenanthroline disulphonate complex; anion-exchange resin thin layer*

As concentrations of trace elements in most natural waters seldom exceed the µg l^{-1} level, the introduction or removal of even small amounts of them produce detectable variations. In order to assess accurately these variations, techniques are needed in which a larger number of samples can be analysed with adequate precision. This demand had spawned a continued research and development effort to expand the scope of methodology. Several years ago, it was pointed out[1] that much of the data on the occurrence of trace elements in sea water is unreliable owing to the use of insufficiently tested analytical methods. In recent years, a number of methods have been introduced for the determination of trace elements in marine environments.[2–9] However, some of these methods are time consuming and often tedious. In view of the above difficulties, we have developed a sensitive, rapid and accurate technique for the determination of trace elements in sea water[10,11] so that variations in the lower concentration range might be accurately identified.

As the determination of iron in sea water is of great importance and most of the literature reflects the considerable difficulties encountered in its determination, the same technique has been extended for the determination of iron in sea water after filtration of the sample through a membrane filter with a pore size of 0.45 µm and a preliminary treatment with hydrochloric acid.

Experimental

Apparatus

A Shimadzu CS-920 Chromatoscanner was used for the reflection absorbance measurements of the red complex on the thin layer of anion-exchange resin. A densitometer was used to linearise the convex calibration graph based on the Kubelka - Munk theory.[12,13] A Hitachi-Horiba Type-F7$_{LC}$ pH meter was used for the adjustment of pH and a Toyo KG-25 filter holder, with TM-1 filter-paper (0.65 µm pore size, 47 mm diameter) were used for the preparation of the thin layer by filtration under suction.

Reagents

Unless stated otherwise all reagents used were of analytical-reagent grade and de-ionised, redistilled water was used throughout.

Hydrochloric acid. Of super special grade (SSG-reagent).

Iron(II) standard solution, 1000 p.p.m. (1 mg ml^{-1}). Prepared by dissolving 7.0216 g of ammonium iron(II) sulphate, FeSO$_4$(NH$_4$)$_2$SO$_4$.6H$_2$O, in 100 ml of water containing 5 ml of sulphuric acid, and diluting the solution to 1 l. A working solution, containing 1 µg ml^{-1}, was prepared daily by appropriate dilution with dilute hydrochloric acid (pH *ca.* 1.5).

Iron(III) standard solution, 1000 p.p.m. (1 mg ml^{-1}). Prepared by dissolving 8.6340 g of ammonium iron(III) sulphate, FeNH$_4$(SO$_4$)$_2$.12 H$_2$O, in 100 ml of water containing 5 ml of sulphuric acid, and diluting the solution to 1 l. A working solution, containing 1 µg ml^{-1}, was prepared daily by dilution with dilute hydrochloric acid (pH *ca.* 1.5).

Bathophenanthroline (4,7-diphenyl-1,10-phenanthroline, BP) solution. Obtained from Dotite (Japan) and used without further purification. A 1 mM solution was prepared by dissolving 33 mg of the reagent in 50 ml of ethanol and then diluting with water to 100 ml.

Bathophenanthroline disulphonate (4,7-diphenyl-1,10-phenanthrolinedisulphonic acid, BPS) solution. The disodium salt was obtained from Dotite (Japan) and used without further purification. A 1 mM solution was prepared by dissolving 53.6 mg of the reagent in 100 ml of water.

Hydroxylammonium chloride solution, 10% *m/V.* Prepared and purified by reacting with BP and extracting the red iron - BP complex with isoamyl alcohol as described by Strickland and Parsons.[14] The purified hydroxylammonium chloride solution was stored in a well stoppered polyethylene bottle.

Sodium acetate solution, 10% *m/V.* Purified according to the procedure described by Strickland and Parsons.[14]

Disodium salt of ethylenediaminetetraacetic acid (EDTA) solution, 10^{-2} mol l^{-1}.

Anion-exchange resin (ARS). A macroreticular type Amberlyst A-27 (Rohm and Haas) in the chloride form was used. The anion-exchange resin suspension with less than 30-µl particles was prepared according to the reported method.[15] The exchange capacity of the suspension, as determined by conductimetric titration, was 7.12 µequiv. ml^{-1}. The working suspension (3.56 µequiv. ml^{-1}) was prepared by dilution. The impurity of iron in ARS was masked using EDTA. When EDTA was previously added to ARS, the formation of iron(II) - BPS complex from the impurity in the

resin was masked effectively and, once formed, the complex was stable in the presence of EDTA.[16] A 25-ml portion of 10^{-2} mol l^{-1} EDTA solution was added to 500 ml of ARS (7.12 μequiv. ml^{-1}) and the final volume was adjusted to 1 l.

Water samples. The water samples were collected from Ishikari Harbour (Japan Sea). The samples were filtered through a 0.45 μm membrane filter, acidified to pH *ca.* 1.5 and stored in pre-cleaned polyethylene bottles.

Polyethylene bottles. Filled with 6 M nitric acid and shaken for 5 d, then rinsed twice with water.

All glassware was immersed overnight in 6 M nitric acid and then rinsed thoroughly with de-ionised redistilled water.

General Procedure

An aliquot containing 0.4 μg of Fe(II) is placed in a 100-ml beaker. A 100-μl volume of 20% hydrochloric acid, 2 ml of 10% hydroxylammonium chloride, 1 ml of 1 mM BPS and 2 ml of sodium acetate solutions are added and the volume is made up to 50 ml with water giving a final pH of 4.5. The mixture is left to stand for 10 min and then 2.0 ml of ARS are added. The mixture is stirred for 10 min by means of a magnetic stirrer and the resin collected on a membrane filter by suction. A disc of coloured thin layer is formed, which is 17 mm in diameter and about 0.2 mm in thickness. It is then kept wet in water. The wet membrane filter holding the resin is placed on a white plastic plate in the densitometer. The integrated absorbance is measured at 550 nm by scanning the thin layer in a range 24 mm wide and 30 mm long. The blank value is obtained by the foregoing procedure without the addition of iron(II).

Determination of Iron in Sea Water

A 50-ml portion of the sample was placed in a 200-ml beaker and diluted to 150 ml with water. A 100-μl volume of 20% hydrochloric acid and 2 ml of 10% hydroxylammonium chloride solution were added. The pH was adjusted to *ca.* 3 by the addition of 5 M ammonia solution, as the reduction of iron(III) by the hydroxylammonium chloride is fairly slow at very low pH.[17] A 1-ml portion of BPS and 2 ml of sodium acetate solutions were added. The pH of the final solution was 4.5. The thin layer was prepared and the absorbance measured as described under General Procedure. The amount of iron was obtained from the calibration graph.

Results and Discussion

Absorption Spectra

The absorption spectra of iron(II) - BPS in the resin phase showed an absorption maximum at 550 nm as shown in Fig. 1. The absorbance was measured against the membrane filter substrate by swinging the thin layer so that the light beam swept back and forth through the centre.

Effect of Amount of ARS

The influence of amount of ARS on the absorbance of iron(II) - BPS complex was examined (Fig. 2). The absorbance increased with increasing amount of ARS, reaching a maximum at 0.5 ml, which then started to decrease with increasing amount of ARS. A 2-ml volume of ARS was chosen, as at this concentration the linearity of the calibration graph was better.

Effect of Amount of BPS

The variation in the absorbance of the iron(II) - BPS complex was investigated as a function of added amount of BPS. A constant absorbance was observed in the presence of BPS solution in the range 0.5–4 ml.
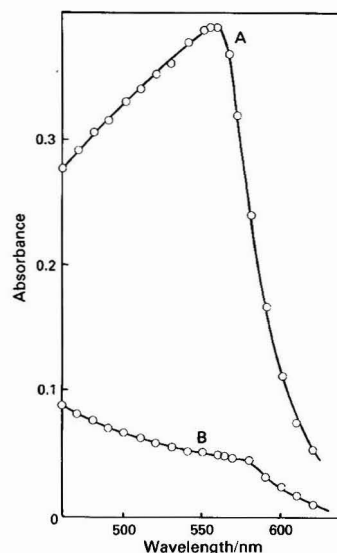
Fig. 1. Absorption spectra of iron(II) - BPS complex in a thin layer of anion-exchange resin on a membrane filter. A, Thin layer prepared from 50 ml of solution containing 0.4 μg of iron(II); reagents added, 100 μl of 20% hydrochloric acid, 2 ml of 10% hydroxylammonium chloride, 1 ml of 1 mM BPS, 2 ml of 10% of sodium acetate and 2.0 ml of ARS (3.56 μequiv. ml^{-1}). B, Thin layer of reagent blank

Effect of Amount of Hydroxylammonium Chloride

The influence of the amount of hydroxylammonium chloride on the reduction of iron(III) to iron(II) was examined. It was found that a complete reduction occurred in the presence of hydroxylammonium chloride solution in the range 0.5–4 ml.

Effect of Sample Volume

The recovery of iron(II) - BPS complex was investigated using different sample volumes. A constant recovery of a 0.4-μg amount of iron(II) was obtained from different sample volumes ranging from 25 to 200 ml. The filtration time was about 10 min for a 200-ml sample volume. As the volume of the thin layer was estimated to be *ca.* 0.045 cm^3, the concentration factor was found to be *ca.* 4400 for 200 ml of the sample.

Calibration Graph

A calibration graph for the determination of iron(II) - BPS was prepared according to the general procedure. A linear calibration graph was obtained for up to 0.5 μg of iron(II) using the lineariser. The regression line was $y = 137902x$, where y is the integrated absorbance in arbitrary units and x the amount of iron(II) in μg. The relative standard deviation was 2.8% ($n = 6$) for 0.3 μg of iron(II). A calibration graph for the determination of iron(III) after reduction to iron(II) - BPS was also prepared. It was found that both calibration graphs have the same slope. As stated above, a constant recovery of the iron(II) - BPS complex was obtained with varying sample volumes up to 200 ml; the regression equation obtained here is also valid for sample volumes up to 200 ml.

Effect of Concentration of Sodium Chloride

As shown in Fig. 3, a constant recovery of iron(II) - BPS complex was found using up to 0.3 M sodium chloride solution.
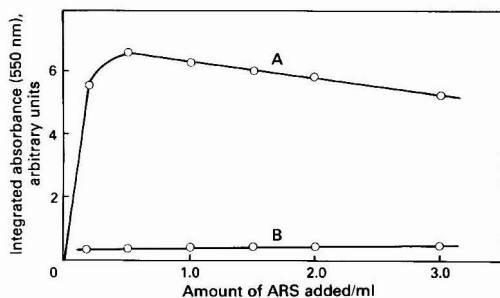
Fig. 2. Influence of amount of ARS on the absorbance of iron(II) - BPS complex. A, Iron(II) - BPS; and B, reagent blank. Except for the amount of ARS, other reagents were added as in Fig. 1
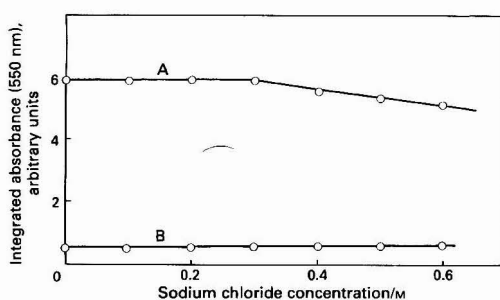

Fig. 3. Effect of concentration of sodium chloride on the recovery of iron(II) - BPS complex. A, Iron(II) - BPS; B, reagent blank. Reagents were added as in Fig. 1

When the concentration of sodium chloride increased above this value, the recovery of iron(II) - BPS was found to decrease, which is due to the incomplete fixation of iron(II) - BPS complex on the resin. The sample effect was found with iron(III) after reduction to iron(II) with hydroxylammonium chloride.

Effect of Foreign Ions

The effect of foreign ions on the determination of iron as the iron(II) - BPS complex has been examined (Tables 1 and 2). The diverse ions may have an effect on the coloration of the thin layer and/or the fixation of the complex on the thin layer. Except for sodium chloride it was found that the cations K(I), Zr(IV), Li(I), Ag(I), Cs(I), Mg(II), Ca(II), Ba(II), Sr(II), Pb(II), Ti(IV), Ni(II), Cu(II), Zn(II), Cd(II), Co(II), Mn(II), Sn(II), Hg(II), Pd(II), Al(III), Bi(III), As(III), V(V), Cr(VI), Mo(VI) and Se(IV), and anions Br^-, I^-, SCN^-, NO_3^-, HCO_3^-, SiO_3^{2-}, SO_4^{2-}, BO_3^{3-} and PO_4^{3-} did not affect the coloration of the thin layer or the fixation of the complex on the thin layer when present at levels similar to or higher than those found in sea water. As previously stated, chloride ions affect the fixation of the iron(II) - BPS complex when present at concentration levels higher than 0.3 M and as the concentration of the chloride ions in sea water is about 0.6 M, it is necessary to reduce the concentration of the chloride ions in sea water samples; this was achieved by dilution of the sea water samples so that a maximum recovery could be obtained. Although some positive interferences have been observed from some cations and anions when examined at very high concentrations, it is believed that such interferences are mainly due to the trace impurities of iron in the reagents used.

Table 1. Effect of foreign cations on the determination of iron(II)*

Cation		Added as	Amount added/ μg	Error,† %
Ca(II)	..	$CaCl_2$	40080	3.6
Mg(II)	..	$MgCl_2$	19450	2.0
			24312	7.4
Sr(II)	..	$SrCl_2$	87620	2.5
K(I) ..	..	KCl	9776	0.9
			19551	6.0
Ba(II)	..	$BaCl_2$	13734	1.6
Pb(II)	..	$PbCl_2$	100	4.8
			50	3.2
Al(III)	..	$K_2Al_2(SO_4)_4.2H_2O$	50	3.0
Li(I) ..	..	LiCl	100	−1.3
Cu(II)	..	$CuCl_2$	10	−1.6
Zn(II)	..	$ZnCl_2$	10	3.4
Ni(II)	..	$NiCl_2$	10	−1.4
Cd(II)	..	$Cd(NO_3)_2.4H_2O$	10	1.3
Co(II)	..	$CoCl_2$	10	1.4
Sn(II)	..	$SnCl_2$	10	3.4
As(III)	..	$NaAsO_2$	10	2.5
Ag(I)	..	$AgNO_3$	10	1.1
Mn(II)	..	$MnCl_2$	10	3.0
Cs(I) ..	..	CsCl	10	0.5
Bi(III)	..	$Bi(NO_3)_3$	10	2.8
Hg(II)	..	$HgCl_2$	10	−2.8
Pd(II)	..	$PdCl_2$	5	2.8
Se(IV)	..	SeO_2	5	2.2
Ti(IV)	..	$Ti(SO_4)_2$	5	4.9
			2.5	1.4
Zr(IV)	..	$ZrOCl_2$	0.9	2.6
Cr(VI)	..	$K_2Cr_2O_7$	5	−0.4
V(V) ..	..	NH_4VO_3	10	20
			5	9.4
			4	2.9
Mo(VI)	..	$(NH_4)_6Mo_7O_{24}.4H_2O$	10	−1.0

* Fe(II), 0.4 μg; sample volume, 50 ml.
† Mean of the relative error of duplicate measurements.

Table 2. Effect of foreign anions on the determination of iron(II)*

Anion			Added as	Added amount/ mmol	Error,† %
SO_4^{2-}	..	..	Na_2SO_4	0.4	11.7
PO_4^{3-}	..	..		0.2	1.0
PO_4^{3-}	..	..	KH_2PO_4	0.5	46.2
				0.2	14.4
				0.1	2.2
NO_3^-	..	..	$NaNO_3$	0.2	1.1
HCO_3^-	..	..	$NaHCO_3$	0.4	12.0
				0.2	4.8
SCN^-	..	..	NH_4SCN	0.005	4.2
				0.003	3.0
SiO_3^{2-}	..	..	Na_2SiO_3	0.072	−6.1
				0.036	−4.5
BO_3^{3-}	..	..	H_3BO_3	46.5	1.2
				27.9	1.0
I^-	..	..	NaI	0.01	4.3
				0.005	0.3
Br^- ..	..	..	KBr	0.01	2.4
				0.005	2.2

* Fe(II), 0.4 μg; sample volume, 50 ml.
† Mean of the relative error of duplicate measurements.

Analysis of Sea Water

The recovery of iron(II) from sea water as the BPS complex was examined. It was found that the maximum recovery of iron(II) - BPS complex was obtained when the dilution ratio exceeded 1:2 as shown in Fig. 4. The reproducibility and accuracy of the method were examined and the results are summarised in Table 3. The results show that the sea water

Table 3. Determination of iron in sea water collected from Ishikari Harbour, Japan Sea

Sample volume/ ml	Iron found/ $\mu g\,l^{-1}$	Standard deviation/ $\mu g\,l^{-1}$	Number of analyses
30	2.61	0.080	4
50	2.55	0.090	11
60	2.62	0.086	8
Mean = 2.59		$S^* = 0.085$	

* Pooled standard deviation.
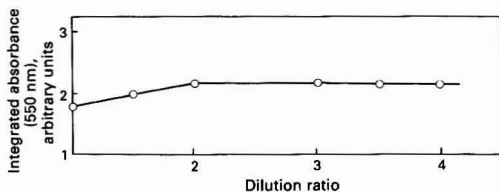
Fig. 4. Effect of dilution on the recovery of iron as iron(II) - BPS complex from a 50-ml portion of sea water. Reagents were added as in Fig. 1

contained 2.55 $\mu g\,l^{-1}$ of iron. The relative standard deviation was 3.5% ($n = 11$) for a 50-ml volume of sea water.

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Gas Chromatographic - Mass Spectrometric Identification of 9,10-Epoxyoctadeca-12-enoate Ester and 12,13-Epoxyoctadeca-9-enoate Ester in Human Blood

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The two epoxides 9,10-epoxyoctadeca-*cis*-12-enoate ester and 12,13-epoxyoctadeca-*cis*-9-enoate ester have been identified as endogenous components in human blood. The epoxy and ester function were reduced with either lithium aluminium hydride or the corresponding deuteride, before trimethylsilylation and subsequent gas chromatographic - mass spectrometric analysis. The fragmentation of the deuterated sample demonstrated the presence of the intact epoxide in the sample prior to chemical reduction. The sum of the identified epoxides in plasma was found to be 4.5–4.6 mg l⁻¹.

Keywords: *9,10-Epoxyoctadeca-12-enoate ester determination; 12,13-epoxyoctadeca-9-enoate ester determination; gas chromatography - mass spectrometry; endogenous components; blood*

During oxygenation of unsaturated compounds in biological systems, epoxides can be formed as reactive intermediates. The formation of the biologically active leukotrienes from eicosa-all-*cis*-5,8,11,14-tetraenoic acid (arachidonic acid) was shown to proceed through an unstable epoxy intermediate in *in vitro* experiments.[1,2] The same epoxide has also been isolated from human polymorphonuclear leukocytes taking account of the instability of the epoxide and development of a suitable isolation procedure.[3] Using an epoxide hydrolase inhibitor in experiments with hepatic monooxygenase, more epoxides of arachidonic acid have been isolated.[4] Also, in phospholipids taken from the lung tissue of rats, different epoxides of fatty acids have been identified.[5]

9,10-Epoxyoctadecanoate ester has been identified[6,7] in freshly drawn human blood and in intravenous infusions stored in poly(vinyl chloride) (PVC) bags. This epoxide is a major component of epoxidised oils used as additives in PVC equipment for medical care. In blood this epoxide could be a contaminant, but the endogenous origin of the epoxide is more probable.[6]

This paper describes the identification of two monoepoxides of octadeca-*cis*-9, *cis*-12-dienoate ester (linoleate ester) in human blood, *viz.*, 9,10-epoxyoctadeca-*cis*-12-enoate ester and 12,13-epoxyoctadeca-*cis*-9-enoate ester. Analogous to

previously published identification methods for epoxides, the ester and epoxy groups were reduced with lithium aluminium hydride before gas chromatographic - mass spectrometric (GC - MS) analysis.[8] To demonstrate the inact epoxy function, the samples were also reduced with the corresponding deuteride before analysis. Finally, the content of the identified epoxides was determined.

Experimental

Apparatus

Mass spectra were obtained using an LKB 2091 mass spectrometer equipped with a Packard Model 438 gas chromatograph. The electron-impact ion source was operated at 70 and 11 eV when scanning the mass spectra and at 20 eV when recording the selected ion traces. A flexible quartz capillary column (DB-1, 30 m × 0.25 mm i.d., 0.25 μm film) was used. The injector, the separator and the ion source were all maintained at 220 °C.

The column oven was temperature programmed from 150 to 194 °C at a heating rate of 4 °C min⁻¹. The epoxy derivatives were then eluted during an isothermal period of 33 min. The temperature was finally increased to 220 °C to elute
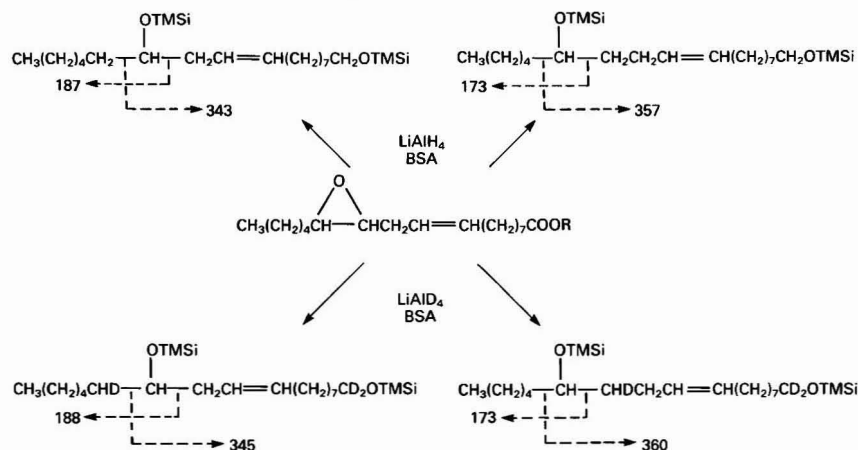
Fig. 1. Expected reaction products formed after reduction with lithium aluminium hydride and deuteride and trimethylsilylation of 12,13-epoxyoctadeca-9-enoate ester. Major α-cleavage fragments in the mass spectrometer and *m/z* values are also shown. R = glyceryl; TMSi = trimethylsilyl; BSA = *N,O*-bis(trimethylsilyl)acetamide
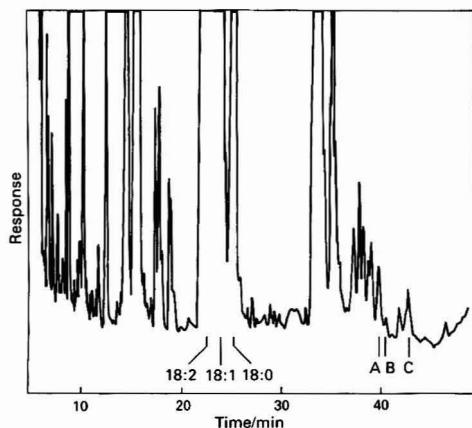
Fig. 2. Total ion chromatogram of a reduced and trimethylsilylated extract of human plasma analysed on a 30-m DB-1 capillary column. Peaks: A, three of the isomers of derivatised epoxyoctadecaenoate esters; B, the fourth isomer of derivatised epoxyoctadecaenoate esters; C, the two isomers of derivatised 9,10-epoxyoctadecanoate ester; 18:0, 18:1 and 18:2, derivatives of octadecanoate ester, octadeca-9-enoate ester and octadeca-9,12-dienoate ester, respectively. Retention times: A, 39 min 45 s; B, 40 min 45 s; and C, 42 min 50 s
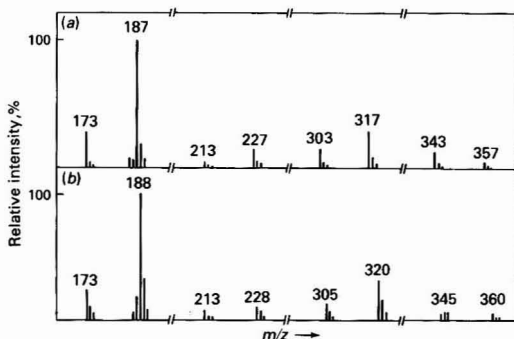
Fig. 3. Sum of partial mass spectra of peaks A and B showing α-cleavage fragments of octadecaenediols in plasma derived by various chemical treatments. (a) LiAlH$_4$, BSA and (b) LiAlD$_4$, BSA, where BSA = N,O-bis(trimethylsilyl)acetamide

the higher boiling components. The flow-rate of helium through the column was 1 ml min^{-1} and that of the make-up gas for the separator was 20 ml min^{-1}. The splitter was opened 30 s after injection.

Reagents and Procedures

All reagents used were of the highest purity available.
Methyl 9,10-epoxyoctadeca-12-enoate and *methyl 12,13-epoxyoctadeca-9-enoate*, cis *and* trans. Synthesised according to the method given previously.[9]
Sampling procedure and extraction of plasma. As described previously.[6]
Reduction and trimethylsilylation. As described previously.[7,8]

Results and Discussion

Linoleic acid can form two monoepoxides, *viz.*, 9,10-epoxyoctadeca-12-enoic acid and 12,13-epoxyoctadeca-9-enoic acid.

Reduction of the oxirane ring in each epoxide involves an approximately equal probability of cleavage of the two carbon - oxygen bonds. Hence octadeca-12-ene-1,9- and -1,10-diol, originating from the 9,10-epoxide, should be formed in about equal amounts during the chemical reduction. The same is valid for octadeca-9-ene-1,12- and -1,13-diol originating from the 12,13-epoxide (Fig. 1).

The trimethylsilyl ethers of octadeca-9-ene-1,12- and -1,13-diol should, in the mass spectrometer, give intense α-cleavage fragments at m/z 173, 187, 343 and 357 (Fig. 1). Correspondingly, the octadeca-12-ene-1,9- and -1,10-diol originating from the 9,10-epoxide after reduction and silylation, should give α-cleavage fragments at m/z 213, 227, 303 and 317 (not depicted). In the samples, these main fragments were recognised in peaks A and B (Fig. 2). In peak A, the 13-hydroxy isomer was eluted first, followed by a mixture of the 12- and 9-hydroxy isomers; in peak B the 10-hydroxy isomer was eluted. Partial mass spectra showing α-cleavage fragments of each isomer are added and shown in Fig. 3. The mass spectra, the retention times and the elution order of the isomers on the column were identical with those of the reduced and silylated synthesised sample in the *cis*-configuration.

The spectra give no proof of the intactness of the epoxides when the blood was drawn. As reduction converts the epoxides into secondary alcohols and the carboxy end to a primary alcohol, the mass spectra could arise from a mixture of octadeca-1-ene-9-, -10-, -12- and -13-diols, ketones or a mixture of both, and with a terminal hydroxy or aldehyde group. To invalidate these possibilities, both the sample and standard were reduced with lithium deuteride. The α-cleavage fragments from the deuterated compounds derived from the 12,13-epoxide were expected to be m/z 173, 188, 345 and 360 (Fig. 1). By analogy, corresponding fragments from the reduced 9,10-epoxide would be m/z 213, 228, 305 and 320. The mass spectra of the deuterated sample and standard showed about the same pattern and the main fragments were found at m/z 173, 188, 213, 228, 305, 320, 345 and 360 as expected (Fig. 3). Further, after deuteration, the weak molecular ion in the sample and reference had moved from m/z 428 to 431 as expected. A minor deviation in the spectra from the 12-hydroxy isomer at m/z 345 was detected in the deuterated sample. The relative increase in the intensity of the fragment at m/z 346 can be explained by the presence of the corresponding 12-keto compound in addition to the 12,13-epoxide. The presence of the ketone cannot be detected in the undeuterated sample as both the ketone and epoxide give the same 12-hydroxy compound after reduction.

The fatty acids in blood consist mainly of *cis*-compounds, although *trans*-compounds have been identified. Both *trans, trans*- and *cis,trans*-octadeca-9,12-dienoic acid have been identified in serum and erythrocyte lipids.[10] The stereochemistry of the epoxide cannot be determined by this identification method, but we could elucidate the configuration of the unoxidised double bond. The two monoepoxides of octadeca-*trans*-9, *trans*-12-dienoate methyl ester were synthesised, then reduced and trimethylsilylated before GC - MS analysis. In the mixture of the four *cis*- and four *trans*-isomers, the three *cis*-isomers (13-,12- and 9-hydroxy) were eluted first (Fig. 2, peak A) without interference from any *trans*-isomers. The *cis*-10-hydroxy isomer (peak B) was then eluted in a mixture with the *trans*-isomers, 10- and 12-hydroxy, followed by the *trans*-isomers, 9- and 13-hydroxy, in well separated peaks. The elution of all isomers lasted for 1 min 40 s. The *cis*-configuration of the double bond for both structures in the sample is shown by elution of the first peak, as the two 9- and 10-hydroxy compounds originating from the same structure must have the same configuration. Further, if appreciable amounts of the *trans*-compounds had been present, the mass spectra of peak B in the sample would have revealed the intense fragment from the *trans*-12-hydroxy isomer at m/z 187.

The leukotriene epoxide detected in *in vitro* experiments is

chemically unstable even at a physiological pH.[1] The identified epoxides of linoleate ester seem to be much more stable. However, there is one main chemical difference between the epoxides—the leukotriene epoxide is in an allylic position to a triene system, whereas the epoxides from the linoleate ester are in a homoallylic position to one double bond. The difference in structure should give an increased chemical stability of the linoleate ester epoxides. Before quantitative measurements could be made, stability-indicating experiments were performed in plasma to which 50 mg l^{-1} of the two synthetic cis-epoxides were added. After storage for 1 h at room temperature, no decomposition was detected by direct gas chromatographic analysis of an extract of the plasma. In blood, however, the stability of the detected epoxides is still unknown. Therefore, the blood sample was cooled in ice a few seconds after collection followed by centrifugation in the cold.

The amount of the epoxides in plasma was then determined by comparison with reduced and silylated standards of the epoxides in a suitable concentration (2.5, 5.0 and 7.5 mg l^{-1}). During the measurements, the most intense fragment in each isomer was used, viz., m/z 173, 187, 227 and 317. The mass fragmentograph gave linear calibration graphs and the quantifications were performed on straight base lines. The recovery of the epoxides (10 mg l^{-1}) added to plasma was 98%.

When blanks were analysed, no contamination by epoxides could be detected. The sum of the epoxides was 4.5–4.6 mg l^{-1} and both were present in about equal amounts.

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Simultaneous Quantitation of Morphine, Monobutanoylmorphine and Dibutanoylmorphine Using Short Capillary Column Gas Chromatography - Mass Spectrometry

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An assay procedure for quantitating 3,6-dibutanoylmorphine, a morphine diester, and its metabolites, 6-monobutanoylmorphine and morphine, has been developed. The method involves a one-step extraction from various biological media, gas chromatographic separation using a short methylsilicone capillary column and sensitive and selective quantitation by single ion monitoring mass spectrometry. The use of short capillary column GC - MS allowed the rapid separation of compounds with dissimilar retentions that are biologically interrelated and must therefore be measured intact in small sample volumes. The application of this method to studies on the metabolism of butanoyl esters of morphine in whole brain homogenates is described.

Keywords: *Dibutanoylmorphine; morphine esters; gas chromatography; mass spectrometry*

3,6-Dibutanoylmorphine (DBM) is a semi-synthetic diester derivative of morphine that has been previously shown to be an opioid agonist *in vitro*,[1] to act as a potent analgesic *in vivo*[2] and to produce less severe behavioural effects than other morphine esters when administered to rats.[3] Investigation of the regional metabolism and disposition of DBM required a method of reliably and rapidly quantitating unchanged DBM and its primary metabolites, 6-monobutanoylmorphine (MBM) and morphine (M), in a variety of tissues and tissue homogenates.

A number of assay procedures have been described for the quantitation of morphine using high-performance liquid chromatography (HPLC) coupled to electrochemical,[4-6] ultraviolet[7] or fluorescence[8] detectors; however, these methods either lacked the required sensitivity or were incapable of measuring unchanged DBM. Similarly, radio-immunoassay (RIA) methods for measuring morphine concentrations have been described,[9-11] but these lack the necessary specificity for distinguishing between such chemically similar compounds as M, MBM and DBM.

The technique of gas chromatography - mass spectrometry (GC - MS) seemed the most appropriate method for the current application as this technique has been successfully applied to the quantitation of morphine[12,13] and allows the detection of DBM without either derivatisation or hydrolysis. The procedure described in this paper involved a one-step extraction procedure for all three compounds (M, MBM and DBM), a rapid chromatographic separation using a very short methylsilicone capillary column and a highly selective method of detection using selected ion monitoring (SIM) mass spectrometry.

Experimental

Equipment

The assay of M, MBM and DBM was conducted using a Hewlett-Packard (Palo Alto, CA, USA) 5985 GC - MS system operated in the electron-ionisation (EI) mode. Default tuning parameters for the mass spectrometer were established using the program Autotune and perfluorotributylamine as the

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calibration standard at a source pressure of approximately 2×10^{-6} Torr. The mass spectrometer was operated at an ion source temperature of 200 °C and the electron multiplier voltage was increased by 600 V over the default voltage.

Drugs and Reagents

Morphine sulphate was purchased from BDH (Toronto, Ontario, Canada). MBM was synthesised as the hydro-chloride salt using the method of May and Jacobson[14] adapted for butanoyl derivatives. DBM was synthesised according to the method of Beckett and Wright[15] and was converted into the hydrochloride salt. MBM and DBM were authenticated by mass spectrometry.

Ethyl acetate, chloroform, butan-1-ol and acetonitrile were HPLC-grade solvents from Fisher Scientific (Whitby, Ontario, Canada). Pyridine and acetic anhydride were of spectrophotometric grade, from Fisher, and anhydrous potassium phosphate (dibasic) was obtained from J. T. Baker (Phillipsburg, NJ, USA).

Homogenate Preparation

Male Sprague-Dawley rats (200–300 g) (Canadian Breeding Farm Laboratories, Montreal, Quebec, Canada) were killed by decapitation and the brains were removed on ice. Whole brains were homogenised in cold Dulbecco's phosphate-buffered saline (pH 7.35) to produce 10% *m/V* homogenates after addition of drug. Homogenates containing drug were incubated at 37 °C in a shaking water-bath (Precision Scientific, Chicago, IL, USA).

Extraction and Derivatisation Procedure

Samples (0.5 ml) of 10% *m/V* whole brain homogenate were removed at selected time points and mixed with 0.5 ml of 100 mM K_2HPO_4 - acetonitrile (50 + 50 *V/V*, pH 8.7) in glass test-tubes. Ethyl acetate - chloroform - butan-1-ol (80 + 10 + 10 *V/V*, 2.0 ml) was then added and the mixture was shaken for 5 min in a horizontal shaker (Eberbach, Ann Arbor, MI, USA) and then centrifuged at 250 *g* for 5 min in a bench-top centrifuge (Model HN-S; Damon/IEC, Needham Heights, MA, USA). An aliquot (1.0 ml) of the upper (organic) phase was then removed and evaporated to dryness under nitrogen in a 1.5-ml polypropylene microcentrifuge tube (Sarstedt, St. Laurent, Quebec, Canada).

M and MBM were derivatised for GC analysis by dissolving the extraction residue in 0.2 ml of pyridine and incubating with 0.5 ml of acetic anhydride for 60 min at 60 °C in a dry bath (Fisher Scientific). Derivatised samples were then dried under nitrogen and were stored at −70 °C until undergoing analysis by GC - MS.

Chromatography

Residues were dissolved in 50 μl of ethyl acetate and an aliquot (1 μl) was injected on to the GC column, which consisted of a 3-m section of methylsilicone glass capillary column (SPB-1, 0.75 mm) (Supelco, Bellefonte, PA, USA). The injection port temperature was 250 °C and immediately following injection a programmed linear temperature gradient was initiated such that the oven temperature increased from 150 to 270 °C at a rate of 12 °C min^{-1}. The carrier gas (helium) was delivered at a flow-rate of 35–40 ml min^{-1}. The GC effluent passed via a jet separator into the ionisation chamber of the mass spectrometer and detection was accomplished using SIM at a mass to charge (m/z) ratio of 268.

Results and Discussion

A chromatogram obtained following extraction, derivatis-ation and injection of a 0.5-ml brain homogenate sample containing M, MBM and DBM is shown in Fig. 1. The peaks corresponding to the appropriate derivatives (3,6-diacetylmorphine, 3-O-acetyl-6-butanoylmorphine and DBM for M, MBM and DBM, respectively) were well resolved and eluted within 7.5 min. Both the column length and the oven temperature gradient were found to be critical for suitable chromatographic separation. DBM has a low vapour pressure and is unstable at temperatures above approximately 300 °C. DBM therefore failed to elute within 20 min using any of a variety of normal 6-m packed columns or a 30-m capillary column at temperatures below 300 °C. Use of the short capillary column, however, resulted in DBM retention times of less than 10 min at these temperatures. Although the peak shapes were acceptable using the short column, a mixture of M, MBM and DBM was inadequately resolved using iso-thermal chromatography; however, this problem could be suitably overcome by using a programmed temperature gradient. Thus, either a temperature gradient or a short column alone was insufficient for the current application, but these two factors in combination resulted in a rapid and effective separation.

Following derivatisation with acetic anhydride, all three analytes were diester derivatives of morphine; consequently, the mass spectrometer fragmentation patterns were very similar. Examination of the mass spectra revealed that all compounds had a strong ion abundance signal at m/z 268, in accord with results reported previously for other morphine diesters.[1] The use of SIM as a means of detection resulted in both good sensitivity (lower limit of detection ≈ 0.3 ng for M, 0.5 ng for MBM and 0.9 ng for DBM based on a signal to noise ratio ≥ 2) and good selectivity, as no interference at the monitored ion was found in blank samples of either blood or brain homogenate, whereas considerable background noise was found in total ion chromatograms of the GC effluent. Calibration graphs (peak area vs. nanograms of drug) for M, MBM and DBM were found to be linear from 10 to 250 ng and passed through the origin. The correlation coefficients for all these drugs were ≥0.9916.

Over-all coefficients of variation (C.V.) following extrac-tion, derivatisation and injection of aqueous standards con-taining 25 ng each of M, MBM and DBM (n = 6) were 5.3%, 9.4% and 10.2% for M, MBM and DBM, respectively, and mean C.V.s for brain homogenate samples (n = 3) over a range of eight drug concentrations were 10.2%, 9.9% and 13.0% for M, MBM and DBM, respectively. The reproduci-bility in the current assay is comparable to that of existing GC assays for morphine alone[16,17] and is superior to that achieved in other assays for morphine and morphine derivatives.[5,13] For these reasons the use of internal standards was considered unnecessary for the current applications, especially as the different physico-chemical properties of M, MBM and DBM would require that an internal standard for each compound be incorporated into the assay. Ideally, stable isotope analogues of M, MBM and DBM might have been used to increase the reproducibility of the procedure. Unfortunately, these were not available to us.

The metabolism of a mixture of butanoyl esters of morphine by rat brain homogenates (n = 4) was studied using the assay procedure as described; the relative amounts of DBM, MBM and M in brain homogenates at selected time points are presented in Table 1. The concentrations of morphine esters were not significantly reduced at any time in buffer controls, but DBM was metabolised to MBM and subsequently to M in brain homogenates ($t_{1/2}$ = 69 min). In previous studies on the metabolism of DBM in both rat and human blood, it was found that whereas DBM concentrations decreased exponen-tially, the metabolism terminated at the level of MBM.[18] Hence brain tissue appears to contain enzymes that can deacylate MBM to M, whereas blood does not. This result is important to an understanding of the actions of DBM within the brain, as both MBM and M, but not DBM, are presumed to be pharmacologically active.[2]

The procedure described in this paper represents a rela-tively simple and effective method of simultaneously deter-mining three biologically interrelated compounds that have
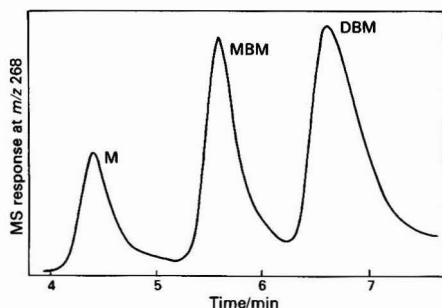

Fig. 1. Chromatogram showing M, MBM and DBM in a sample of brain homogenate that had been incubated with 7.5 × 10^{-4} M DBM and 2.5 × 10^{-4} M MBM for 60 min

Table 1. Biotransformation of a mixture of DBM and MBM by rat brain homogenates. DBM and BMB were added to 10% m/V rat brain homogenates to initial concentrations of 7.5 × 10^{-4} and 2.5 × 10^{-4} M, respectively. At the time indicated, samples were taken for analysis as described under Experimental. Data are presented as percentages of the total opioid concentration ± standard deviation (n = 4); asterisks indicate p ≤ 0.01 relative to time zero by repeated measure ANOVA

| Time/ | Concentration, % | | |
min	DBM	MBM	M
0	75.9 ± 2.9	23.3 ± 2.4	0.8 ± 0.6
5	76.4 ± 3.8	20.8 ± 2.7	2.8 ± 1.2
15	74.0 ± 4.0	19.0 ± 1.9	7.0 ± 2.1
30	62.1 ± 1.7*	25.4 ± 0.9	12.4 ± 1.6*
60	46.4 ± 5.8*	35.1 ± 2.7*	18.4 ± 3.6*
90	35.6 ± 6.8*	39.2 ± 1.7*	25.2 ± 6.7*
120	23.8 ± 6.6*	44.2 ± 4.6*	32.0 ± 11.2*
180	15.7 ± 5.1*	45.0 ± 2.9*	39.4 ± 7.8*

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dissimilar chromatographic properties. The use of a very short capillary column allows the chromatographic separation of three chemicals with relatively low vapour pressures, and circumvents the need for harsh chemical treatments that might distort the relative concentrations of these drugs in biological samples. As such, the use of similar chromatographic manipulations may provide a solution to comparable problems in the analysis of other biologically active compounds where small sample sizes and rapid analysis times are priorities.

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Problems in the Application of Ion-selective Electrodes to Serum Lithium Analysis

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Three types of lithium ion-selective electrode (ISE) have been studied in microconduit flow injection analysis for their response to lithium ions in serum samples containing between 0.21 and 2.00 mM lithium. The effects of possible interferents, such as proteins and sodium chloride, in samples have been studied with regard to the poor quality of data.

Adjustment of the serum sodium chloride level to 153.9 mM improved the ISE lithium data to match more closely those obtained by flame photometry. Nevertheless, even the best of the three lithium ISEs studied, namely that based on an electroactive component of barium propoxylate, produced data that matched flame photometric data for only five serum samples out of the ten examined.

The other two electrode types studied were based on PVC matrix membranes of a commercial lithium ISE (Philips Cat. No. 561-LI) and dodecylmethyl-14-crown-4

Keywords: *Ion-selective electrodes; lithium analysis; serum lithium*

The development of lithium ion-selective electrodes (ISEs) for monitoring lithium ions in blood serum has recently received considerable attention, as lithium salts are prescribed for the treatment of manic depression.

Several crown ethers,[1–5] lipophilic diamides[6–8] and other diverse materials[9–11] have been investigated as sensors for lithium ISEs. Most of these sensors lack satisfactory sodium selectivity for the determination of lithium in blood serum where the sodium level is typically over 1400 times higher than the lowest lithium levels of clinical interest. So far, lithium ISEs reported to have good selectivity, *e.g.*, $k_{Li,Na}^{pot} \approx 0.002$, have been restricted to lithium analysis in artificial sera,[5,6] which is unrealistic as proteins and most other organic materials found in blood serum have been excluded.

A new PVC lithium ISE based on the tetraphenylborate of a barium polypropoxylate, $Ba(PPG-1025)_{0.69}TPB_2$, and dioctyl-phenyl phosphonate (DOPP) solvent mediator has a linear slope of 57 mV decade[-1] down to 10^{-4} M lithium.[12] The qualities of this inexpensive PVC electrode motivated this investigation of its behaviour for the determination of lithium in sera.

Experimental

Apparatus and Materials

Microconduits made from PVC blocks were used as a sample cell in the manner described previously.[12] Compressed air was used to propel the carrier solution through the microconduit at 1.0 cm[3] min^{-1}. A Radiometer PHM64 millivoltmeter was used for e.m.f. measurements and the response was recorded on a Linear Instrument Corp. Model 0555-0000 chart recorder.

Reagents and Chemicals

The barium polypropoxylate, $Ba(PPG-1025)_{0.69}TPB_2$, was synthesised as reported by Jaber *et al.*[13] Dioctylphenyl phosphonate was obtained from Lancaster Synthesis, Morecambe, UK, polypropylene glycol (PPG-1025) from BDH Chemicals, Poole, UK, sodium tetraphenylborate and dialysis membranes from Aldrich, Gillingham, Dorset, UK, polycarbonate and cellulose acetate protein exclusion membrane from Millipore, Molsheim, France, α-globulin, β-globulin, albumin (bovine) and glucose from Sigma, Poole, UK, and a lyophilised powder mixture of cholesterol (free and total),

triglyceride, phospholipids and β-lipoproteins from Boehr-inger Mannheim, East Sussex, UK.

The commerical lithium PVC membrane (Type No. 561-LI) was obtained from Philips Scientific and Analytical Equipment, Cambridge, UK, and dodecylmethyl-14-crown-4 was a gift from Professor T. Shono, Osaka University, Japan.

All solutions were prepared from analytical-reagent grade salts using distilled, de-ionised water.

Electrode Fabrication

Three different lithium cocktails were utilised in the fabrication of otherwise identical PVC lithium ISEs.

The barium complex, $Ba(PPG-1025)_{0.69}TPB_2$, (0.04 g) was dissolved in dioctylphenyl phosphonate (0.36 g) and the sensor cocktail completed by adding PVC (0.17 g) in tetrahydrofuran (6 cm[3]). Electrodes (Type 1) were fabricated as reported earlier.[12]

One Philips lithium PVC membrane disc (7.0 mm) was dissolved in tetrahydrofuran (1 cm[3]) and the cocktail used to fabricate Type 2 electrodes in an analogous fashion to the above PVC barium model.[12] Type 3 electrodes were similarly fabricated from a cocktail consisting of dodecylmethyl-14-crown-4 (3.6 mg), *o*-nitrooctylphenyl ether (0.25 g), PVC (0.1 g), potassium tetra(4-chlorophenyl)borate (2.3 mg) and trioctylphosphine oxide (3.6 mg) in tetrahydrofuran (3 cm[3]).

Each exclusion membrane was carefully attached to the surface of the particular lithium PVC membrane and sealed around the edges to the microconduit channel with PVC glue.

Serum Analysis Procedure

Analyses were carried out by flow injection analysis employing microconduits.[11] Serum samples (1–10) were obtained from the University Hospital of Wales. Of these, samples 1 and 10 were diafiltrated serum with added lithium carbonate, and samples 2–9 were mixes of 1 and 10 to give a graded lithium content. Each sample (1–10) was previously analysed for lithium and other cations (Table 1). Serum samples from patients, containing between 0.1 and 2.8 mM lithium (analysed by flame photometry), were also briefly studied. Aliquots (100 mm[3]) of the serum samples were injected into the carrier stream of artificial serum electrolyte A, specified below, and the change in potential was recorded. Calibrations were made using lithium standards in artificial serum electrolyte A.

Table 1. Cation concentrations in serum samples determined by previous analysis at the University Hospital of Wales

Serum sample No.	Cation concentration/mM			
	Li	Na	K	Ca
1	0.21	105.3	1.77	1.62
2	0.40	110.7	2.36	1.78
3	0.61	116.1	2.95	1.97
4	0.79	122.0	3.57	2.16
5	1.00	126.9	4.17	2.35
6	1.20	132.3	4.78	2.53
7	1.40	137.7	5.38	2.72
8	1.58	143.5	6.03	2.90
9	1.75	148.7	6.60	3.09
10	2.00	153.9	7.18	3.27
Analytical technique	Flame photometry	ISE	ISE	Spectrophotometry

The artificial serum electrolyte, A, consisted of sodium chloride (140 mM), potassium chloride (2.8 mM), potassium dihydrogen phosphate (1.3 mM), calcium chloride (2.5 mM) and magnesium sulphate (2.3 mM). Its pH was adjusted to *ca.* 7.3 with potassium hydroxide solution (1.0 M).

A modified artificial serum electrolyte B, used as an alternative carrier for the pre-treated real sera, differed only in that it comprised 153.9 mM sodium chloride (to match that in sample 10 of Table 1) instead of the 140 mM in the case of serum A.

Investigation of Interference effects

Albumin bovine (0.2889 g), α-globulin (0.0771 g), β-globulin (0.0705 g), γ-globulin (0.12 g) and glucose (0.0067 g) were each dissolved in 5 cm³ of 0.1 M lithium chloride solution made up in artificial serum electrolyte A. Another solution consisting of all five compounds in 0.1 M LiCl artificial serum electrolyte A (5 cm³) was also prepared.

The Boehringer Mannheim powder, consisting of cholesterol, triglyceride, phospholipids and β-lipoproteins, was dissolved in 10 cm³ of 0.1 M lithium chloride/artificial serum electrolyte A.

Each of these protein- or protein/fat-based sera was also injected into the carrier stream and the response was compared with that from 0.1 M lithium chloride in solution A.

Results and Discussion

Clearly, the Type 1 electrode responds reproducibly to lithium, even at 0.1 mM in artificial serum A (Fig. 1). The results from the serum samples (confirmed by flame photometry to contain between 0.21 and 2.0 mM lithium; see Table 1) were unexpected in that either negative or positive FIA electrode responses were obtained for some samples whereas others (Nos. 4, 5 and 7) gave both negative and positive peaks (Fig. 2). Moreover, most responses were not reproducible. Similar erratic responses were obtained for patients' serum samples shown by flame photometry to contain up to 2.8 mM lithium. However, as a wider range of electrolyte data were available for samples 1–10, the remainder of this study was focused on these.

The response patterns mentioned above could relate to effects such as pH, anions, proteins, lipoproteins, the nature of the electrode itself and/or the varying electrolyte concentrations of the serum samples (Table 1). The pH of the serum samples analysed ranged from 7.1 to 7.8, but the PVC barium electrode suffers little hydrogen ion interference.[12] The negative response is also unlikely to be due to chloride and phosphate anions (the predominant anions in serum), as similar concentrations of these anions were present in the carrier solution A.
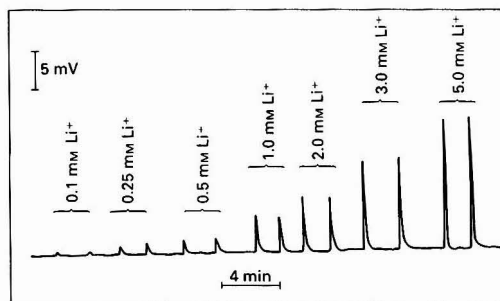
Fig. 1. Flow injection profiles of the Type 1 electrode to lithium standards in artificial serum electrolyte A. Carrier: artificial serum electrolyte A at pH 7.1
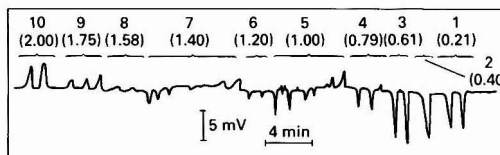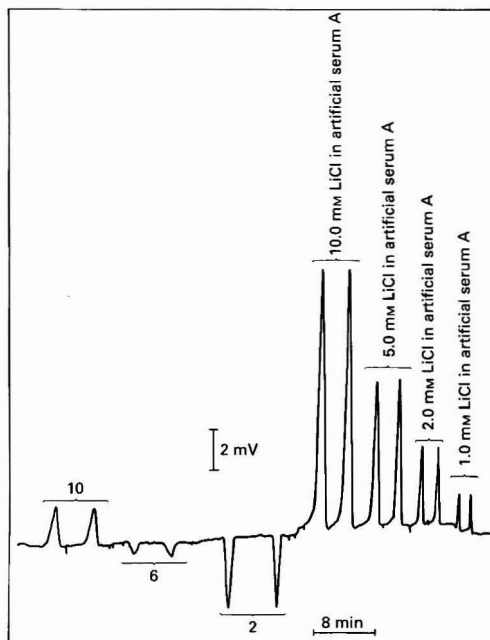

**Fig. 2.** Flow injection analysis profiles of the Type 1 electrode for ten serum samples. Carrier: artificial serum electrolyte A at pH 7.1. Numbers in parentheses are lithium levels (mM) found by flame photometry

Effect of Natural Products on the Electrode

The electrode response was unaffected by α-, β- and γ-globulin, albumin (bovine) and glucose, both individually and all five together. The same applied to cholesterol (free and total), triglyceride, phospholipids and β-lipoproteins, both individually and in admixture in artificial serum A. Of course, many other organic materials in serum (not studied here) could affect the electrode response.

In clinical applications of commercial ISEs protein (or high relative molecular mass) exclusion membranes are often applied to the sensor surfaces to offset large molecule effects. Thus, such exclusion membranes were attached to the Type 1 electrode surface as described. Three different exclusion membranes were used, namely a dialysis membrane capable of excluding molecules with relative molecular mass >2000, a polycarbonate protein exclusion membrane with a pore size of 0.4 µm and cellulose acetate with a pore size of 0.22 µm. The respective exclusion membranes resulted in 50, 0 and 26% reduction in response when 0.1 M lithium chloride in artificial

serum electrolyte A (*i.e.*, no protein or large molecule components were present) were injected into the carrier stream. The reductions of 50 and 26% are most unfavourable. Indeed, even at the 5 mM lithium chloride level, the reduction of 59% observed for cellulose acetate was even worse than the 50% noted for 0.1 M lithium chloride.

With the polycarbonate protein exclusion membrane, where no reduction of artificial serum lithium response was observed, the response to serum lithium was still unsatisfactory, *i.e.*, the peaks were irreproducible and often showed negative profiles. This suggests that either the polycarbonate protein exclusion membrane was inefficient in excluding interfering large molecules from the electrode, or the effect on the electrode is due to one or more unidentified low relative molecular mass organic component(s) of the serum. Fortunately, this anomalous response is temporary, as the FIA base line is quickly restored after passage of sample and the PVC electrodes showed their usual Nernstian response in lithium standards.

Nature of Sensor

To establish that these FIA electrode behaviour patterns are not due to the nature of the sensor in the electrode, the responses of the other two lithium ISEs were similarly investigated. Their respective responses to real serum lithium were similar to those illustrated in Fig. 1. Therefore, these strange FIA patterns seem to relate to component(s) in serum rather than to the sensors.

Effect of Varying Electrolyte Levels in Serum Samples

The sodium content of the serum samples studied varied from 105.3 to 153.9 mM (Table 1). It is frequently assumed that the average concentration of sodium in serum is about 140 mM and the carrier artificial serum electrolyte A was accordingly set at 140 mM. Serum samples containing less sodium than the carrier solution A generally resulted in negative peaks and serum samples containing more sodium than the carrier solution A usually resulted in positive peaks (Fig. 2). This was investigated by injecting mock serum samples (free of organic components) containing lithium, sodium, potassium and calcium ions in amounts equivalent to real serum samples Nos. 2, 6 and 10 (Table 1) into the carrier electrolyte stream A.

As suspected, the low-sodium system gave negative peaks (Fig. 3), supporting the idea that the variable sodium in the serum samples is partly responsible for the strange response of the electrodes. It should be noted, however, that the responses obtained using the mock serum samples are reproducible, unlike the results when real serum samples were used. In addition to the varying sodium effect in the serum, there must be other parameters in the serum that affect the electrode response.

Serum Electrolyte Correction

To attempt a correction for the effect of varying sodium concentration on the lithium response (see Fig. 2), serum samples (1 cm^3) were spiked with appropriate volumes of sodium chloride solution (1.0 M) to bring the concentration of sodium in each serum to the same level as that in the carrier solution B, namely 153.9 mM. These spiked samples were then injected into the non-protein based artificial serum electrolyte B. The results obtained using the three different electrodes showed improvement over the previous analysis. The results, however, are not statistically satisfactory. For the serum samples analysed with each electrode, only three results out of ten samples examined showed any agreement with the actual flame photometric (Table 1) lithium values obtained using the Type 3 electrode (Fig. 4). No response was obtained for samples 2, 3 and 4 in Table 1. Sample 10 (Table 1) containing 2

Fig. 3. Flow injection analysis profiles of Type 2 electrode to three mock serum samples. Carrier: artificial serum electrolyte A at pH 7.1
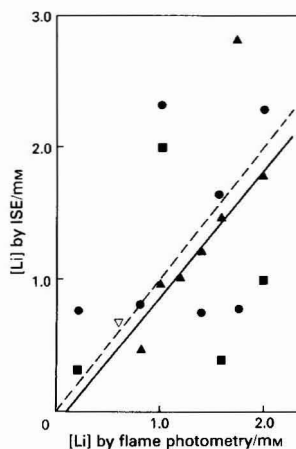
Fig. 4. Comparison of lithium ISE data with flame photometric data for serum samples. Samples for ISE data were adjusted to 153.9 mM sodium chloride, except for Type 1 (special case), which was used as it was. Carrier for ISE data: artificial serum electrolyte B at pH 7.1 except for Type 1 (special case), where the carrier solution was 116.1 mM sodium chloride, 2.95 mM potassium chloride, 1.97 mM calcium chloride and 1.01 mM magnesium chloride. Broken line: theoretical line for $[Li^+]_{ISE} = [Li]_{flame\ photometry}$. Electrodes: ▲, Type 1; ■, Type 2; ●, Type 3; and ▽, Type 1 (special case)

mM lithium as determined by flame photometry is to be compared with the value of 2.3 mM (standard deviation 0.49 mM; $n = 4$) obtained with the Type 3 electrode. For the Type 2 electrode only one result out of five samples examined showed some agreement. However, only five serum samples were analysed with the Type 2 electrode owing to a lack of sample.

For the Type 1 electrode, six results out of ten samples examined showed relatively close agreement (Fig. 4). No response was obtained for samples 1, 2 and 3.

The best six results for the Type 1 electrode gave the line (full line in Fig. 4) $[Li^+]_{Type\ 1\ electrode} = 0.93[Li^+]_{flame\ photometry} - 3.77 \times 10^{-5}$, with a correlation coefficient of 0.992 and a standard error of the estimate of 5.89×10^{-5}.

This modified procedure for serum lithium analysis requires a prior knowledge of the sodium level in the serum samples in order to increase the sodium concentration to that of the carrier B before injection. Nevertheless, as seen above, the quality of the results obtained, even for the Type 1 electrode, is still poor but mainly because no response is obtained for the samples low in lithium. The high sodium level in the carrier solution (153.9 mM) can interfere with the electrode response at low lithium concentrations and is one reason for the inability to obtain data for certain serum samples. To offset such problems, a different carrier solution containing the same amount of sodium, potassium and calcium as the serum sample was tried for the analysis of serum sample No. 3 using the Type 1 electrode. Thus, the lithium standards were prepared in a protein-free carrier solution containing 116.1 mM sodium chloride, 2.95 mM potassium chloride, 1.97 mM calcium chloride and 1.01 mM magnesium chloride. A value of 0.68 mM lithium was obtained (Fig. 4), compared with 0.61 mM by flame photometry. However, this method of different carrier solutions for each sample, even though it allows low levels of lithium to be measured, is too laborious and not practicable.

From Fig. 4, it appears that there is a greater serum component effect on the Type 2 and 3 electrodes than on the Type 1 electrode.

Conclusion

It can be concluded that potentiometric analysis for serum lithium requires further research, firstly regarding a lithium ISE with $k_{Li,Na}^{pot} < 10^{-3}$, which can be used without sample pre-treatment for adjusting sodium levels. Here, the respective separate solution selectivity coefficients for Type 1, 2 and 3 electrodes[12] at 4.4×10^{-2}, 1.0×10^{-2} and 9.4×10^{-3} are higher. However, a recently described[7] lithium ion sensor based on cis-cyclohexane-1,2-dicarboxamide (ETH 1810) with a selectivity coefficient of 5.0×10^{-3} is a step towards the ideal value of $<10^{-3}$.

This study has shown that lithium ISEs are subject to serious interferences from other serum components, which have not been identified despite eliminating the more obvious proteins and lipids as the source of error. The different sensors were affected by serum to different extents, and this aspect of performance may need as much attention as the selectivity over sodium.

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Determination of Sodium in Salted Foods Using an Ion-selective Electrode

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There is a need to control our daily intake of sodium via the national diet. A fast and reliable method for the determination of sodium in individual foods would satisfy dietician and consumer interests. This work investigated the use of the sodium ion-selective electrode in the analysis of a wide variety of cheese, butter and other salted foods. Good agreement was found when the results for cheese were compared with those found by reference method but a bias of +7–9% was found for results of ion-selective electrode over the results from reference method for butters.

Keywords: *Salted foods; sodium determination; ion-selective electrode*

The Medical Research Council has linked high sodium intake with hypertension in certain sensitive individuals. A fall in blood pressure is observed when salt intake is reduced. Therefore, the dietician requires precise information on the sodium content of individual foods when formulating a low-sodium diet. Some food manufacturers include sodium in their nutrient labelling of products but this is not a statutory requirement according to the Food Labelling Regulations.[1] Guidelines are given by the DHSS[2] for infant formulae, recommending minimum and maximum levels for sodium intake in liquid feed. The World Health Organization Expert Committee on Prevention of Coronary Heart Disease[3] recommend that adult intake should not exceed 5 g of salt ($\equiv$ 2 g of sodium) per day. It is estimated that only 12% of the daily intake of sodium is provided by table salt and that the bulk of sodium intake is via processed foods. The contributions of foods to sodium intakes were investigated by Bull and Buss,[4] who gave a calculated intake of 2.6 g of sodium per person per day with most of this (2.2 g) contributed via processed foods. Consequently, it is desirable for the manufacturer to have a rapid, reliable and inexpensive method for monitoring sodium levels in manufacturing processes. In this work we investigated the use of the sodium ion-selective electrode for the determination of sodium in salted foods and assessed how well the procedure satisfies the aforementioned criteria.

Chapman and Goldsmith[5] determined sodium in salted foodstuffs by using an ion-selective electrode. The "dry sample addition" method was used to measure the increase in electrode potential found after adding known masses of solid sample to a standard sodium solution. Results from a collaborative study of the determination of sodium in dietetic foods using ion-selective electrodes were reported by McNerney.[6] Standard additions of sodium were made to the buffered food sample, electrode potential readings plotted on Gran's paper and the concentration in the food sample evaluated from the intercept of the extrapolated straight line. Statistical analysis of the results showed good precision of measurement for a range of dietetic foods and the procedure has been adopted as official final action by the AOAC. Moxon[7] gave a rapid method for the determination of sodium in salted and unsalted butters. The sample is treated with hydrochloric acid and the filtered extract is analysed for sodium content by atomic absorption spectrometry. Variability of the sodium results for salted butter samples analysed on five consecutive days gave coefficients of variation of 2–4% on the over-all mean values. However, the method involves at least 2 h for sample preparation and requires the use of expensive equipment.

Kindstedt and Kosikowski[8] developed a rapid, inexpensive method for the determination of sodium chloride in cheese using a sodium ion-selective electrode. They tested the procedure using a variety of cheeses and found good agreement between the results determined with the electrode and those determined by the standard method employing Volhard titration. Their method, with minor modifications, was adopted in this work for the determination of sodium in samples of cheese, butter and other salted foods.

Experimental

Apparatus

Specific ion meter. Orion Model 407A/F, supplied by MSE Scientific Instruments, Crawley, Sussex.

Sodium ion-selective electrode, glass body. Orion Model 97–11, supplied by MSE Scientific Instruments.

Double-junction reference electrode. Orion Model 90–02, supplied by MSE Scientific Instruments.

Waring blender. Two-speed, supplied by Christison Scientific Equipment Ltd., East Gateshead Industrial Estate, Gateshead.

Reagents

These were of analytical-reagent grade wherever possible and were obtained from BDH Chemicals, Poole, Dorset. Distilled, de-ionised water was used in the preparation of solutions where required.

Buffer. A 40-g amount of ammonium chloride was weighed into a 200-ml calibrated flask and dissolved in approximately 100 ml of water; 54 ml of concentrated ammonia solution (sp.gr. 0.88) were added and the solution was made up to the mark with water.

Electrode rinse solution. The buffer was diluted by measuring 20 ml into a 1-l calibrated flask and making up to the mark with water.

Electrode storage solution, 5 M sodium chloride. A 29.2-g amount of NaCl was weighed into a 100-ml calibrated flask and dissolved in and made up to the mark with water.

Table 1. Recovery of sodium added to cheese as determined with the ion-selective electrode

| Sodium added/ mg | Meter read-out, p.p.m. | | Recovery, % |
	Expected	Found	
0	—	50.0	—
1.0	55.0	54.5	99.1
2.5	61.2	62.5	102.1
5.0	75.0	72.8	97.1

Table 2. Determination of sodium in cheese: comparison of ion-selective electrode (ISE) with atomic absorption spectrometry (AAS)

| | | | | | Sodium/mg per 100 g | | | | |
| | | | | | ISE | | AAS | | |
Cheese					Mean*	S.d.	Mean*	S.d.	Difference
Cheddar	..	..	..	..	720	6	722	2	2
Cheshire	..	..	..	..	526	4	530	7	4
Leicester	..	..	..	..	498	5	528	10	30
Double Gloucester		..	..	..	515	11	516	4	1
Caerphilly	..	..	..	..	632	10	610	14	22
Lancashire	..	..	..	..	544	7	522	13	22
Wensleydale		..	..	..	570	12	570	9	0
Derby sage	..	..	..	..	725	8	721	17	4
Blue Stilton		..	..	..	1144	28	1200	33	56
Over-all mean		..	..	..	653	—	658	—	16
$r = 0.996$ ($p > 0.10$)									

* Mean of six determinations.

Reference electrode filling solution (inner), 90-00-02. Supplied by MSE Scientific Instruments.

Reference electrode filling solution (outer), 0.5% m/V ammonium chloride. A 0.5-g amount of NH_4Cl was weighed into a 100-ml calibrated flask and dissolved in and made up to the mark with water.

Sodium standard solutions. Sodium chloride was oven-dried at 250–350 °C for 2 h. An accurate mass of 2.542 g of the dried salt was dissolved in water, transferred quantitatively into a 1-l calibrated flask, made up to the mark with water and mixed well. Working standard solutions were prepared from this stock solution by diluting 10 ml to 1 l and 100 ml to 1 l to give concentrations of 10 and 100 p.p.m. of sodium, respectively.

Procedure

Calibration

The double junction reference electrode was filled with inner and outer solutions and kept in the storage solution together with the sodium electrode between usages. This is necessary to maintain electrode response. Between each measurement the electrodes were thoroughly rinsed using the electrode rinse solution. They were blotted dry with tissue rather than wiped dry. For fatty deposits the electrodes were first rinsed with acetone and blotted dry. The meter was calibrated with working standard solutions for the required range of concentrations, *i.e.*, 10–100 or 100–1000 p.p.m. of sodium. An 8-ml aliquot of buffer was added to 200 ml of working standard solution and the electrode potential of the mixture measured after 5 min. Calibration was carried out periodically during analysis to check for variation in temperature and electrode response.

Determination of Sodium

All determinations were carried out in duplicate unless stated otherwise.

Reference method A

Samples were dry-ashed and hydrochloric acid extracts analysed for sodium by atomic absorption spectrometry as described by Florence *et al.*[9]

Reference method B

Samples were analysed for salt content by Volhard titration according to the British Standards.[10,11] Sodium concentrations were calculated from the results for salt content.

Table 3. Determination of sodium in butter: comparison of ion-selective electrode (ISE) with reference methods A (AAS) and B (British Standard, BS)

| | | | | Sodium/mg per 100 g | | |
Butter sample No.				ISE	AAS	BS
1	..	..	..	766	722	731
2	..	..	..	440	413	428
3	..	..	..	684	627	652
4	..	..	..	782	711	715
5	..	..	..	935	824	857
6	..	..	..	943	865	896
7	..	..	..	959	890	892
8	..	..	..	896	800	849
9	..	..	..	519	450	452
10	..	..	..	582	545	534
11	..	..	..	880	799	833
12	..	..	..	1089	1023	1053
Over-all mean		..	..	790	722	741

Cheese

The meter was calibrated for the range 10–100 p.p.m. of sodium. A Cheddar cheese sample was prepared according to the British Standard.[10] A 2 g ($\pm$ 50 mg) aliquot of the prepared sample was weighed to the nearest milligram and placed in the blender jar together with 8 ml of buffer solution and 198 ml of water. This volume of water was obtained by withdrawing 2 ml by pipette from a 200-ml volume in a calibrated flask. The mixture was blended at high speed for 1 min and then transferred into a 400-ml beaker. A magnetic PTFE-coated follower was added and the beaker placed on a magnetic stirrer. A gentle vortex was induced and the electrodes plus thermometer immersed in the mixture. The temperature was noted and, when necessary, adjusted to ± 1 °C of the temperature measured during calibration. The direct read-out of sodium concentration (p.p.m.) was taken after 5 min. A preliminary investigation determined that the electrode time response to reach equilibrium was between 4 and 5 min.

Butter

A sample for analysis was prepared according to the British Standard.[12] A 5 g ($\pm$ 50 mg) aliquot was weighed to the nearest milligram and 95 ml of water were added. The mixture was warmed to approximately 50 °C to liquefy the fat completely, mixed and cooled to ambient temperature. A 4-ml aliquot of buffer was added and mixed in thoroughly. The mixture was analysed for sodium content as for cheese but with the meter calibrated in the range 100–1000 p.p.m. of sodium.

Table 4. Determination of sodium in butter: statistical analysis of results found by ion-selective electrode (ISE) and reference methods A (AAS) and B (BS)

Method	Mean sodium concentration/mg per 100 g	Differences between methods*/mg per 100 g	
AAS	722	—	
ISE	790	+67 ($p < 0.001$)	—
BS	741	+19 ($p < 0.01$)	−49 ($p < 0.001$)
Method		AAS	ISE

* Mean differences in results of analysis on twelve butters.

Table 5. Comparison of methods for the determination of sodium in foods

Food	Sodium/g per 100 g		
	ISE	AAS	BS
All-bran	1.08	1.35	1.10
Custard powder	0.35	0.36	0.34
Self-raising flour	0.52	0.58	0.24
Gravy powder	9.09	8.85	9.67
Oxo	9.98	10.26	9.80
Salad cream	1.02	1.04	1.08

Other salted foods

All measurements were made with the meter calibrated in the range 100–1000 p.p.m. of sodium.

For water-soluble foods of high sodium content ($\geq 9\%$) a 2-g aliquot was weighed to the nearest milligram and dissolved in 1 l of water. A 200-ml aliquot of this solution was treated and analysed as for cheese.

For water-soluble foods of lower sodium content a 6-g aliquot was weighed to the nearest milligram into a 200-ml calibrated flask and dissolved in and made up to the mark with water. This solution was treated and analysed as for cheese.

For cereal-type foods a 5-g aliquot was weighed to the nearest milligram and blended at high speed for 1 min in 195 ml of water and 8 ml of buffer solution. This mixture was analysed as for cheese.

Results and Discussion

Tests were carried out to determine the recovery of sodium added to cheese. Various concentrations of sodium were added to Cheddar cheese samples prior to blending. The results are given in Table 1. The recovery is good over the concentration range used (10–100 p.p.m. of sodium). Test samples of nine varieties of cheese selected for a wide range of sodium content were prepared for analysis according to the British Standard.[10] Six replicates of each sample were analysed for sodium content by the proposed ion-selective electrode method and reference method A. The results are given in Table 2. There is good agreement between results for all cheeses with a high correlation coefficient (0.996).

Test samples of twelve varieties of butter were analysed for sodium content by the proposed ion-selective electrode method and by reference methods A and B. The results are given in Table 3. There is an obvious bias where values measured by the electrode method are higher than those found by both reference methods. A statistical analysis of the results is given in Table 4. There is reasonable agreement between the results for the reference methods, as expected. However, the electrode results are 9% and 7% higher than the reference method A and B results, respectively. The electrode response time for butter was not as definitive as for cheese and it was difficult to elect a point of stability. This needle drift may be associated with enhanced activity of the free sodium

ion in presence of oil. This was tested by measuring sodium in total butter mixtures and in oil-free filtrates. A test sample was analysed as such in quadruplicate. The test sample was also analysed by reference method B. Mean values of 691 (s.d. 13) and 682 (s.d. 10) mg of sodium per 100 g of butter were found for total butter mixtures and oil-free filtrates, respectively. Reference method B gave a value of 642 mg of sodium per 100 g of butter. The electrode results are 8 and 6% higher than this value for total butter mixtures and oil-free filtrates, respectively.

A selection of salted foods was analysed by the proposed electrode method and by reference methods A and B. The results are given in Table 5. There is good agreement between the results of the three methods for all foods, with the exception of the result for the analysis of all-bran by reference method A (AAS), which is 25% higher than that found by electrode measurement. The sodium content of self-raising flour is dependent on the level and nature of the raising agent used and the low analytical value found by reference method B (British Standard) is associated with salt content only.

Conclusion

A rapid and inexpensive method has been developed for the determination of sodium in cheese, butter and other salted foods. The total time of analysis is ≤ 10 min. Good precision was achieved for a variety of cheese and other salted foods with reference to a standard method. For butter a positive bias was found ($\leq 9\%$) for measurements made with the ion-selective electrode over those using the reference method.

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SHORT PAPERS

Gas Chromatographic Identification of Adamantanes in Some Iraqi Crude Oils

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Adamantane was determined in three Iraqi crude oils using adductive crystallisation with thiourea and capillary gas chromatographic and infrared spectrometric methods. The amount of adamantane determined in these crude oils was 1–3 p.p.m. *m/m*. Certain derivatives of adamantane were also identified from their retention indices calculated from literature data.

Keywords: *Adamantanes identification; crude oil; thiourea; gas chromatography*

Adamantane, tricyclo[3.3.1.1^{3,7}]decane and some of its homologues were first isolated from petroleum fractions by adductive crystallisation with thiourea.[1–4] Recently, because of their importance as active fuels for jet engines, thermostable plastics, insecticides and fungicides,[5–9] adamantane and its derivatives, which occur in trace amounts in crude oil (1–300 p.p.m.), have been synthesised.[10] Generally, thiourea forms complexes with branched-chain and cycloalkane hydrocarbons, but does not form complexes with straight-chain compounds, aromatics and terpenes.[11,12]

Adductive crystallisation with thiourea is a well known selective method for the isolation and determination of adamantane and its homologues in petroleums of various origins and compositions.[4] Some polyakylated adamantanes that have been identified by other methods, for example thermal diffusion,[13] do not pass into the interstices of the crystal lattice of the thiourea. In all instances, a gas - liquid chromatographic (GLC) methods have been used to calculate the amount of adamantane and its homologues in crude oils.

In this work, adamantane and some of its derivatives were identified in three Iraqi oils using adductive crystallisation with thiourea and capillary GLC, with confirmation by infrared spectroscopy.[18–20]

Experimental

The 150–250 °C fractions of East Baghdad, Kirkuk and Qayara crude oils were filtered through 2 g of 35–70 mesh silica gel. The mass of each fraction obtained (170 g) was mixed with thiourea and methanol in the ratio of 10 : 1 : 1 *m/m* and the mixture was stirred at room temperature for 2 h.

The adduct was filtered through a Buchner funnel, washed with a few millilitres of cold pentane and dried by air suction. It was then transferred into a flask containing 300 ml of

distilled water and 100 ml of the mixture were distilled over.[2] The oily distillate was separated from the water and dried with anhydrous calcium chloride. The adamantanes in each extract were examined using capillary GLC and IR spectroscopy.

A Pye Unicam Model 4500 gas chromatograph, equipped with a flame ionisation detector and an SP 4100 computing integrator, was used. The stainless-steel columns were 100 m × 0.25 mm i.d. and coated with squalane or Apiezon L (Pye Unicam). The column temperatures were 110 and 140 °C, respectively. Helium was used as a carrier gas with a flow-rate of 1 ml min$^+$ and a splitting ratio of 1 : 50. The temperature of the flame-ionisation detector was 250 °C. Samples (10^{-3} ml) were injected with a Hamilton syringe. The infrared spectrophotometer was a Model SP3-300. All calculations of retention indices were carried out on a Hewlett-Packard 97 processing calculator.

A reference sample of adamantane was obtained from Fluka.

Results and Discussion

The masses of the 150–250 °C fractions and extracts and the amounts of adamantane in the crude oils are shown in Table 1. A calibration graph of peak height *versus* concentration of standard adamantane was found to be linear and was used to

Table 1. Amount of adamantane in crude oil samples

Crude oil	Mass of 150–250°C fraction in crude oil, %	Mass of extract/g	Extract in crude oil, % *m/m*	Adamantane in the extract, % *m/m*	Adamantane in the crude oil, p.p.m.
East Baghdad	19.3	6.3	0.67	0.851	3.59
Kirkuk	18.5	4.5	0.49	0.961	2.12
Qayara	7.5	4.5	0.23	1.482	1.53

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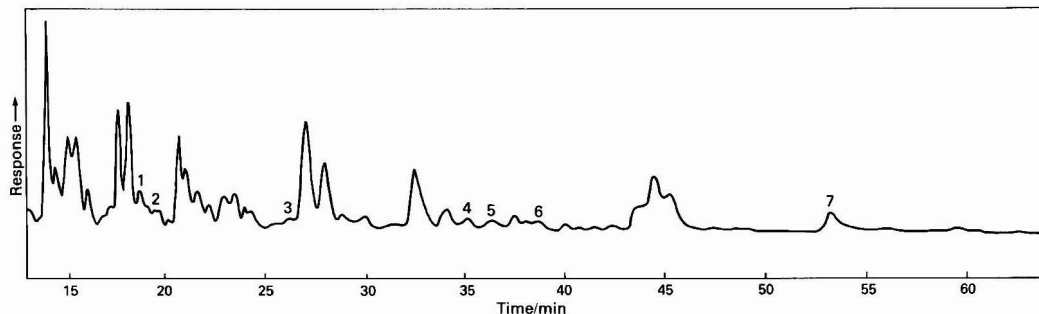
Fig. 1. Chromatograms of the thiourea extract from East Baghdad crude oil. Peak numbers as in Table 2

Table 2. Retention indices of some adamantane derivatives identified in the extract

		Retention indices			
		Squalane (110 °C)		Apiezon L (140 °C)	
Peak No.	Compound	Calculated	Literature	Calculated	Literature
1	Adamantane	1095.2	1095	1162.2	1162
2	1-Methyladamantane	1115.3	1116	1178.9	1179
3	2-Methyladamantane	1176.2	1175	1243	1242
4	1-Ethyladamantane	1241.9	1240	1307	1309
5	Homoadamantane	1235.2	1234	1312.9	1313
6	2-Ethyladamantane	1262	1262	1328.9	1329
7	Tetracyclododecane	1299.8	1300	1388	1386
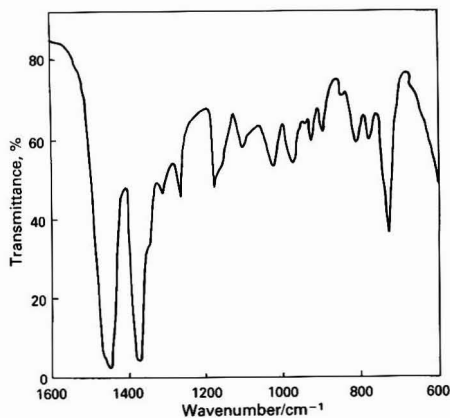
Fig. 2. Infrared spectra of the thiourea extract from East Baghdad crude oil

calculate the amount of adamantane in the crude oils. A chromatogram for one of the isoalkane - naphthenic extracts is shown in Fig. 1. From Fig. 1 it is clear that the extract consists of many mono-, bi-, tri- and polycyclic naphthenes together with some polycyclic aromatics, but the homologues of adamantane are the dominant compounds. To identify some of these homologues, literature data for the elution times of adamantane and its derivatives were employed to calculate Kovát's retention indices.[14-17] From the mean adjusted retention times obtained from three measurements, the compounds listed in Table 2 were identified. An infrared spectrum is shown in Fig. 2. An absorption near 1100 cm^{-1} is typical for adamantane and its derivatives, corresponding to a deformation vibration of CH in the top part of the adamantane nucleus.

Conclusion

This study has shown that adductive crystallisation using thiourea can be used for the isolation and determination of adamantane and its homologues in Iraqi crude oils of various composition.

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Separation and Determination of Food Colours in Pharmaceutical Preparations by Column Chromatography

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Food colours were extracted from pharmaceutical preparations on an Amberlite XAD-2 resin. From the food colours retained, selective elutions of disulphonic acid derivatised (DSD) and trisulphonic acid derivatised (TSD) food colours were carried out using two different strengths of methanolic sulphuric acid. A quantitative determination of these separated food colours was carried out spectrophotometrically.

Keywords: *Food colours separation and determination; column chromatography; spectrophotometry; pharmaceutical preparations*

There are many methods[1-9] available for the separation of food colours, of which adsorption chromatography[1-4] has been the most widely used. In the past, Scoggins and Miller[8] have separated monosulphonic acid derivatives from disulphonic acid derivatives and sulphuric acid using an Amberlite XAD-2 resin. They have also reported that 4-biphenylsulphonic acid is strongly held by the resin and is only eluted with methanol. This resin separates out water-soluble organic compounds, and because food colours are water-soluble sulphonic acid derivatives, their separation can be achieved on this resin. Previously, Takayoshi *et al.*[9] used a pre-coated resin with triethylammonium hydrogen carbonate for retaining the food colours.

In this work it was observed that food colours were retained by the column bed only when the eluent was neutral; under acidic conditions the food colours were not retained by the column. Two different concentrations of methanolic sulphuric acid were used for the selective elution of disulphonic acid derivatised (DSD) and trisulphonic acid derivatised (TSD) food colours and quantitative determinations were subsequently carried out spectrophotometrically.

Experimental

Materials and Solutions

Amberlite XAD-2 resin, 20–50 mesh size. Fluka.
Glass-distilled water.
Methanol. Analytical-reagent grade.
Sulphuric acid, 95–98%. Analytical-reagent grade.
Food colours. Available commercially and listed in Table 1.
Standard food colour solutions. Prepared in distilled water to contain 100 µg ml^{-1} of each food colour.
Solutions of liquid samples. A known amount of liquid was taken to contain the equivalent of 100 µg of food colour.
Solutions of solid samples. Prepared by dissolving in water a known amount of solid (tablet or dry powder) containing the equivalent of 100 µg ml^{-1} of food colour.
Eluent A, 0.1% sulphuric acid in methanol.
Eluent B, 4.0% sulphuric acid in methanol.

Apparatus

Spectrophotometer. A Hitachi Model 220S UV - visible spectrophotometer was used.
Column. A glass column of 600 × 10 mm i.d. was fitted with a small glass-wool plug at the bottom and then 30 ml of resin, suspended in methanol, were poured into the column. The column bed was washed with water to remove any methanol present in the column and to make the column bed neutral.

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Procedures

Separation

A sample containing the equivalent of 100 µg of food colour was poured on to the neutral column bed. The column was first washed with water to remove other unretained pharmaceutical excipients. A coloured band was visible at the top of the column bed, owing to the retention of the food colours. The separation of DSD and TSD food colours was then achieved using two different eluents (0.1 and 4.0% sulphuric acid in methanol). The DSD food colours were eluted from the column with eluent A. During this wash TSD moved slowly but its zone was distinctly separated from the DSD food colour zone. All the DSD food colour zone was collected for spectrophotometric determination. The TSD food colour was eluted with eluent B and also collected for quantitative determination.

Table 1. Categorisation of food colours and their ionic groups

Food colour	Colour Index (C.I.) No.	Ionic groups
Trisulphonate compounds—		
Amaranth	16 185	$-SO_3Na(3), -OH$
Ponceau 4R	16 255	$-SO_3Na(3), -OH$
Disulphonate compounds—		
Fast Red	16 045	$-SO_3Na(2), -OH$
Sunset Yellow FCF .	15 985	$-SO_3Na(2), -OH$
Carmoisine	14 720	$-SO_3Na(2), -OH$
Brilliant Blue.. ..	42 090	$-SO_3Na(2), -SO_3^- = \overset{+}{N} =$
Tartrazine	19 140	$-SO_3Na(2), -OH, -COONa$

Table 2. Percentage recoveries of food colours from an Amberlite XAD-2 column

Food colour	λ_{max}/nm	Recovery, %
Trisulphonate compounds—		
Amaranth	520	99.2
Ponceau 4 R	510	100.1
Disulphonate compounds—		
Fast Red	526	97.2
Sunset Yellow FCF	482	98.3
Carmoisine	510	97.6
Brilliant Blue	627	98.6
Tartrazine	430	99.9

Table 3. Amount of food colours obtained from pharmaceutical products

Sample	DSD food colour	Amount found, %	TSD food colour	Amount found, %
Liquids—				
A	Caramel	96.1	Amaranth	98.3
B	Sunset Yellow FCF	98.8	Ponceau 4R	101.2
C	—	—	Amaranth	100.6
D	Brilliant Blue	99.4	—	—
Tablets—				
A	Tartrazine	100.3	—	—
B	Sunset Yellow FCF	101.2	—	—
Dry powder—				
A	Sunset Yellow FCF	99.6	—	—

* This percentage was calculated from the amount of food colour added to the product and the amount obtained experimentally.

Spectrophotometric determination

The absorbance of each food colour was measured at their wavelengths of maximum absorption, $\lambda_{max.}$, which are given in Table 2. The amounts of food colours in pharmaceutical preparations were calculated by comparing the percentage recoveries obtained with those obtained using a standard solution (containing a known amount of food colour) eluted in the same way.

Results and Discussion

This method was developed to provide a routine fast and efficient method for determining food colours in different pharmaceutical preparations. Food colours are present in small amounts in pharmaceutical preparations and hence quantitative determinations can be achieved by retention on a column with selective elution. The column used was re-usable and most of the pharmaceutical excipients did not interfere, except caramel, which was eluted together with DSD, but not TSD food colours. Erythrosin was separated from TSD food colours, but it produced a colour change under acidic conditions and so a quantitative determination of this compound was not carried out.

Recovery experiments for each food colour were carried out individually and also with a mixture of each DSD and TSD food colour. The recovery was more than 95% in all instances as shown in Table 2. The amounts of food colours in the pharmaceutical preparations were calculated against stan-dards. Generally, the amounts of food colours were not indicated on the label on the pharmaceutical preparations. Hence only with our products was the percentage of each food colour compared with the stated amounts and the results are shown in Table 3.

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Spectrodensitometric Analysis of Penicillin V Benzathine Oral Suspension

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A rapid spectrodensitometric method is described for the determination of penicillin V in penicillin V benzathine oral suspension. Penicillin V is separated from benzathine, common additives and closely related degradation products on HPTLC-F_{254} plates using acetone - chloroform - acetic acid $(10 + 9 + 1)$. Quantitative determinations are made using a spectrodensitometer at 230 nm. The proposed method is more sensitive than the official method.

Keywords: *Penicillin V determination; spectrodensitometry; penicillin V benzathine oral suspension*

The iodimetric assay is one of the official methods for the analysis of penicillin V benzathine and its formulations.[1] However, it has been reported that benzathine reacts with iodine, resulting in reduced thiosulphate titres and values for the penicillin V content that are 5–10% too low.[2] Lebelle *et al.*[3] reported a method for the determination of penicillin V in oral suspensions using HPLC with 53% *V/V* methanol in phosphate buffer as the mobile phase and 1,3,5-trimethoxybenzene as an internal standard. Manni *et al.*[4] reported a method for the determination of penicillin G on silica gel plates using acetone - chloroform - glacial acetic acid $(10 + 9 + 1)$, which measured the intensity at 230 nm in the reflectance mode.

The purpose of this study was to develop a simple, rapid and specific spectrodensitometric method for the determination of the penicillin V content in penicillin V benzathine after separation from closely related degradation products and benzathine on HPTLC-F_{254} plates using the Manni *et al.* method.

Experimental

Apparatus

A Carl Zeiss KM$_3$ chromatogram spectrophotometer and a Transferpettor displacement micropipette, continuously adjustable to 10 μl, were used.

Materials

Penicillin V potassium solution, 0.1%. Prepared by dissolving penicillin V obtained from Grünental in the minimum volume of distilled water and diluting to volume with methanol.

Penicillin V benzathine solution, 0.1% *in methanol.* Prepared from penicillin V benzathine obtained from Biochemie.

Ospen 200 syrup labelled to contain 200 000 IU per 5 ml *of penicillin V benzathine (corresponding to* 120 mg *of penicillin V acid).* Obtained from Biochemie.

HPTLC-F_{254} plates, 10 × 10 cm^2. Obtained from Merck.

Penicilloic and penillic acids. Prepared by standard methods.[5]

Methods

Analysis of penicillin V benzathine

Aliquots of 1 μl of standard penicillin V benzathine equivalent to 1–10 μg of penicillin V were transferred, by micropipette, on to HPTLC-F_{254} plates scored into 1 cm lanes. Alternate lanes were spotted, permitting the use of reference lanes for each sample lane. The solvent system used for development

was acetone - chloroform - acetic acid $(10 + 9 + 1)$. All plates were developed for about 7 cm from the base line (about 15 min). The spots were air-dried, and measured at 230 nm in the reflectance mode at an attenuation output of 0.2 A full scale. The concentrations of penicillin V in penicillin V benzathine were calculated from calibration graphs prepared by the same procedure using standard penicillin V solutions.

For Ospen oral suspension

The powder content of the bottle was well mixed. An accurately weighed amount, equivalent to 10–100 mg of penicillin V, was placed in 10-ml calibrated flasks and about 5 ml of methanol were added. The flasks were swirled to disperse the suspension. Methanol was added to make up to volume and insoluble materials were allowed to settle. The method was continued as described under *Analysis of penicillin V benzathine.*

Results and Discussion

The proposed method has been used for efficiently separating penicillin V from benzathine, various common excipients and

Table 1. R_F values of penicillin V, various common excipients and penicillin V degradation products

Compound	R_F value
Penicillin V	0.44
Benzathine	0.71
Sodium benzoate	0.64
Methyl paraben	0.60
Penicillin V penicilloic acid	0.35
Penicillin V penillic acid	0.38

Table 2. Determination of penicillin V in penicillin V benzathine using the proposed method and the official BP method[7]

| Penicillin V taken/mg | 1973 BP method | | Proposed method | |
	Penicillin V found*/mg	Recovery, %	Penicillin V found/mg	Recovery, %
4.00	4.07	101.75	4.01	100.75
5.00	5.05	101.00	5.00	100.00
6.00	6.03	100.50	5.98	99.66
8.00	8.00	100.00	8.00	100.00
10.00	10.21	102.10	10.10	101.00
Mean ($P = 0.05$)		101.13 ± 0.83		100.18 ± 0.48
N		5		5
T				2.12 (2.31)
F				2.96 (6.39)

* Average of three determinations.
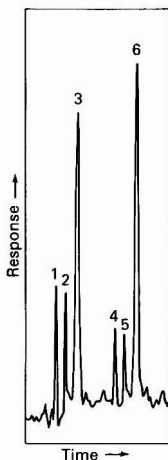
Fig. 1. Spectrodensitometer chromatogram of: 1, penicillin V penicilloic acid; 2, penicillin V penillic acid; 3, penicillin V; 4, methyl paraben; 5, sodium benzoate; and 6, benzathine

Table 3. Determination of the precision of the proposed method on penicillin V benzathine oral suspension

Penicillin V benzathine taken/mg	Penicillin V benzathine found/mg	Recovery, %
2.00	2.00	100.00
3.00	3.02	100.66
4.00	3.98	99.50
6.00	6.02	100.33
7.00	6.96	99.43
8.00	8.00	100.00
9.00	9.04	100.44
10.00	10.06	100.60
	Mean ($P = 0.05$)	100.12 ± 0.33
	N	8
	Standard deviation	0.471
	Standard error	0.166

penicillin V degradation products (Fig. 1). The R_F values for penicillin V lie within the range 0.25–0.75, which is desirable for quantitative spectrodensitometric analysis[6] (Table 1).

The minimum detectable concentration is 1 µg µl^{-1} for penicillin V. The statistical equation relating peak area to concentration is

$$Y = 1345.92 + 22419.21x \qquad r = 0.999$$

where Y is the peak area by integration, X is the concentration in micrograms and r is the correlation coefficient.

The suggested procedure was applied to the determination of penicillin V in penicillin V benzathine and the results obtained were compared with the official iodimetric method. A statistical analysis of the results shows good agreement

Table 4. Determination of penicillin V in penicillin V benzathine oral suspension using the proposed method and the official BP method[7]

	1973 BP method		Proposed method	
Penicillin V taken/mg	Penicillin V found*/mg	Recovery, %	Penicillin V found*/mg	Recovery, %
5	5.06	101.2	5.02	100.4
6	6.04	100.66	5.98	99.66
8	8.10	101.25	8.05	100.62
9	9.045	100.5	9.074	100.82
10	10.15	101.5	10.15	101.5
Mean ($P = 0.05$)		101.02 ± 0.4		100.6 ± 64
N		5		5
T				1.18 (2.31)
F				2.49 (6.39)

* Average of three determinations.

between the suggested method and the official method with respect to accuracy and precision (Tables 2 and 3).

Penicillin V benzathine oral suspension was analysed by the suggested method and the results obtained reveal that the suggested procedure is as accurate as the official method[7] (Table 4).

From the above study, it can be concluded that the proposed method is more sensitive (*i.e.*, up to 1 µg) than the iodimetric method and in addition it is more selective as it depends on the measurement of the intact penicillin molecule and not its hydrolytic products. Therefore, it can be used as a stability-indicating assay. Moreover, from the results obtained it has been shown to be reproducible, in contrast to the iodimetric method, which suffers from problems due to the lower pH.[8]

In addition the proposed method is more sensitive than the HPLC method, the sensitivity ranges being 1–10 and 10–40 µg, respectively. A further advantage over the HPLC method is that smaller volumes of solvent are required.

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Simultaneous Spectrophotometric Determination of Strychnine and Yohimbine in Pharmaceutical Preparations

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A spectrophotometric method is proposed for the simultaneous spectrophotometric determination of strychnine.HCl and yohimbine.HCl in pharmaceutical preparations. No preliminary separation step is required. The proposed method involves the use of the modified Vierordt equation. Accurate and reproducible results were obtained. The mean recoveries (six determinations) for strychnine.HCl and yohimbine.HCl were 98.8 ± 0.5% and 100.3 ± 0.8%, respectively. The method was applied to the determination of the two compounds in dosage forms and the results obtained were accurate and precise.

Keywords: *Strychnine determination; yohimbine determination; pharmaceuticals; spectrophotometry*

Strychnine, the main alkaloid obtained from the seeds of *Strychnos nux-vomica*, is a widely prescribed drug and several methods have been reported for its quantitation. Titrimetric methods include non-aqueous,[1] complexometric,[2] potentiometric,[3] amperometric[4] and radiometric titration.[5] Numerous spectrophotometric methods have also been described.[6-9] Chromatographic methods include paper,[10] gas[11] and high-performance liquid chromatography.[12]

Yohimbine is the principal alkaloid of the bark of the yohimbe tree, *Pausinystalia yohimbe*. Comparatively few methods have been described for the determination of yohimbine, *viz.*, non-aqueous titration,[1] paper chromatography,[13] gas chromatography[14] and fluorimetry.[15] Strychnine and yohimbine are frequently co-formulated in aphrodisiac and nerve tonic preparations, but the analysis of such mixtures has not been reported in the literature. It was therefore desirable to develop a procedure for the analysis of mixtures of the two compounds in dosage forms. The method proposed here depends on the use of a modified Vierordt equation as developed by Glenn,[16] and the two compounds can be determined simultaneously without prior separation. The method was applied successfully to the determination of the compounds in commercially available and laboratory-made preparations. The interferences produced by other ingredients, if present, could be overcome by simple chemical means. The results obtained are accurate and precise.

Experimental

Apparatus

A Pye Unicam SP 1800 spectrophotometer was used.

Materials

Yohimbine hydrochloride (yohimbine.HCl) and strychnine hydrochloride (strychnine.HCl). Chemically pure and satisfying the requirements of the Swiss Pharmacopoeia.[1]

Pharmaceutical preparations. Preparations containing the two drugs were obtained from commercial sources or were laboratory made.

Procedure

Authentic compounds

Prepare separate stock solutions containing 1.0 mg ml⁻¹ of strychnine.HCl and yohimbine.HCl in 0.1 N H₂SO₄. Prepare mixtures of the two compounds, and dilute with 0.1 N H₂SO₄ so that the concentration of strychnine.HCl is in the range

0.4–3.2 mg per 100 ml and that of yohimbine.HCl is in the range 0.8–4.8 mg per 100 ml. Measure the absorbance as described below.

Tablets containing strychnine.HCl and yohimbine.HCl

Weigh and pulverise 20 tablets. Extract an accurately weighed amount of the powder with three 30-ml volumes of water, filter, combine the filtrates and dilute to 100 ml. Transfer an aliquot of the filtrate into a 100-ml calibrated flask so that the concentrations of the compounds are in the ranges indicated above. Dilute to the mark with 0.1 N H₂SO₄. Measure the absorbance as described below.

Ampoules containing strychnine.HCl and yohimbine.HCl

Mix the contents of 20 ampoules in a 100-ml calibrated flask and dilute to the mark with water. Transfer an aliquot into a 100-ml calibrated flask so that the concentrations of the compounds are in the ranges indicated above. Dilute to the mark with 0.1 N H₂SO₄. Measure the absorbance as described below.

Calculation

Calculate the concentrations of the compounds from the equations

$$c_x = \frac{A_1}{\alpha_1}[(b - m)/(b - a)]$$

and

$$c_y = \frac{A_2}{\beta_2}[b(m - a)/m(b - a)]$$

where c_x and c_y are the concentrations of strychnine.HCl and yohimbine.HCl, respectively, in grams per 100 ml, *A* is the

Table 1. Application of the modified Vierordt method to the determination of strychnine.HCl and yohimbine.HCl in mixtures

	Added/mg per 100 ml		Recovery, %	
No.	Strychnine. HCl	Yohimbine. HCl	Strychnine. HCl (255 nm)	Yohimbine. HCl (272 nm)
1	1.2	0.8	99.5	101.8
2	1.6	1.6	98.5	100.6
3	2.0	1.2	99.0	100.7
4	0.8	2.0	99.1	99.9
5	1.5	4.5	98.5	99.2
6	0.5	3.0	99.1	99.7
			Mean = 99.0 ± 0.5	100.3 ± 0.8

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Table 2. Application of the proposed method to the determination of strychnine.HCl and yohimbine.HCl in dosage forms

No.	Composition	Recovery, %*	
		Strychnine.HCl	Yohimbine.HCl
1	Strychnine.HCl 0.5 mg + yohimbine.HCl 3 mg per tablet	101.6 (0.5)	101.3 (0.5)
2	Strychnine.HCl 1.5 mg + yohimbine.HCl 4.5 mg + monoethyl arsenate 4 mg per ampoule	99.6 (1.0)	100.2 (0.7)
3	Strychnine.HCl 0.5 mg + yohimbine.HCl 3 mg + zinc phosphide 4 mg per tablet	100.5 (0.4)	98.7 (0.3)

* The results given are averages of six separate determinations. The figures in parentheses are relative standard deviations.

absorbance of a 1-cm layer of the measured solution and represents the values of $A_{1\,cm}^{1\%}$ for strychnine.HCl and yohimbine.HCl, the subscripts 1 and 2 refer to 255 and 272 nm, respectively, $m = A_2/A_1$, $a = \alpha_2/\alpha_1$ and $b = \beta_2/\beta_1$.

Discussion

The modified Vierordt method, as developed by Glenn,[16] was adopted for the simultaneous spectrophotometric determination of strychnine and yohimbine in pharmaceutical preparations.

Derivation of the Equations

The method whereby a mixture of two absorbing substances A and B may be determined was first applied by Vierordt,[17] and involves measurement of molar absorptivities at a pair of suitable wavelengths. The concentrations of A and B can be determined as follows:

$$A_1 = \alpha_1 C_A + \beta_1 C_B \quad .. \quad .. \quad .. \quad (1)$$

$$A_2 = \alpha_2 C_A + \beta_2 C_B \quad .. \quad .. \quad .. \quad (2)$$

where the subscripts 1 and 2 refer to wavelengths, A denotes the molar absorptivity of a 1-cm layer of the solution of the mixture, C_A and C_B are the concentrations of A and B and α and β are their molar absorptivities.

The formulation of equations (1) and (2) in terms of ratios of molar absorptivities was achieved by Glenn[16] by substituting the expressions $m = A_2/A_1$, $a = \alpha_2\alpha_1$ and $b = \beta_2\beta_1$ into equation (2). Noting that m refers to the mixture, a to substance A and b to substance B, this leads to the following equation:

$$mA_1 = a\alpha_1 C_A + b\beta_1 C_B \quad .. \quad .. \quad (3)$$

which can be solved in the usual way to give

$$C_A = \frac{A_1}{\alpha_1}[(b - m)/(b - a)]$$

and

$$C_B = \frac{A_2}{\beta_2}[b(m - a)/m(b - a)]$$

As is evident from Fig. 1, 255 and 272 nm are suitable wavelengths for the measurement. The results obtained when this method was applied to authentic mixtures are given in Table 1. Mixtures of the two drugs in different proportions were successfully analysed. Special interest was given to mixtures of the two drugs in the medicinally recommended proportions (mixtures 5 and 6). In all instances, accurate results were obtained.

The method also gave accurate and reproducible results when applied to the assay of pharmaceutical preparations (Table 2). Interferences produced by other drugs commonly dispensed with this mixture could be eliminated, e.g., zinc
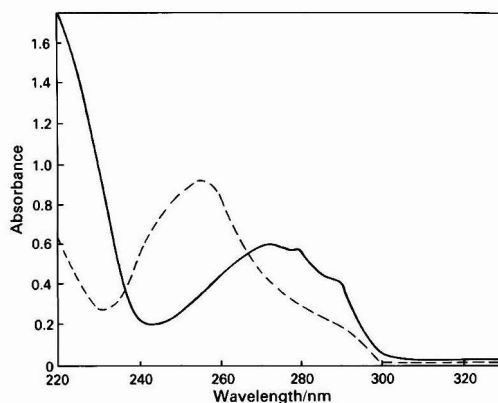
Fig. 1. Absorption spectra for strychnine.HCl (broken line) and yohimbine.HCl (solid line) in 0.1 N H_2SO_4 (3 mg per 100 ml)

phosphide, arsenic oxide and camphor are insoluble in water and can be easily removed. Monoethyl arsenate, although soluble in water, did not affect the results. Tablet excipients such as talc, starch, lactose and magnesium stearate did not interfere.

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Spectrophotometric Determination of Available Copper in Acidic Soils Using 2-(5-Bromo-2-pyridylazo)-5-diethylaminophenol

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A method is described for determining the available copper in acidic soil. The absorbance is measured at 556 nm; the molar absorptivity of the coloured complex is 6.87×10^4 l mol^{-1} cm^{-1} and the Beer - Lambert law is valid for copper concentrations between 0 and 20 µg per 25 ml. The colour of the complex is stable for at least 2.5 h. The ions normally present in acidic soils do not interfere when ammonium citrate, nitroso-R salt and Chugaev's reagent are added as masking agents. Soils from several sources have been analysed by this method, and the results obtained were satisfactory.

Keywords: *Copper determination; spectrophotometry; 2-(5-bromo-2-pyridylazo)-5-diethylaminophenol; acidic soils*

Copper is an important trace element in plants. In China, the recommended method for the determination of available copper in acidic soil is the sodium diethyldithiocarbamate method,[1] after preliminary separation of copper by extraction with 0.1 M hydrochloric acid. However, this method is insensitive and troublesome. Recently, more sensitive methods have been proposed based on azo reagents such as 2-(5-nitro-2-pyridylazo)-1-naphthol,[2] 1-(2-thiazolylazo)-2-naphthol,[3] picramine-epsilon[4] and other diazo derivatives of chromotropic acid[5]; the 1,5-diphenylcarbazide method[6] is also worth noting.

In this paper a simple, sensitive and selective colour reaction for the spectrophotometric determination of copper by means of the copper - 2-(5-bromo-2-pyridylazo)-5-diethylaminophenol (5-Br-PADAP) is described. The available copper can be determined directly in a water - acetone medium, with no need for extraction into an organic solvent.

It has been applied satisfactorily to the determination of available copper in acidic soils from South Jiangxi.

Experimental

Apparatus

The following apparatus was used: a Model 721 spectrophotometer (3rd Analytical Instrument Factory, Shanghai, Peoples Republic of China) and a Model 25 Leichi pH meter (Shanghai Leichi Instruments, Shanghai) equipped with a combined electrode, for measuring the pH.

Reagents

All reagents used were of analytical-reagent grade and obtained from Beijing or Shanghai Chemical Industries Mfg., unless stated otherwise. All water used had a specific conductance of at least 15 Mohm^{-1} cm^{-1}.

Copper standard solution, 100 µg ml^{-1}. Dissolve 196.5 mg of copper(II) sulphate pentahydrate, $CuSO_4.5H_2O$, in 100 ml of 0.1 M hydrochloric acid, and dilute the solution with 0.1 M hydrochloric acid to 500 ml. Dilute the stock solution suitably to give a standard solution containing 2 µg ml^{-1} of copper with the above concentration of hydrochloric acid.

5-Br-PADAP solution, 0.02% *m/V* in 95% *ethanol*.
Triethanolamine (TEA) solution (1 + 2).
Ammonium citrate solution, 50% *m/V*.
Nitroso-R salt solution, 0.1% *m/V*.
Chugaev's reagent, 1% *m/V* in 95% *ethanol*.

General Procedure

Place a volume of the working standard solution corresponding to 10 µg of copper in a 25-ml calibrated flask. Add 8 ml of 0.1 M hydrochloric acid, 1 ml of 3 M ammonia solution, 4 ml of acetone and 4 ml of 0.02% *m/V* 5-Br-PADAP solution. Dilute with water to the mark, and allow to stand for 20 min. Measure the absorbance at 556 nm against a reagent blank, carried through the above-described procedure. Use 1-cm cells.

Results and Discussion

Spectral Characteristics and Effects of Experimental Variables

The absorbance curves of the reagent and its copper complex are shown in Fig. 1. The maximum absorbance of the reagent is at 445 nm, and that of the Cu - 5-Br-PADAP system is at 556 nm. $\Delta\lambda = 91$ nm.

Variation of the pH of the reaction medium revealed that the complex formation remained maximal and constant over the pH range 9.4–10.1. Therefore, a pH value of 9.7 was selected for the procedure.

At the optimum pH, absorbance measurements were carried out by varying the reagent concentration. It was
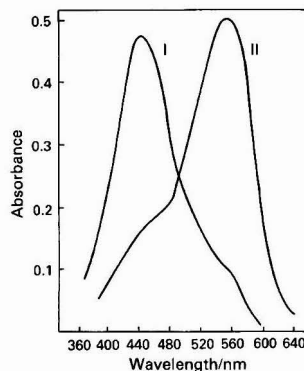
Fig. 1. Absorption spectra of 5-Br-PADAP and its copper complex. I, 5-Br-PADAP (against water as reference), 0.5-cm cell; and II, Cu - 5-Br-PADAP complex (against reagent blank as reference), 20 µg of Cu taken, 0.5-cm cell

Table 1. Results for the determination of available copper in acidic soils

Soil type	Individual results determined, p.p.m.	Average, p.p.m.	Standard deviation, p.p.m.	Coefficient of variation, %	Confidence limits of the mean (at $p = 0.05$)
Red soil	6.30, 6.70, 6.50, 6.70, 6.60, 6.50, 6.70	6.571	0.1496	2.28	6.571 ± 0.139
Alluvial soil ..	9.30, 9.30, 9.30, 9.21, 9.21, 9.30, 9.30	9.274	0.0439	0.47	9.274 ± 0.041
Purple soil ..	8.30, 8.30, 8.10, 8.30, 8.10, 8.30, 8.10	8.214	0.1069	1.30	8.214 ± 0.099
Red soil	6.10, 5.80, 5.90, 5.80, 5.90, 5.70, 5.90	5.874	0.1254	2.13	5.874 ± 0.116

Table 2. Recovery results for copper

Soil type	Source of parent material	Original content in sample/µg	Copper content added/µg	Found/µg	Error/µg	Recovery, %
Red soil	Quaternary Period red clay soil	5.23	2	7.36	+0.13	101.8
			4	9.57	+0.34	103.7
			6	11.25	+0.02	100.2
Alluvial soil ..	River drift	10.2	2	12.5	+0.30	102.5
			4	14.7	+0.50	103.5
			6	16.9	+0.70	104.3
Purple soil	Purple sandstone and shale weathering products	3.65	2	5.80	+0.15	102.7
			4	7.68	+0.03	100.4
			6	9.69	+0.04	100.4
Red soil	Granite weathering product	1.99	2	4.00	+0.01	100.3
			4	6.00	+0.01	100.2
			6	8.20	+0.21	102.6

observed that the addition of more than 3 ml of 0.02% reagent solution was required to obtain a maximum and reproducible absorbance for 10 µg of copper. Smaller amounts gave incomplete complex formation. Therefore, 4 ml of a 0.02% m/V solution of the 5-Br-PADAP were used throughout the study.

The copper - 5-Br-PADAP complex was insoluble in water but dissolved in acetone. The effect of variation in the acetone concentration was examined in the range 1–6 ml. It was found that 3–6 ml were necessary and 4 ml were selected as the most suitable amount.

The minimum time for complete colour development of the complex was found to be 20 min at room temperature (15–30 °C). The absorbance was then stable for at least 2.5 h.

Beer's Law

Once the optimum conditions for the determination of copper with 5-Br-PADAP were established, the concentration range in which Beer's law is obeyed was studied. This range was between 0 and 20 µg per 25 ml with a calibration graph represented by

$$A = 0.0426x + 0.004$$

where A is the absorbance at 556 nm and x is the concentration of copper (µg per 25 ml). The correlation coefficient was 0.999 94 and the molar absorptivity 6.87×10^4 l mol^{-1} cm^{-1}. Sandell's sensitivity of the colour reaction as calculated from Beer's law was 0.925 ng cm^{-2} of copper at 556 nm for $\log(I_0/I)$ = 0.001.

Composition of the Metal Complex

The equilibrium shift method[7] and Asmus method[8] were employed to establish the composition of the complex. The results indicate the formation of a 1 : 1 complex between the metal and 5-Br-PADAP. Further, this conclusion is supported by the Sanchez method.[9]

Apparent Stability Constant of the Complex

The apparent stability constant of the metal complex was calculated by the method of Garcia-Vargas et al.[10] The value thus obtained was 6×10^6.

Interferences

The effects of some ions that often accompany copper were studied by adding different amounts to 0.4 p.p.m. of copper in solution. The colour was developed as outlined in the procedure. When ammonium citrate, nitroso-R salt and Chugaev's reagent were added as masking agents, the following ions did not interfere (amounts given in mg): Fe(III) (3), Al(III) (5), V(V) (0.1), Sb(III) (1); As(V) (1), Cr(III) (1), Sn(II) (0.5), Ca(II) (0.5), Mg(II) (0.3), Pb(II) (0.1), Ag(I) (0.1), Mo(V) (0.2), Co(II) (0.015), Ni(II) (0.01), Zn(II) (0.02), Mn(II) (0.035) and Cd(II) (0.03).

On the basis of these data, the selectivity of the method is good.

Application

The recommended method has been applied satisfactorily to the determination of available copper in acidic soils.

Recommended procedure

Weigh 5.00 g of air-dried soil sample (crushed to pass a 20-mesh sieve), transfer into a 150-ml conical flask and add 50 ml of 0.1 M hydrochloric acid. Place the flask on a vibrator and shake for 1.5 h, then filter or centrifuge the solution.

Pipette 10 ml of this extract into a 25-ml calibrated flask. Add in sequence, with swirling, 1 ml of TEA (1 + 2), 1 ml of 3 M ammonia solution, 1 ml of ammonium citrate solution (50% m/V), 4 ml of acetone, one drop of 0.1% m/V nitroso-R salt solution and one drop of 1% Chugaev's reagent. Then continue as already described.

Simultaneously, prepare a calibration graph, using the same procedure, with aliquots of the working standard solution for 0–20 µg of Cu.

Results for Soil Samples

The results obtained for soil samples are shown in Table 1. Good precision was observed. As no standard soil samples containing copper were available, the standard additions method was applied and a mean recovery of 101.9% (12 determinations) (Table 2) was obtained.

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Determination of Copper in Wine by Potentiometric-stripping Analysis

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The application of potentiometric-stripping analysis (PSA) to the determination of copper in wine is described. The need for some form of sample pre-treatment is demonstrated and a procedure, based on a nitric acid pre-treatment, has been developed. The results obtained by this method compare favourably with those obtained by atomic absorption spectrometry. The usefulness of the PSA technique for the routine determination of copper in a wine laboratory is discussed.

Keywords: *Copper determination; potentiometric-stripping analysis; nitric acid treatment; wine analysis*

Jagner and Westerlund[1] have proposed a method, based on potentiometric-stripping analysis (PSA), for the determination of cadmium, copper and lead in wine in the concentration range 1–1000 ng ml^{-1}. Their method involves the determination of the trace elements in untreated wine, the only requirement being the addition of sufficient hydrochloric acid to acidify the sample to 0.1 M prior to the analysis. Sample pre-treatment can be omitted, as potentiometric-stripping analysis is essentially free from interference from organic couples and as such has a significant advantage over voltammetric techniques such as anodic-stripping analysis.[1]

In the work of Jagner and Westerlund, however, the results obtained for the determination of copper in wine differed from those obtained using atomic absorption spectrometry by up to 15%. The accurate determination of the copper content is an essential part of good wine making practice, as too high a level of copper can cause an increase in the rate of oxidation of the wine and the precipitation of a "protein haze."[2] This study was undertaken in an attempt to improve the agreement between PSA and AAS and to determine whether the PSA technique could be developed into a suitable procedure for the routine determination of copper in a winemaker's laboratory.

Experimental

Reagents

Hydrochloric acid (Merck, pro analysi grade), nitric acid (Merck, pro analysi grade), mercury(II) chloride (BDH Chemicals, AnalaR grade) and doubly distilled, de-ionised water were used. Standard solutions of copper were prepared from the BDH Chemicals AAS standard (1000 mg l^{-1}).

Instrumentation

A Radiometer ISS820 ion-scanning system was used for all potentiometric-stripping measurements. The basic design of this system has been described elsewhere.[1,3] Atomic absorption measurements were carried out on Hitachi Z6000 (flame) and Z7000 (graphite furnace) atomic absorption spectrophotometers, both instruments employing the polarised Zeeman effect as the method of background correction. Calibration by standard additions was used with both instruments.

Wine Samples

Wine samples were obtained commercially and were used as received. The majority of wines investigated were non-vintage wines, which, for the most part, were retailed only a few months after release.

Sample Pre-treatment

Method I: adjustment of pH only

The wine sample was adjusted to 0.1 M in hydrochloric acid.[1]

Method II: heat pre-treatment with hydrochloric acid

Wine (25 cm^3) and hydrochloric acid (25 cm^3 of 0.2 M) were warmed in a water-bath at 50 °C for 1 h. On cooling, the sample was transferred into a 50-cm^3 calibrated flask and made up to volume using doubly distilled de-ionised water. This solution was used for analysis.

Method III: heat pre-treatment with nitric acid

Wine (25 cm^3) and concentrated nitric acid (0.8 cm^3) were warmed in a water-bath at 50 °C for approximately 30 min. On cooling, the sample was diluted to 50 cm^3 in a calibrated flask with 0.2 M hydrochloric acid. This solution was used for analysis.

Preparation of the Mercury Film Electrode

The procedure described by Mannino[3] was followed.

Analytical Procedure

Using the method described by Jagner and Westerlund,[1] the treated sample was transferred into the electrochemical cell of the ion-scanning system and 1.0 cm^3 of 0.002 M of mercury(II) chloride added. After de-aeration for 15 min, repeat enrichment-stripping curves were recorded using a deposition time of 4 min (8 min for copper levels less than 200 ng ml^{-1}) at a potential of −0.9 V *versus* an SCE. Calibration was achieved by making three standard additions to the electrochemical cell. Copper was oxidised at approximately −0.18 V *versus* an SCE and the stripping plateau was measured as described by Jagner[1] and Mannino.[3]

Blanks were measured using doubly distilled de-ionised water in place of the wine.

Results and Discussion

An examination of the results given in Table 1 for the direct determination of copper in wine shows that the method proposed by Jagner[1] (*i.e.*, acidification of the wine to 0.1 M with hydrochloric acid) is not capable of detecting the total amount of copper in the majority of wines studied in this work. In fact, the discrepancy between the values found by PSA and AAS approaches 50% in some instances. Obviously, the simple acidification step is not sufficient to liberate all the copper in these wines.

Table 1. Copper content in untreated wine

				Copper content/ng ml⁻¹	
				PSA	AAS
Riesling 1	..	..	..	770	820
Riesling 2	..	..	..	152	147
Riesling 3	..	..	..	52	109
Riesling 4	..	..	..	65	122
Claret 1	..	..	..	210	200
Claret 2	..	..	..	120	110
Claret 3	..	..	..	487	769
Claret 4	..	..	..	787	1079
Shiraz	..	..	..	131	127
Dry red 1	..	..	..	636	786

Table 2. Copper content after HCl treatment

			Copper content/ng ml⁻¹		
			PSA		
			Method I*	Method II†	AAS
Riesling 3	..	..	52	84	109
Riesling 4	..	..	65	110	122
Claret 3	..	..	487	751	769
Claret 4	..	..	576	774	1079
Sweet white 1	..	..	488	632	813

* Method proposed by Jagner.[1]
† After HCl heat treatment.

Following the procedure described by Mannino[3,4] for the measurement of lead and tin in fruit juices and soft drinks using PSA, equal volumes of wine and 0.2 M hydrochloric acid were warmed at 50 °C before the analysis. The results (Table 2) show that whereas this procedure is successful for some wines, it is not capable of releasing the total amount of copper in all samples.

Several problems were also encountered with this method of pre-treatment. At 50 °C, the amount of copper released was dependent on the time of heating, increasing to a maximum after 2 h and falling to zero after 4 h. Long heating periods (up to 4 h) at 40 °C were not sufficient to release all the copper. Boiling the wine with an equal volume of 0.2 M hydrochloric acid caused the formation of a precipitate and the amount of copper detected by PSA decreased to zero. When the wine sample was adjusted to 0.1 M with concentrated hydrochloric acid and the mixture warmed at either 40 or 50 °C, the copper stripping plateau decreased with repeated scans and a constant measure of the amount of copper could not be obtained. The use of hydrochloric acid at other concentrations (e.g., 2 M) did not prove to be of value. The best results with this method of pre-treatment were achieved by taking equal volumes of wine and 0.2 M hydrochloric acid and heating at 50 °C. However, this procedure is suitable for the routine determination of the copper content in certain situations only.

Treatment of the wine with concentrated nitric acid prior to the analysis has proved successful in liberating the total amount of copper (Table 3). In this method of pre-treatment, nitric acid concentrated to 0.5 M in the final solution is added to the wine and the mixture is heated at 50 °C for 30 min. Hydrochloric acid is added to give a concentration of 0.1 M before the enrichment - stripping cycle is commenced. The agreement between the copper found by AAS and by PSA following this pre-treatment procedure is good, the maximum discrepancy being 3%, rather less than the 15% noted by Jagner.[1]

Table 3. Copper content after nitric acid treatment

			Copper content/ng ml⁻¹		
			PSA		
			Method I*	Method III†	AAS
Riesling 5	..	..	452	671	673
Riesling 6	..	..	95	130	134
Dry white 1	..	..	269	472	466
Dry white 2	..	..	44	96	98
Sweet white 1	..	..	488	805	813
Sweet white 2	..	..	566	777	770
Claret 5	..	..	610	743	748
Claret 6	..	..	186	280	288
Dry red 2	..	..	85	137	134
Dry red 3	..	..	86	272	282

* Proposed by Jagner.[1]
† With HNO₃ heat treatment.

Repeatability

The repeatability of the method was tested by performing repeat analyses on various samples of wine. Typically, relative standard deviations were between 2 and 8% (10 runs), similar to those noted by Jagner.[1]

Application of the Method

In the search for a method that would be suitable for the routine determination of copper in wine laboratories, PSA would appear to have many advantages: it is simple, relatively fast and the instrumentation is inexpensive. Further, as PSA is free from interferences from organic couples,[1] there should not be a need for extensive sample pre-treatment, as is required with voltammetric methods.[5,6] Elimination of sample pre-treatment has the added advantage of reducing the possibility of contamination of the sample.

This work has shown that the use of PSA in its simplest form, i.e., adjusting the sample to 0.1 M in HCl, is not always sufficient to liberate all the copper in the wine. The sample must be warmed with nitric acid to ensure that the total copper content can be determined. The need for this sample pre-treatment restricts the widespread application of the technique in wine laboratories. With sample pre-treatment, the time required for each analysis approaches 1 h, which is too long for a routine application.

Some dependence of the results obtained by PSA for the determination of copper in wine on the methods of pre-treatment would be expected from the complexity of the solution matrix and the conclusions reached in this work are in agreement with recent results obtained by voltammetric methods.[7] If precipitation or protein-haze formation has taken place in the wine, then it is probable that some copper will be tightly bound within the precipitate and nitric acid pre-treatment is necessary for its release. In old wines, which show extensive precipitation, approximately half of the total copper content is contained within the precipitate.[8] The nature of this precipitate is, however, not certain. The copper may be deposited as a complex with proteins or peptides,[2] although Coppola[9] argues that the precipitate is essentially copper(I) sulphide formed as a consequence of highly reducing conditions within the wine. Work is continuing in an attempt to determine whether precipitation is the cause of the apparent speciation effect observed in this study.

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Determination of Molybdenum in Steels by Differential-pulse Polarography of the 8-Hydroxyquinoline - Molybdenum(VI) Complex

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The 8-hydroxyquinoline - Mo(VI) complex has a reduction half-wave potential of -0.380 V *vs.* SCE in 1 M $NaNO_3$ - 0.01 M HNO_3. Iron only interferes at concentrations higher than 1.5×10^{-4} M in the sample. This effect can be avoided with acetic acid. A method for the determination of molybdenum in steel based on catalytic reduction of the 8-hydroxyquinoline - Mo(VI) complex is described. A linear calibration is obtained for a concentration range from 10^{-8} to 10^{-6} M. The detection limit is about 10^{-10} M of Mo(VI).

Keywords: *Molybdenum determination; steels; differential-pulse polarography; 8-hydroxyquinoline complex*

Several methods have been described for the determination of molybdenum, the most useful being spectrophotometric analysis of the Mo(SCN) complex[1] after solvent extraction and electrothermal atomic absorption spectrometry.[2] These methods are sensitive but they are subject to interferences. Several polarographic methods for the determination of molybdenum have also been developed, the most sensitive being anodic stripping voltammetry of the hydrate form of molybdenum, pulse polarography with catalytic reduction of nitrate in acidic solution[3,4] and pulse polarography of the 8-hydroxyquinoline - Mo(VI) complex in organic solution after extraction.[5]

In acidic solutions molybdenum gives two waves due to the reactions

$$Mo(VI) + e^- \rightarrow Mo(V) \quad .. \quad .. \quad (1)$$
$$Mo(V) + 2e^- \rightarrow Mo(III) \quad .. \quad .. \quad (2)$$
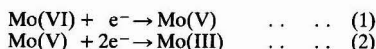
According to Kolthoff and Hodara,[6] in acidic solution containing nitrate, the molybdate ion catalyses nitrate reduction and Mo(V) is reactivated. Under these conditions the polarographic current reaches high values and can be used for analytical applications.

Molybdenum(VI) forms a well known complex with 8-hydroxyquinoline; it has a reduction half-wave potential of -0.30 V *vs.* an Hg pool in isobutyl methyl ketone or ethyl acetate and of -0.46 V *vs.* an Hg pool in chloroform. In nitric acid - sodium nitrate medium the 8-hydroxyquinoline - Mo(VI) complex is reducible and the nitrate reduction process enhances the polarographic current.[7]

This paper describes a direct method for the determination of molybdenum by differential-pulse polarography of the 8-hydroxyquinoline - Mo(VI) complex in nitric acid solution and its application to the determination of molybdenum in different steels.

Experimental

Apparatus

Differential-pulse polarograms were obtained with a Tacussel Model PR G 5 polarograph and a Tacussel EPL 1 B recorder. The polarograph cell (CPRA type) requires a three-electrode system: a dropping-mercury electrode, a platinum counter electrode and a saturated calomel reference electrode.

The differential-pulse polarographic conditions were scan rate 2 mV s^{-1}, pulse amplitude 20 mV, drop time 2 s, initial potential -0.0 mV, final potential -650 mV and current range 125 nA to 2.5 µA. Samples must be deoxygenated for 10 min with pure nitrogen before the polarographic analysis.

Reagents

All chemicals used were of analytical-reagent grade (Merck and Prolabo).

Results

In nitric acid medium containing sodium nitrate the 8-hydroxyquinoline - Mo(VI) complex is reducible and with a supporting electrolyte composed of 1 M sodium hydroxide, 1 M nitric acid (pH 2) and 8-hydroxyquinoline, the polarographic currents reach exceptionally high values. Fig. 1 illustrates the different shapes of the molybdenum peaks in various nitrate
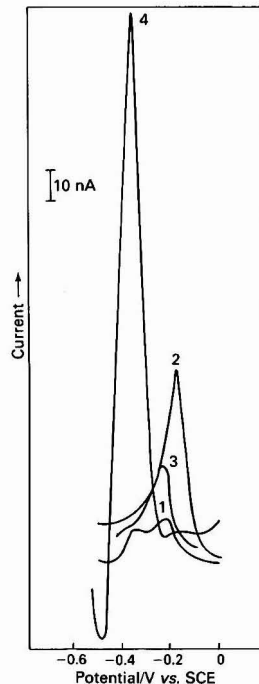
Fig. 1. Differential-pulse polarograms of 10^{-8} M Mo in different supporting electrolytes. 1, 0.25 M HNO_3 - 2 M NH_4NO_3; 2, 1 M HNO_3 - 1 M NH_4NO_3 - 0.1 M NaOH; and 3, 0.1 M H_2SO_4 - 1 M $NaNO_3$. 4, Pulse polarograms of 10^{-9} M Mo in 1 M NaOH - 1 M HNO_3 - 3×10^{-3} M 8-hydroxyquinoline (pH 2.0)

Table 1. Molybdenum contents of steels

Sample			Molybdenum content, p.p.m.	Composition %	Mo found, p.p.m.	Standard deviation, p.p.m.	Relative standard deviation, %
IRSID 012.1	..		20	C 0.082, P 0.083, S 0.255	23.4	0.4	1.7
IRSID 080.1	..		40	C 0.452, P 0.028, Mn 1.116, Cr 0.025, Sn 0.006	40.5	0.4	1.0
IRSID 081.1	..		60	C 0.099, Mn 0.605, Cr 0.012, Ni 0.042	58	2	3.5
IRSID 106.2	..		540	C 0.153, Mn 0.727, Cr 1.03, Ni 1.43	560	10	1.8
IRSID 204.1	..		200	C 0.0888, Mn 0.261, Cr 15.6, Ni 1.606	213	5	2.4
IRSID 206.1	..		100	C 0.019, Mn 1.19, Cr 18.22, Ni 11.30	97	3	3.0
IRSID 1723	..		1 500	C 0.356, Mn 0.758, Cr 0.91, Ni 0.255	1 360	100	7.4
BCS 320	..	..	2 200	C 0.22, Mn 0.19, Cr 0.131, Ni 0.022	2 120	100	4.7
BCS 324	..	..	1 700	C 0.29, Mn 0.28, Cr 0.077, Ni 0.050	1 615	100	6.2
ARC 2266	..	..	28 000	Cr 19.3, Ni 10.4	27 700	1000	3.6

media and the high performance obtained with the 8-hydroxy-quinoline complex. 8-Hydroxyquinoline was used in the form of a 4% (m/V) solution in 0.1 M HCl. The best polarograms were obtained with an 8-hydroxyquinoline concentration of 3×10^{-3} M. Under these conditions, the molybdenum peak occurs at -380 mV and the current values are proportional to the concentration of molybdenum.

Interferences

Fluoride, sulphate, phosphate, nitrate and perchlorate ions have no influence on the determination of molybdenum. However, the SCN^- ion forms a complex with the molybdate ion and the molybdenum peak disappears. Chloride reacts with molybdenum to form MoO_2Cl_2. A slight depressing effect (1%) is observed on the molybdenum peak in the presence of 1 M chloride.

Various metals that may be present in steels cause no interference; iron(III) interferes as it is complexed by 8-hydroxyquinoline at the expense of Mo(VI). In steels, iron is the major component and it can be readily complexed with acetic acid. Hence the interference of iron(III) can be avoided. The working supporting electrolyte was modified to contain 1 M HNO_3, 1 M NaOH, 2.1 M acetic acid and 3×10^{-3} M hydroxyquinoline (pH 2). Under the experimental conditions Mn and Ni present in the investigated samples are not reducible in the range 0 to -700 mV vs. SCE. The reduction of Cr(VI), Cu(II) and Bi(III) occurs between -10 mV [Bi(III)] and -100 mV [Cr(VI)]. Cd(II) is present in only trace amounts ($\leqslant 1$ p.p.m.) in the samples and its reduction potential (-590 mV vs. SCE) does not interfere with the catalytic wave.

Procedure for Determination of Molybdenum in Steels

Weigh 10–100 mg of steel into a PTFE beaker. This material is used for safety reasons when complete dissolution of the sample requires the use of perchloric acid, e.g., in the analysis of steels with a high carbon content ($\geqslant 0.5\%$) for which the formation of an insoluble molybdenum carbide occurs. Add 5 ml of HNO_3 - HCl (1 + 1), heat until complete sample dissolution and evaporate to dryness. Add 12.5 ml of concentrated HNO_3 and dilute to 100 ml with distilled water.

The sample is then in 2 M nitric acid medium. Place in the polarographic cell an aliquot of the sample containing about 5 $\times 10^{-9}$ M of molybdenum. Dilute with 2 M HNO_3 to 20 ml, add 5 ml of concentrated acetic acid and adjust the pH to 2.0 with 2 M NaOH using a pH meter. With a micropipette, add 0.4 ml of the 8-hydroxyquinoline solution. Record the differential pulse polarogram from 0.0 to -700 mV (SCE) after deoxygenation for 10 min.

The standard additions technique must be applied. The procedures described were applied on several standard steels. Table 1 gives the mean values of replicate determinations ($n = 5$).

Conclusion

The determination of molybdenum by differential-pulse polarography of the 8-hydroxyquinoline - Mo(VI) complex is suitable for the analysis of steel. The method can be applied to samples with a wide range of molybdenum concentrations and should be of value because of its high sensitivity (10^{-10} M) and its easy application.

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Ion Exchange of Some Metal Ions on Poly(ethyleneimine) Resin

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The sorption behaviour of several metal ions on poly(ethyleneimine) resin cross-linked with ethylene dibromide was examined. The resin was applied to the separation of Cu and Ni, the determination of Cu and Ni in $CoSO_4.7H_2O$ and the concentration of copper from dilute solutions by column operation.

Keywords: *Copper determination; nickel determination; metal sorption behaviour; ion-exchange chromatography; poly(ethyleneimine) resin*

Cross-linked poly(ethyleneimine) (PEI) resins have been prepared by many workers in a reaction between the uncrossed-linked resin and a variety of cross-linking agents. These resins have the ability to sorb some metal ions such as copper, nickel and cobalt in the order Cu > Ni > Co.[1,2] Walker[3] reported the preparation of PEI resin, which was then cross-linked by reaction with epichlorohydrin. The product was a viscous liquid even after being kept at 60 °C for 10 d. Walker also reported the preparation of PEI resin using ethylene dibromide as a cross-linking agent, the resin obtained having a low mechanical strength and a high moisture content (80–90%). Other workers[4-6] have also prepared PEI resin cross-linked with ethylene dibromide and/or formaldehyde. The resins formed had an acceptable mechanical strength.

In this work, PEI resin was prepared and then cross-linked by reaction with ethylene dibromide. The resin was assessed in terms of water regain (WR) and total capacity (TC) for the metal ions Fe(II), Ni(II), Co(II), Cu(II), Cd(II), Fe(III), Ga(III), V(V), Cr(VI) and Mo(VI). Using this resin, some elemental separations were achieved in different media.

Experimental

The polymer (PEI), cross-linked with ethylene dibromide, was prepared by the method described by Mohammed[6] and purified by washing. Analytical-reagent grade nickel nitrate, copper chloride, cobalt nitrate, cadmium chloride, iron(III) nitrate, iron(II) ammonium sulphate, potassium dichromate, ammonium metavandate, ammonium molybdate, gallium chloride, sodium acetate, acetic acid, hydrochloric acid and sulphuric acid were used. De-ionised water was used throughout.

The ion concentrations were determined using a Perkin-Elmer AA-460 atomic absorption spectrophotometer.

Sorption of Metal Ions Using the Batch Technique

The total capacities of the resin for Fe(II), Ni(II), Co(II), Cu(II), Cd(II), Fe(III), Ga(III), V(V), Cr(VI) and Mo(VI) were determined by equilibrating about 0.5 g of the moist resin (of known water content) with metal ion solution of known concentration in the appropriate medium. For the adjustment of pH, an acetate buffer and hydrochloric acid were used in the pH ranges 3–7 and 1–2, respectively. A Radiometer Model pH-82 pH meter was used for pH measurement. The resin was then filtered and the metal ion concentration in the filtrate was measured by AAS. The TC of the resin for each ion was calculated from the difference in metal ion concentration before and after equilibration.

Separation of Copper and Nickel

The moist resin was packed into a glass tube (20 cm × 1.2 cm i.d.) and conditioned with 30 bed-volumes of acetate buffer

(pH 3). After conditioning the resin, 500 ml of a mixed solution of Cu(II) and Ni(II) were allowed to pass through the column at a flow-rate of 1 ml min^{-1} and the column was washed with acetate buffer (pH 3). Copper retained on the column was eluted with 100 ml of 2 M H_2SO_4.

Determination of Cu(II) and Ni(II) in $CoSO_4.7H_2O$

A solution of $CoSO_4.7H_2O$ (general-purpose reagent) was prepared by dissolving 2.5 g of the salt in de-ionised water and adjusting the pH to 4.5 using acetate buffer and diluting to 250 ml. A 100-ml volume of this solution was passed through a bed of the resin, which was prepared as stated previously at a flow-rate of 1 ml min^{-1}. The effluent was collected in a 150-ml calibrated flask and then 50 ml of acetate buffer (pH 4.5) were added. The sorbed metal ions were eluted with 100 ml of 2 M H_2SO_4. The copper(II), nickel(II) and cobalt(II) contents in the influent, effluent and eluate were measured by AAS.

The previous procedure was followed for the analysis of a solution of $CoSO_4.7H_2O$ (general-purpose reagent) spiked with 10 mg each of copper(II) and nickel(II).
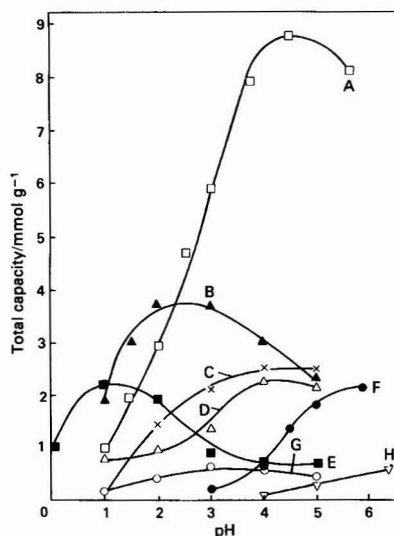
Fig. 1. Total capacities of PEI resin for some metal ions as a function of pH. A, Vanadium; B, molybdenum; C, copper; D, gallium; E, chromium; F, nickel; G, cadmium; and H, cobalt

Table 1. Separation of Cu(II) and Ni(II) by PEI resin at pH 3

Metal ion	Amount of metal in solution/mg	Amount in effluent/mg	Amount in eluate/mg	Separation, %
Cu(II)	25.00	0.00	25.00	100
Ni(II)	25.00	25.00	0.00	100

Table 2. Determination of Cu(II) and Ni(II) in $CoSO_4.7H_2O$

Metal ion	Amount of metal in influent/mg	Amount of metal in effluent as a percentage of influent, %	Amount of metal in eluate/mg	Recovery, %
Unspiked salt—				
Cu(II)	0.0119	0.00	0.012	100.8 ± 4
Ni(II)	0.063	0.00	0.062	98.4 ± 4
Co(II)	219.5	99.0	0.25	0.11 ± 3
Spiked salt—				
Cu(II)	10.125	0.00	9.850	97.3 ± 4
Ni(II)	13.75	0.00	13.50	98.2 ± 3
Co(II)	220	99.09	0.28	0.12 ± 2

Table 3. Extraction of Cu(II) from dilute solutions

Amount of Cu(II) in solution/mg	Volume of the solution/ml	Amount of Cu(II) in the effluent/mg	Amount of Cu(II) in the eluate/mg	Recovery, %
0.1	1000	0.000	0.103	103 ± 5
1.0	1000	0.000	0.99	99 ± 5
5.0	500	0.000	5.05	101 ± 3

Extraction of Cu(II) from Dilute Solution

Three columns of 1.2 cm i.d. were packed to a height of 20 cm with moist resin. The resin was then buffered to pH 4 by washing with acetate buffer. A 1-l volume each of 0.1 and 1 p.p.m. and a 500-ml volume of 10 p.p.m. copper(II) solutions were passed through the resin at about 1 ml min⁻¹. The resin was then washed with acetate buffer (pH 4). The sorbed copper was eluted with 100 ml of 2 M H_2SO_4 and the copper(II) contents of the effluent, washing solution and eluate were determined by AAS.

Results and Discussion

As a preliminary experiment, the sorption behaviour of some metal ions such as Fe(II), Ni(II), Co(II), Cu(II), Cd(II), Fe(III), Ga(III), V(V), Cr(VI) and Mo(VI) on PEI resin was examined from different media using a batch method. The resin did not show any sorption of Fe(II) and Fe(III). The capacities for the other ions are shown in Fig. 1. The sorption capacities for Co(II) and Cd(II) were not high in comparison with those of Ni(II), Cu(II) and V(V), which were high and increased sharply with increase in the pH of the solution; this is in agreement with previous results.[6,7] The maximum sorption of V(V) was found at pH 4.5 and for Cu(II) and Ni(II) at the maximum pH obtainable before metal hydroxide began to precipitate. Molybdenum showed a reasonable uptake over a wide range of pH. The maximum sorption capacity for Cr(VI) and Ga(III) were similar in value but were obtained with media of different pH, the highest sorption of Cr(VI) being obtained at low pH and that of Ga(III) at higher pH.

The graphs shown in Fig. 1 indicate that more than one mechanism can be suggested for the sorption of the ions by PEI resin. The resin is a weak base anion exchanger that can also form stable complexes with some metal ions such as

Cu(II) and Ni(II) and this is accompanied by chelate formation of the following type[1,5]:
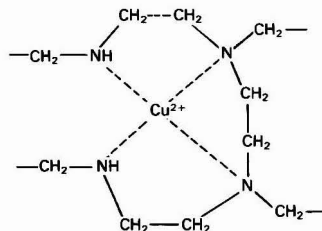
This mechanism is probably valid for some of the ions that are strongly held by the resin at high pH and have a lower affinity for the resin at low pH. The observed sorption behaviour of these ions can be explained by the protonation of the resin at low pH and a decrease in the degree of protonation as the pH increases. If the imino group is less protonated, which occurs at high pH values, more opportunities exist to form stronger complexes with the metal ions. Therefore, the capacity of the resin for these ions increases with increase in pH. Other ions that have high capacities at low pH such as Mo and Cr are probably sorbed as negatively charged complexes, *i.e.*, sorption is by a simple anion-exchange process.

The selectivity coefficient of the resin for Cu(II) over Ni(II), E_{Ni}^{Cu}, was 15, *i.e.*, the extent of separation was more than 99%. Therefore, Cu - Ni separation would appear to be possible. At the same time the capacities of the resin for Cu(II) and Ni(II) at pH 4.5 were *ca.* 2.25 and 1.00 mmol g⁻¹ of dry resin, respectively, whereas Co(II) showed a capacity of less than 0.1 mmol g⁻¹. Therefore, separation of Cu(II) and Ni(II) from Co(II) would again be possible. The resin has been applied to separate Cu(II) and Ni(II) in pH 3 acetate buffer. The mixed solution of Cu(II) and Ni(II) ions was passed through the resin

bed on which Cu(II) was retained and the results in Table 1 indicate that an efficient separation was obtained.

An analysis of $CoSO_4.7H_2O$ (general-purpose reagent) gave contents of Cu(II) and Ni(II) of 4.78 and 25.2 p.p.m., respectively (Table 2), and the recoveries of both ions were about 100%. To examine the efficiency of the resin in recovering ions at a higher concentration, about 10 mg each of both ions were spiked with 1 g of $CoSO_4.7H_2O$ and the procedure was repeated. No significant change in the resin efficiency for the recovery of Cu(II) and Ni(II) was observed.

Finally, the extraction of Cu(II) from dilute solutions was examined. Copper(II) was sorbed from dilute solutions adjusted to pH 4 by acetate buffer and the results are shown in Table 3. These results indicate that the method can be applied to the extraction and determination of copper in sea water.

Conclusion

The resin shows a high capacity for most of the ions studied and the sorption behaviour was found to be dependent on pH. Nickel and copper were efficiently separated from each other and from cobalt using the resin in pH 4 and 4.5 media, respectively. PEI also shows a good ability for the pre-concentration of copper from dilute solutions.

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BOOK REVIEWS

Handbook of Tritium NMR Spectroscopy and Applications
E. A. Evans, D. C. Warrell, J. A. Elvidge and J. R. Jones. Pp. xiv + 249. Wiley. 1985. Price £25.50. ISBN 0 471 90583 6.

This short book, which summarises the work of the first decade and a half of tritium NMR spectroscopy, is organised into three chapters: Experimental aspects of tritium NMR spectroscopy (20 pages); Labelling patterns and methods for tritium labelling (135 pages); and Applications (51 pages). Appendix I summarises the international system of units for radioactivity and Apendix II contains standard proton NMR correlation tables widely included in undergraduate texts on spectroscopy.

The text seems to be aimed primarily at students and "generalists" rather than NMR specialists. However, the topic has also been featured in a recent review by the same authors published in a specialist NMR journal.[1]

The book is well produced and it provides an excellent compilation of tritium NMR work up to the end of 1984. The comprehensive list of over 200 literature references is particularly valuable. The chapter on applications includes interesting uses of the technique for the study of organic reaction mechanisms and structural, biochemical and environmental studies. In some ways this chapter, which is a good reason for reading the book, could have usefully been expanded to give more examples. This monograph will be a useful starting point for students and others interested in this challenging field. The detailed subject and compound indexes will be invaluable to the casual reader.

In summary, the book provides a good overview of tritium NMR spectroscopy with interesting applications in chemistry, biochemistry and medicine.

Reference

1. Elvidge, J. A., *et al.*, "Practical Aspects of Tritium Magnetic Resonance," *Prog. NMR Spectrosc.*, 1983, **16**, 99.

D. P. Leworthy

Analytical Chemistry. An Introduction. Second Edition
Gerald F. Lewis. Pp. xii + 91. Macmillan. 1985. Price £4.50. ISBN 0 333 38567 5.

The prime objective of this monograph is to supplement the practical training of laboratory technicians. It also satisfies the second objective of giving those with GCE A and similar levels an intelligent grasp of the scope and purpose of analytical chemistry. Within its 11 chapters it systematically considers sampling, weighing, preliminary sample treatment, physical properties, classical analysis, which includes non-aqueous, EDTA and potentiometric titrations, spectroscopic methods, chromatography, polarography, automated analysis and statistical concepts. There is no reference to flow injection analysis and some other topics that might have been briefly

considered, but the plasma torch is there and so is electrophoresis.

Encased in a rainbow-coloured jacket, the book is of invitingly attractive appearance, as are the contents with the help of clear print, well drawn diagrams and eight good colour plates. These together give a good first impression of analytical chemistry, as does reading the book. The price is moderate, but need the book have been printed in far away Hong Kong for a British publisher and sponsor?

J. D. R. Thomas

Selected Methods of Trace Metal Analysis: Biological and Environmental Samples
Jon C. Van Loon. *Chemical Analysis, Volume* 80. Pp. xiv + 357. Wiley–Interscience, 1985. Price £56.25. ISBN 0 471 89634 9.

This is a valuable review of the state of the art of atomic absorption (AAS) and emission spectrometry (AES) in determining trace metals. After brief survey chapters on instrumentation, sampling and contamination problems and sample decomposition, there are six chapters describing proven methods. These refer to biological samples, foods, chemical samples, natural waters, air particulates and soils, and quote detailed procedures selected from the author's own work and recent literature. A final chapter, on metal speciation, reviews the problems of interfacing GLC and HPLC techniques with AAS and AES. Hence the book is of particular interest to practising analysts concerned with the reliable determination of trace metals, and is recommended to them.

The presentation is clear, although the mass of detail is sometimes tedious. The author does not emphasise the importance of the residence time of atoms in the light beam that makes furnace AAS so much more sensitive than flame AAS. There is a good description of PTFE decomposition vessels, but surprisingly no mention of Bock's excellent work on sample decomposition.[1] Sections on ion exchangers and laboratory safety could have been improved; for example, the author recommends the use of 4-methylpentan-2-one in solvent extraction without a caution as to its unfortunate physiological effects on many indviduals. I noticed few scientific errors, although "quartz" is sometimes used for "silica" and heparin is recommended as an anticoagulant for blood despite earlier warnings as to its high content of trace metals. SI units are used throughout; in the detailed methods, operating conditions are mostly described for US or Canadian instruments.

The format and printing are good, but the worst feature of the book is the rather large number of misprints, which could have been set right by careful proof-reading. For example, Cr(VIII), referred to on p. 169, is unknown. As techniques develop fast a book of this type is bound to become out of date in approximately 5 years, hence the price of 15.8 pence per page is rather high.

Reference

1. Bock, R., "A Handbook of Decomposition in Methods in Analytical Chemistry," Blackie, Glasgow, 1979.

H. J. M. Bowen

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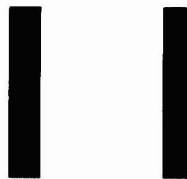
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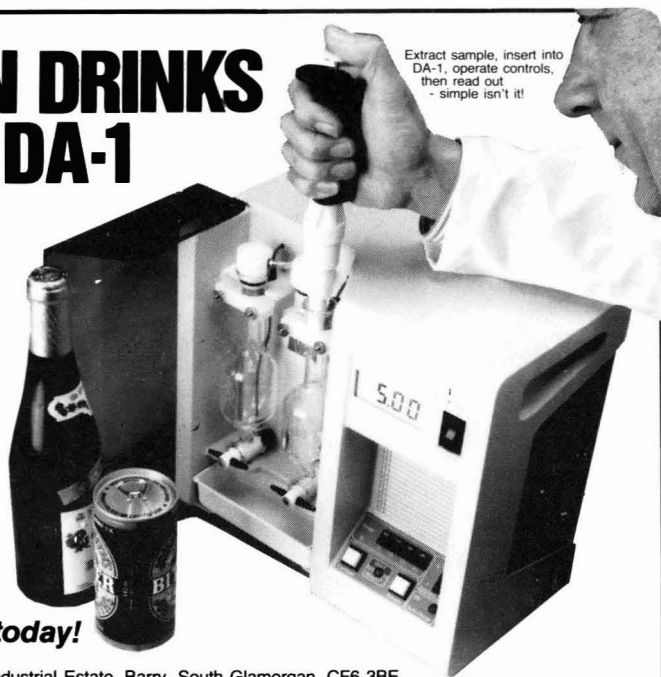
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