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Cambridge, 1991.

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Application of Total-reflection X-ray Fluorescence Spectrometry to Elemental Determinations in Water, Soil and Sewage Sludge Samples

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Total-reflection X-ray fluorescence (TXRF) spectrometry represents a relatively new instrumental analytical technique for the determination of trace elements in liquid samples. In this study, a range of sample types common to the water industry have been analysed for their elemental composition by TXRF and inductively coupled plasma (ICP) spectrometry. The TXRF method was found to offer, in general, lower limits of detection than are possible with ICP spectrometry, 4–5 orders of magnitude range in calibration, a sample preparation precision of <3% and an instrumental precision of <1%. Results obtained by TXRF compared favourably with those acquired by ICP and with the reference values that were available. Preparation of the digested and aqueous-based samples for TXRF analysis offered a very simple internal standard method for calibration and required only very small volumes (≈10 μl).

Keywords: *Total-reflection X-ray fluorescence; inductively coupled plasma; soil; water; sewage sludge*

X-ray fluorescence (XRF) spectrometry has, over the past two decades, become an established non-destructive technique for multi-element determinations, applicable to a wide range of matrix types.[1] In trace element analysis, the major disadvantage of conventional XRF has been the poor elemental sensitivity, which is mainly a consequence of high background noise levels, resulting from instrumental geometries and sample matrix effects.[2] Total-reflection X-ray fluorescence (TXRF) spectrometry is a relatively new multi-element technique with a potential to achieve trace element determinations for a variety of sample types.[3]

The problem in detecting elements at the nanogram or sub-ppb level is basically one of being able to obtain a signal that can be clearly distinguished from the background. The detection limit is typically specified as the signal that is equivalent to three times the standard deviation of the background counts for a given unit of time.[4] In XRF, the background is essentially caused by interactions of radiation with matter resulting from an intense flux of elastic and Compton-scattered photons. The background, especially in the low-energy region (<3 keV), is due mainly to Compton scatter of high-energy Bremsstrahlung photons from the detector crystal itself.[5] In addition, the background might be elevated owing to impurities on the specimen support contributing to Compton scatter in the higher-energy region (17–20 KeV).[6] The Auger effect does not contribute to an increased background, as the emitted electrons, of different but lower energy, are absorbed either in the Be foil of the detector entrance windows or in the air path of the spectrometer.[6]

A reduction in the spectral background can be effectively achieved by X-ray total reflection at the surface of a smooth reflector material such as quartz. If a collimated X-ray beam impinges onto the surface of a plane, smooth and polished reflector at an angle less than the critical angle, then total reflection occurs. In this instance the angle of incidence is equal to the angle of reflection, and the intensities of the incident and totally reflected beams should be equal (Fig. 1).

The principles of TXRF were first reported by Yoneda and Horiuchi[7] and further developed by Aiginger and Wobrauschek.[8] In TXRF, the exciting primary X-ray beam impinges at angles of incidence in the region of 2–5 minutes of arc below the critical angle on the specimen prepared as a thin film on an optically flat support. In practice, the primary radiation does not (effectively) enter the surface of the support, but skims the surface and irradiates any sample placed on the support surface. The scattered radiation from the sample support is virtually eliminated, thereby drastically reducing the background noise. A further advantage of the TXRF geometry is that the solid-state energy-dispersive detector can be accommodated very close to the sample (0.3 mm), which allows a large solid angle of fluorescent X-ray collection, thus enhancing signal sensitivity and enabling the analysis to be carried out in air at atmospheric pressure.

Since Yoneda and Horiuchi[7] first reported the use of TXRF, various versions have been developed, in particular the use of a cut-off filter to reduce the background from

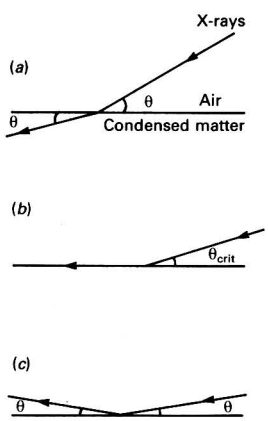

Fig. 1 Total reflection phenomena: (*a*) $\theta > \theta_{crit}$; (*b*) $\theta = \theta_{crit}$; and (*c*) $\theta < \theta_{crit}$

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high-energy photons (>20 keV).[9-14] Recently, an X-ray generator with a fine-focus tube and multiple-reflection optics has been developed by Seifert and coupled with an energy-dispersive spectrometer fitted with an Si(Li) detector and a multi-channel analyser supplied by Link Analytical. Results obtained with such an instrument known as the EXTRA II are described in this paper. These results include an evaluation of the instrumental performance and the suitability of the instrument for multi-elemental quantification of trace elements in a wide range of liquid-based sample matrices.

Experimental

Instrumentation

TXRF

Element determinations were carried out using an EXTRA II TXRF spectrometer fitted with a multiple-total-reflection unit. A schematic diagram of the instrument is shown in Fig. 2.

The EXTRA II is equipped with a Seifert (Hamburg, Germany) Model ID 3000 high-voltage generator fitted with the appropriate control and regulation hardware. The output voltage can be varied from 1 to 60 kV in steps of 1 kV, and output currents from 1 to 80 mA, in steps of 1 mA, can be selected. The total-reflection chamber has two reflector units, one optimized for Mo Kα and the other for W white spectrum radiation. The X-ray tubes were two fine-focused Model SF60-K's (with Mo and W as anode materials), each with a maximum output of 2000 W and a line profile of 8 mm width and 0.4 mm depth.

The X-ray detector and multi-channel analyser, supplied by Link Analytical (now Oxford Analytical, Abington, UK), consisted of an AN 10/55 analyser system. The Si(Li) detector crystals used had an active area of 80 mm^2 with a resolution of ≤155 eV at 5.9 keV. The detector was fitted with a pulsed optical feedback pre-amplifier and pulse processor with dead-time correction, pulse pile-up rejection, and a 100 MHz ADC data-handling system consisting of a 512 kbyte CPU (20 MHz clock), and two floppy and one hard disk running software for auto-acquisition, data processing and instrumental controls.

ICP spectrometer

A Jobin–Yvon JY 38 PI ICP (EDT Research) sequential-emission spectrometer, fitted with a Hook and Tucker auto-sampler, was used. The specifications of the spectrometer were as follows.

Generator. Durr JY2200W; frequency, 55.5–56.6 MHz.

Argon flow-rates. These were 18, 0.4, 0.6 and 0 $dm^3\ min^{-1}$ for plasma, coating, carrier and auxiliary, respectively.

Torch. Jobin–Yvon three-piece demountable type; observation height, just above the torch outer tube.

Nebulizer. Glass (Meinhard), with the sample pumped at 1.0 ml min^{-1} with use of a peristaltic pump.

Monochromator. Czerny–Turner 1000M: focal length, 1 m; aperture, *f*/6.8; master holographic grating, 2400 grooves mm^{-1}; resolution, 0.01 nm at 200 and 400 nm; and wavelength step size, 0.002 nm. The monochromator was purged with nitrogen at a flow-rate of 18 $dm^3\ min^{-1}$ when measurements were made below 200 nm (*i.e.*, for determination of Hg and As). Duplicate peak scans were performed for samples and triplicate peak scans for standards. A peak area calculation mode was used for all elements except Pb, Ca and K, when peak height measurement was used. A background correction (one side of the peak) was used for Pb, and a two-sided correction was used for As and Hg. Re-calibration was performed after analysis of five samples. The choice of wavelengths used was as follows: Cd, 228.80; Cr, 267.72; Cu, 224.70; Ni, 231.60; Pb, 220.35; Zn, 213.86; As, 193.69; Hg, 194.16; K, 766.49; Ca, 317.93; Fe, 259.94; and Se, 196.03 nm.

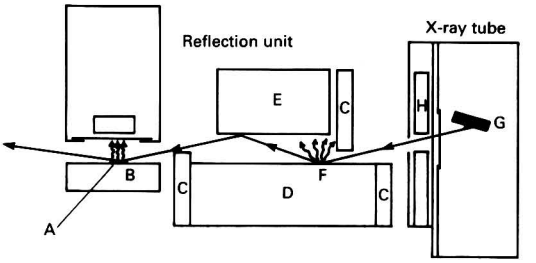

Fig. 2 Schematic diagram of the apparatus. A, Sample; B, sample support; C, diaphragm; D, reflector 1; E, reflector 2; F, primary reflection zone; G, X-ray tube anode; and H, slit-type collimator

Digestion Apparatus

A CEM MDS-81D microwave digestion system and a Tecam Dri-Block DB-4S electrically heated aluminium block were used.

Reagents and Gases

All reagents were of AnalaR or SpectrosoL grades (Fisons or BDH), and the water used throughout was distilled and doubly de-ionized. All the standards were prepared from 1000 mg dm^{-3} SpectrosoL solutions and were freshly made up in de-ionized water. For the inductively coupled plasma (ICP) spectrometry work, dilute solutions of metal standards were prepared by mixing 5.0 ± 0.02 ml of a standard solution (in 10% v/v nitric acid) of Hg^{II}, Cd, Cu, Cr^{III}, Pb, Ni or Zn (10.0 mg dm^{-3}) with 50 ± 1 ml of nitric acid (sp. gr. 1.41) and diluting to 500 ml in a calibrated flask. A standard solution of As^{III} or Se^{IV}, 10.0 mg dm^{-3}, in 10% v/v hydrochloric acid, was similarly prepared using hydrochloric acid (sp. gr. 1.19). A mixed alkali–alkaline earth metal standard containing 200 mg dm^{-3} of Ca and 20.0 mg dm^{-3} of K was prepared by mixing 100.0 ± 0.1 ml and 10.00 ± 0.02 ml of stock Ca and K solutions, respectively, with 50 ± 1 ml of nitric acid and diluting to 500 ml in a calibrated flask. Blank solutions of 10% v/v nitric and hydrochloric acids were prepared with omission of the stock metal solutions.

The 10% acid concentrations were found to be suitable for the analysis of samples in both 12% nitric acid and 10% *aqua regia* [HCl–HNO_3 (3 + 1)] matrices. For the analysis of liquid samples stabilized in 1% nitric acid, all of the above standard solutions and blanks were duplicated by substituting 1% for 10% acid matrix.

The following Certified Reference Materials (CRMs) were used. Community Bureau of Reference (BCR) (Belgium) CRMs 143 Sewage sludge-soil; 144 Sewage sludge—domestic; and 142 Soil-light sandy.

BOC 'Vac Spec' grade argon and nitrogen were used.

Digestion Procedure

Sludges (block digestion)

A pre-weighed fraction of dried sludge (0.500 ± 0.002 g) was transferred into a 50 ml borosilicate tube. De-ionized water (1.0 ± 0.1 ml) was added to wet the sample, then nitric acid (6.0 ± 0.1 ml) was added carefully and any initial reaction was allowed to subside. The tube was then placed in an electric heating block fitted with a polytetrafluoroethylene (PTFE) stopper and heated gently under reflux for 15 min. After cooling, the sample was transferred quantitatively through an acid-rinsed filter into a 50 ml calibrated flask, and then made up to the mark with de-ionized water. The final acid strength was 12% in nitric acid.

Metal standards and blanks (1.0 ml of H_2O + 6 ml of HNO_3 only) were subjected to the same procedure.

Soils, sediments (microwave oven digestion)

A pre-weighed fraction of the dried sample (0.500 ± 0.002 g) was transferred into a Teflon PFA digestion vessel (acid-cleaned and dried). De-ionized water (1.0 ± 0.1 ml) was added to wet the sample, then 3.75 ± 0.02 ml of hydrochloric acid and 1.25 ± 0.02 ml of nitric acid were carefully added and the initial reaction was allowed to subside. The pressure-relief valve and cap were fitted and tightened to the correct torque.

The following three-stage digestion programme was operated. Stage 1: 10 min at 20% power; 2: 10 min at 70% power; and 3: 25 min at 50% power.

The digestion vessel was removed, allowed to cool, then manually vented. The contents were transferred quantitatively through an acid-rinsed filter into a 50 ml calibrated flask, then made up to the mark with de-ionized water. Final acid strength: 10% *aqua regia*.

Preparation of Samples for Analysis by TXRF

Reflector plate preparation. All the sample solutions were transferred by pipette (Gilson microman; 25 µl adjustable pipette) directly (10 µl) onto highly polished quartz reflector plates (30 mm diameter) previously analysed to establish the plate background. The quartz plate was treated prior to sample addition with 5 µl of a pure silicone solution (Serva Feenbiochemical) to prevent excessive spreading of the sample solution.

In the analysis for As, Hg and Se it was necessary to add a further 10 µl of 1% ammonium pyrrolidin-1-yldithioformate (ammonium pyrrolidinedithiocarbamate) to the sample on the quartz plate to minimize losses of the volatile analytes during drying.

Aqueous samples. A 10 µl aliquot of the aqueous sample was transferred by pipette onto a quartz plate and mixed on the plate with a 10 µl aliquot of an internal standard (30 ppm of Co). The plate was dried under an infrared lamp for 10 min prior to analysis. The Mo X-ray tube excitation conditions used were 50 kV and 10–50 mA, with a data acquisition time for each sample of 1000 s.

Acid-digested samples. A 10 µl aliquot of the digested sample was transferred by pipette onto the quartz plate and mixed with 10 µl of an internal standard (30 ppm of Co). The plate was then dried under an infrared lamp for 10 min prior to analysis.

Results and Discussion

Optimization of X-ray Excitation Radiation

As the EXTRA II is supplied with fixed reflector optics, the only variables requiring attention from the analyst are tube voltage, tube current and sample presentation on the quartz reflector. An investigation of the spectral distribution in terms of energy profiles for the determination of most elements of interest, identified 50 kV and 38 mA as the most suitable tube setting. Tube conditions are investigated more fully and relevant results are discussed under Detection Limits. The results shown in Table 1 confirm that the best compromise tube conditions are in the range 40–50 kV. Changes in the tube current were found to be necessary in order to minimize scattered radiation entering the detector. For a pure standard solution, low scatter was observed and a typical current setting of 38 mA was suitable. However, the presence of dissolved solids on drying caused increased scatter and produced a corresponding increase in the dead time of the counting statistics, and thus it became necessary to reduce the excitation current. For example, with the acid-digested samples used, a tube current of 10 mA would produce a typical dead time of 40%. The ideal dead time for the system was found to be in the region of 30 to 50%; however, at present, there is no automated facility for self-adjusting the current output to match changes in the scatter from samples. Work is currently being undertaken to enable this aspect of instrumental optimization to become fully automated.

As indicated above, the dissolved solids content of the sample will affect the degree of scatter observed by the detector. To date, samples containing 2% dissolved solids have been analysed and indeed fine powders[15] and biological tissues[16] have been analysed directly. The distribution of the sample on the quartz surface does not appear to affect the quantification or the precision of the analysis, provided that the sample is spotted within the envelope of the excitation beam profile (8 mm width). A small PTFE jig enables spots to be easily placed within the zone of excitation.

Table 1 Variation of MDL (µg dm^{-3}) as a function of tube voltage and current in ambient atmosphere for aqueous standards

Tube conditions	S	Ca	V	Mn	Co	Zn	Se	Sr
59 mA; 20 kV	260	63	39	21	17	12	11	24
59 mA; 25 kV	233	54	28	16	12	8	8	1
59 mA; 30 kV	121	31	16	10	8	5	4	5
28 mA; 40 kV	118	29	17	9	7	5	4	4
20 mA; 50 kV	135	30	16	10	7	5	4	4
12 mA; 50 kV	136	32	17	10	7	5	4	4

Calibration

Multi-element standards covering five orders of magnitude, ranging from ppb to ppm concentrations, were prepared for Se, V and Zn. Each standard was spotted (10 µl) onto a quartz reflector plate, and K line intensities were measured relative to Co K lines. The three elements were chosen to represent a range of emission energies in the Mo X-ray excitation range. All three elements were found to yield good linear relationships, according to the following equations, where y is the normalized intensity count and x the concentration. Se: $y = 1.02x - 0.0311$; V: $y = 1.022x - 0.192$; Zn: $y = 1.00x + 0.0267$.

As the main thrust of this work was to determine trace element levels, no further examination of higher concentrations was carried out. It seems probable, however, that higher concentrations could be determined by the TXRF technique.

Detection Limits

The investigation into detection limits examined not only aqueous samples, but also acid-digested sample matrices. The elements selected for this section of the work were chosen to represent low (S) to high (Sr) range X-ray emission energies typically obtained with use of Mo X-ray excitation (2–20 keV).

Detection limits with use of aqueous standards

A multi-element standard was prepared containing 10 ppm each of Ca, Mn, S, Se, Sr, V and Zn, with Co acting as the internal standard at 10 ppm. A 10 µl aliquot of this standard solution was transferred by pipette onto a quartz plate and air-dried. The signals were then collected for a 1000 s period for both the standards and a blank. The minimum detection limit (MDL) was calculated from the following equation:

$$\mathrm{MDL} = \frac{3 \times c}{I} \times \sqrt{\frac{I_B}{t}} \quad (1)$$

where c is the concentration of standard, I is the total peak counts for the standard, I_B is the total background peak count, and t is time.

The MDLs for various elements in an ambient atmosphere over a range of tube voltages and currents are shown in Table 1.

The same procedure as above was then followed, except that a helium flush was introduced into the cavity between the

detector and sample plate, and the MDLs for the same range of elements were determined as a function of tube voltage and current (Table 2). From the data in Tables 1 and 2, it is clear that the MDL can be improved for most elements with the introduction of a helium flush. This effect was particularly pronounced in the lower energy emission range, as seen for S and Ca. As a general trend it was found that elements in the higher energy range (Sr and Se) afforded improved detection limits as the tube voltage was increased, with a corresponding reduction in tube current. This characteristic is thought to be associated with a reduction of the base line in this region of the spectrum, which normally results from the target of the Mo X-ray tube when high currents are used. However, a reduction in tube current will also lead to a loss of emission intensity, hence the apparent effect will be limited to the current ranges indicated; further reduction in tube current will subsequently lead to a loss in fluorescence yield. In the element range V–Zn, MDLs are seen to improve when a tube voltage of 30–40 kV and a current of 28–59 mA are used. For lighter elements at the lower end of the energy range, lower X-ray tube voltages and high currents appear to give the best MDL. As the technique is designed to be a simultaneous multi-element method of analysis, a suitable compromise of intermediate tube voltage and current would seem to be appropriate. However, for specific regions in the spectrum, optimum conditions of sensitivity could be better achieved by selecting the appropriate X-ray tube conditions. The MDL values obtained by TXRF were considered to be acceptable for a technique required for trace element analysis and comparable to other widely used trace element techniques.

Detection limits in acid media and digested material

The MDLs were determined for various elements in an acid matrix and for acid-digested reference materials. The internal standard (3 ppm of Co) was mixed with 10 μl of the acid-digested material, and the MDLs were calculated by using eqn. (1) and are summarized in Table 3.

From the results obtained, various conclusions can be drawn. The most general trends are that the MDLs for all the elements examined are similar for both the acidic and aqueous standards and that MDLs improve for higher Z elements, which corresponds to an increase in X-ray emission energies when using K lines. However, when the MDLs were calculated for a reference material present in an acid-digested matrix it was obvious that a deterioration of the values obtained had occurred. This decrease in detection limits is approximately twice that for nitric acid-based matrices, except for some of the light elements in saline water, but the detection limit can be seen to improve by approximately five times for *aqua regia* matrices. The apparent determinator in detections limits observed for digested samples is thought to be a function of dissolved solids and scatter from the sample. As a general conclusion, it would appear that nitric acid used at strengths of up to 12% v/v represents the most suitable acid matrix for sample digestion.

Table 2 Variation of MDL (μg dm^{-3}) as a function of tube voltage and current in a helium atmosphere

Tube conditions	S	Ca	V	Mn	Co	Zn	Se	Sr
59 mA; 20 kV	230	68	39	21	16	13	11	23
59 mA; 25 kV	140	36	22	13	9	7	6	7
59 mA; 30 kV	103	32	17	10	7	5	4	4
28 mA; 40 kV	96	28	16	9	6	5	4	4
20 mA; 50 kV	104	28	17	9	2	5	3	3
12 mA; 60 kV	117	31	17	9	7	5	4	3

Precision of Sampling Methodology

Ten quartz reflector plates, previously analysed for background, were spotted with a solution of mixed standards, and, after drying, each plate was analysed ten times. The typical relative standard deviation (RSD) values of 2–3% for most elements were found to be similar to those for other manual injection techniques such as electrothermal atomic absorption spectrometry. The precision for Se was, however, considered to be unacceptably high (6.7% RSD) and this was found to be a feature of the drying step when samples were dried under an infrared lamp. During this stage the surface temperature of the quartz plate can reach 170 °C and this will be sufficient for significant amounts of volatile analytes, such as Se, Hg and As, to be lost. Air- or vacuum-drying has been found to reduce this effect, and a precision of 2–3% for the most volatile elements can be achieved in this way. The position of the 10 μl sample spot on the surface appeared to have little effect on the precision obtained, provided that the entire spot was contained within the 8 mm profile width of the excitation beam: this is not difficult to achieve practically.

Instrumental Precision

The evaluation of the instrumental precision was carried out in two ways. The first way was to introduce (*via* the autosampler) the sample plate ten times into the instrument, while the second way was to take ten consecutive measurements, with the plate remaining in the analytical position. From the RSD values obtained for both experiments it was found that an instrumental precision of <1% RSD could be obtained, and this was considered to be acceptable as an instrumental procedure and compared favourably with similar techniques.

Accuracy

Various reference materials were examined in order to assess the accuracy of the results obtained with TXRF. In addition,

Table 3 MDLs for a range of elements determined in acid medium and for various matrix types (all values are in μg dm^{-3})

Sample	Ca	K	Fe	Cu	Zn	Ni	Cr	Pb	Se	Hg	As
1. Low concentration (1% HNO_3)	260	80	45	1	21	13	13	5.8	2.9	—	3.9
2. High concentration (12% HNO_3)	130	41	23	4	11	6.4	6.4	2.9	1.4	3.4	2.0
3. First sludge (12% HNO_3)	160	97	27	4.8	13	7.7	7.6	3.5	3.5	1.7	—
4. Second sludge (12% HNO_3)	110	70	19	2.4	9.0	5.5	5.4	2.5	—	—	—
5. Composite potable water (1% HNO_3)	72	43	12	2.2	5.6	—	3.5	1.6	—	1.8	—
6. Composite river water (1% HNO_3)	69	42	12	2.0	5.5	—	—	1.5	—	1.7	—
7. Saline water (1% HNO_3)	84	510	140	—	65	—	—	1.9	—	2.1	—
8. Digested sludge (12% HNO_3)	110	70	20	3.5	9.1	5.3	5.5	2.5	1.2	2.9	—
9. Digested sludge (12% HNO_3)	100	62	17	3.1	8.0	4.9	4.7	2.2	1.1	2.5	—
10. High concentration (10% *aqua regia*)	160	140	25	2.0	12	7.3	7.5	5.9	1.5	5.2	2.3
11. Certified soil (10% *aqua regia*)	120	110	19	3.0	8.7	5.5	5.5	4.4	1.1	3.8	1.7
12. River sediment (10% *aqua regia*)	230	200	35	5.7	17	10	10	8.2	8.2	7.2	3.2
13. River sediment (10% *aqua regia*)	280	250	43	6.9	20	13	13	10	—	9.2	4.0
14. Composite soil (10% *aqua regia*)	280	250	43	6.9	20	12	—	10	—	—	3.8

Table 4 Comparison of TXRF and ICP results for a variety of sample types

Sample		Ca	K	Fe	Cu	Zn	Cr	Ni	Pb	Se	As	Hg
Synthetic standards (μg dm^{-3})—												
1. 1% HNO_3	ICP	200	20	5	5	5	5	5	5	1	1	5
	TXRF	260	23	5	5	5	4.7	5	5	1	1	5
2. 10% *aqua regia*	ICP	200	20	50	20	20	20	20	20	5	5	10
	TXRF	270	22	50	20	20	19.8	20	22	5	4.6	12
3. 12% HNO_3	ICP	200	20	50	20	20	20	20	20	5	5	10
	TXRF	274	25	50	20	20	20	20	20	5	5.4	9.0
Reference solids (μg dm^{-3})—												
4. BCR CRM 143 (12% HNO_3)	ICP	588	50	190	2.3	12	1.5	0.79	12.2	ND*	0.11	ND
	REF	—	—	214	2.36	13.01	2.08	0.93	13.12	0.006	0.16	—
	TXRF	625	—	212	3.3	13	1.6	0.90	13.7	ND	0.21	ND
5. BCR CRM 144 (12% HNO_3)	ICP	361	14.1	440	6.8	30.6	4.31	9.30	4.4	ND	0.012	ND
	REF	410	14.0	460	6.94	30.55	4.94	9.47	4.79	0.023	0.09	—
	TXRF	408	—	420	6.3	27.7	4.50	8.6	3.8	ND	ND	ND
6. BCR CRM 142 (10% *aqua regia*)	ICP	300	19	136	0.27	0.80	0.31	0.26	0.25	ND	0.20	ND
	REF	—	—	190	0.25	0.80	0.44	0.28	0.30	0.008	0.17	—
	TXRF	360	—	160	0.27	0.81	0.26	0.28	0.20	ND	0.10	ND
Environmental solids (μg dm^{-3})—												
7. Digested sludge (12% HNO_3)	ICP	240	19	58	5.22	7.36	1.70	1.40	4.09	—	0.09	
	TXRF	280	20	74	5.21	7.20	1.50	1.20	4.20	0.02	ND	
8. Digested river sediment (10% *aqua regia*)	ICP	560	15	320	0.27	1.0	0.16	0.17	0.60	ND	0.12	
	TXRF	570	18	340	0.30	1.1	0.21	0.21	0.36	ND	0.27	
9. Digested river sediment (10% *aqua regia*)	ICP	1290	32	230	0.24	0.80	0.24	0.19	0.28	0.12	0.14	
	TXRF	1340	31	260	0.26	0.94	0.29	0.24	0.28	ND	0.18	
10. Composite soil (10% *aqua regia*)	ICP	48	36	340	0.38	1.34	0.54	0.30	0.48	ND	0.14	
	TXRF	50	22	330	0.40	1.34	—	0.31	0.49	ND	0.20	
11. Digested sludge (12% HNO_3)	ICP	300	23	89	68	9.8	1.4	2.0	5.3	0.18	0.16	
	TXRF	360	25	94	70	9.7	1.6	2.0	5.6	0.03	ND	
Environmental liquid samples (μg dm^{-3})—												
12. Composite potable water sample (1% HNO_3)	ICP	59	32	0.12	0.02	ND	ND	ND	ND	ND	ND	
	TXRF	75	36	0.28	0.02	ND	ND	ND	0.03	ND	ND	
13. Composite river sample (1% HNO_3)	ICP	81	43	0.31	ND	0.03	ND	ND	ND	ND	ND	
	TXRF	100	52	0.44	0.02	0.05	ND	ND	0.04	ND	ND	
14. Saline water (1% HNO_3)	ICP	330	460	0.01	ND	ND	ND	ND	ND	ND	ND	
	TXRF	280	163	0.73	ND	ND	ND	ND	ND	ND	ND	

* ND = not detected.

the elemental composition of each sample was also determined by ICP, and the results are summarized in Table 4.

From the data set obtained there can be seen to be, in general, good agreement between the ICP and TXRF values and close agreement with available reference values. There appear to be one or two spurious values, which tend to be associated with the lower Z elements (*e.g.*, Ca and K). In addition, Pb appeared to give a low value in sample 8 for the TXRF, with Pb values, for both the TXRF and ICP methods, in sample 6 being lower than the reference value. This latter effect could be due to the poor recovery from the extraction medium as results by both methods show reasonable agreement. Poor recovery could also account for the variation in the Cr values in sample 6. Within the data set, one or two disagreements between the two measurement techniques are apparent, *e.g.*, Se in sample 9, As in sample 8, and Fe in samples 12 and 14. There appeared to be no apparent reason for the variations observed and these can only be attributed to random experimental error. In general, the extraction procedures used for the soil and sewage sludge samples proved to be satisfactory, as did the instrumental method of analysis.

Conclusions

The aim of this work was to evaluate the analytical performance of TXRF as a suitable multi-element method for a range of liquid-based matrix types. The results indicate that TXRF is a suitable method for multi-element determinations for the range of liquid-based matrices studied. The performance characteristics of the technique have proved to be satisfactory, offering a simple and rapid method for sample preparation when performing TXRF analysis. In addition, the

small samples required for analysis were not decomposed and remained stable on the quartz reflector plate surface. The technique affords a reliable and simple method of calibration, and satisfactory results (within normal experimental error), in comparison with those of the ICP method and with reference values, have been obtained.

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Interlaboratory Determination of Copper, Chromium and Arsenic in Timber Treated With Preservative

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Precision analyses of timber treated with preservative are an important part of quality control in the New Zealand timber preservation industry. Determinations of Cu, Cr and As remain important criteria in this industry. Repeatability and reproducibility values for two interlaboratory trials are calculated. The repeatability for the Rotorua interlaboratory trial ranged from 0.0109 to 0.0497% for Cu, 0.0190 to 0.600% for Cr and 0.0121 to 0.0346% for As, while reproducibility values ranged from 0.0121 to 0.0663% for Cu, 0.0389 to 0.1951% for Cr and 0.0342 to 0.1298% for As. The Queensland interlaboratory trial also produced repeatability and reproducibility values similar to those for the Rotorua trial. Significant differences between laboratories were found for the determination of Cu, Cr and As. Overall, the data from both interlaboratory trials were similar. Increased awareness of quality assurance programmes is seen as essential to improve the performance of laboratories involved in the timber preservation industry.

Keywords: *Copper, chromium and arsenic determination; wood; interlaboratory trial; precision; quality assurance*

Copper, chromium and arsenic (CCA) have excellent fungicidal (Cu) and insecticidal (As) properties in the treatment of wood.[1] The primary role of Cr^{VI} is in the fixation of Cu and As in the wood. These properties have been responsible for CCA preservatives being the most used worldwide for several decades.

The importance of CCA preservatives in the New Zealand timber preservation industry is shown by the New Zealand Forest Service statistics.[2] Out of a total of 1.4×10^6 m^3 of timber treated, over 1×10^6 m^3 were treated with CCA preservatives. The industry, therefore, places a high reliance on accurate determinations of the CCA components.

The maintenance of national or industry standards of timber preservation is monitored by quality management programmes involving treatment plants, the industry, auditors and analytical laboratories.

Analytical techniques that have been employed to determine CCA in the preservation industry include atomic absorption spectrometry (AAS),[3–5] iodimetry,[6] spectrophotometry,[7] X-ray fluorescence spectrometry,[8,9] inductively coupled plasma atomic emission spectrometry[10] and neutron activation analysis.[11] There are also several national standard methods available.[12–17] The micro-distribution of CCA in various wood tissues has also been studied using energy dispersive X-ray analysis.[18]

Industrial reliance on determinations of CCA has economic ramifications. To support the timber preservation industry, it was decided to conduct an interlaboratory trial on the determination of CCA, in various laboratories, primarily to establish precision data and variability of analytical methods. The data analysis was carried out following two methods in order to compare their usefulness for application to analysing interlaboratory trials. Data from a second interlaboratory study on CCA in wood were also analysed.

Experimental

Design of the Interlaboratory Programme for the Determination of CCA in Treated Timber

The design of the interlaboratory trial followed recommendations contained in the International Standard ISO 5725.[19] Fourteen New Zealand, Australian and Fijian laboratories involved with the timber preservation industry were invited to participate. A written method was supplied and laboratories were asked to follow this method closely if it was similar to their normal laboratory method. If their normal method was dissimilar (*e.g.*, methods based on different physical principles), they should use their own method and provide a brief description of it.

Eighteen samples of *Pinus radiata* sapwood treated with CCA compounds [0.05–0.48% of Cu, 0.09–0.84% of Cr and 0.06–0.60% of As (all m/m)] were analysed. These concentrations span the range of preservative retentions expected in timber when treated with CCA in accordance with current New Zealand specifications.[20] The samples represented six concentrations to be determined in triplicate under repeatable conditions.[19] (Raw analytical data are available on request.)

Preparation of Treated Timber Samples

A solution containing AnalaR grade $CuSO_4 \cdot 5H_2O$, $Na_2Cr_2O_7 \cdot 2H_2O$ and As_2O_3 was prepared. Six aliquots of this solution were adjusted to a constant volume (75 ml) before addition to 150.0 g of milled (Wiley mill), oven-dried (105 °C) *Pinus radiata* sapwood. Each mass of timber was then mixed, frozen and freeze-dried, before being ground in a large stainless-steel ring mill to produce a timber flour and finally riffled ten times through a 12-channel riffle to ensure the homogeneity of each sample (see below). Sample lots of 3–6 g were distributed in plastic screw-cap jars identified by a laboratory/sample code. The laboratories were informed of the broad concentration ranges of the samples.

Analytical Methods Used by Participating Laboratories

Eleven laboratories responded to the invitation to participate in the interlaboratory trial. Three of these laboratories requested extra sample sets and performed the analyses by two methods. A brief description of the methods used by each laboratory for the determination of CCA is given in Table 1.

The method used by laboratory C was the method supplied. Six laboratories followed this method and two others, F and L, had seemingly minor deviations. The techniques employed included digestion followed by titration, digestion or extraction, followed by AAS and X-ray fluorescence spectrometry.

Results and Discussion

Method Validation

For many years the supplied method of analysis (*e.g.*, laboratory C, Table 1) has been used satisfactorily in our laboratory. The method consists essentially of two parts:

digestion of the sample and determination of the CCA analytes. Although several standard methods exist for each part of the analysis, and these standards constitute a method validation in themselves, there are several points that should be considered in the context of the present interlaboratory trial. Two of these are the limit of detection and 95% confidence intervals.

Limit of detection

Fifty analyses of a homogenized bulk of treated wood (means of 0.095% of Cu, 0.166% of Cr and 0.108% of As) gave relative standard deviations (RSDs) of 1.8, 2.9 and 3.2% for Cu, Cr and As, respectively. The RSD is an estimate of variability attributable to sample heterogeneity and the use of the method of analysis at the operational level. For single subsequent analyses involving use of these precision data, the limits of detection are 0.008% for Cu, 0.023% for Cr and 0.017% for As.[21]

Confidence limits

Based on the standard deviation of the 50 analyses the 95% confidence interval can be calculated.[21] The values are ±0.0034% of Cu, ±0.0096% of Cr and ±0.0070% of As for a single analysis (at the specified concentrations).

Sample contamination during preparation

A check was made on possible contamination of samples from the iron-alloy ring mill (86% Fe, 12% Cr, 2% Si/Mn and Cu <0.05%; classified as a D3 mill by the American Iron and Steel Institute) by determining Fe in the wood after grinding. Successive 50 g lots of Wiley milled *Pinus radiata* were ring-milled from 2 to 8 min. There was no trend in the Fe content of the milled wood ($n = 8$, mean = 34 μg g^{-1}, standard deviation = 5 μg g^{-1}). When the wood was not ground, the Fe content was 14 μg g^{-1}. Therefore, maximal Cr contamination (based on the mill's composition) would be less than 5 μg g^{-1} (with Cu and As being much less). These levels are negligible compared with CCA concentrations in wood treated with preservative.

Table 1 Brief description of the methods of analysis for each laboratory—Rotorua trial

Laboratory	Method
A, B	Cu, iodimetric titration with thiosulphate following digestion of sample with H_2O_2–H_2SO_4 (3 + 1) Cr, back-titration of excess of ammonium iron(II) sulphate with standard dichromate following digestion of sample with H_2O–H_2SO_4 (3 + 1) As, titration with standard cerium(IV) nitrate following digestion of sample with H_2O_2–H_2SO_4 (3 + 1)
C, D, I, J, M, P	Modification of American Wood Preservers Association Standard A7-75.* Digestion of sample with H_2O_2–H_2SO_4 (3 + 1). No perchloric acid added to digested sample. Determination by AAS
E	Portable XRF (Asoma LCA). Calibrated each day with manufacturer's calibration disk
F	As for Laboratory C, except different preparation of standards used for AAS
O	Digestion with HNO_3–$HClO_4$ (5 + 1) on temperature-programmable digestion block. Analysis by AAS
H, N	British Standard 5666: Part 3. Method 1†
L	As for Laboratory C except temperature-programmable digestion block used. Require H_2O_2–HNO_3 than expected to affect digestion

* Reference 14.
† Reference 13.

These points indicate that the method supplied to the laboratories can produce precise results and will, therefore, be a suitable method with which to compare the performance of the methods employed by the various laboratories.

Data From Queensland Interlaboratory Trial

For comparative purposes, data generated by the Queensland Forest Service[22] have been included in this paper. In this trial, duplicates of six samples were sent to nine laboratories worldwide, all of which analyse large numbers of treated timber samples annually. Sample treatment included grinding to pass through a 1 mm mesh. The sample concentration range was less than that for the Rotorua interlaboratory trial. Analyses by participating laboratories involved a range of methods; there was no recommended method of analysis.

Statistical Analyses

Statistical analyses, based on the ISO 5725[19] and the Analytical Methods Committee (AMC)[23,24] procedures for calculating the repeatability (for within-laboratory variation, denoted by subscript e) and reproducibility (for between-laboratory variation, denoted by subscript L) and for checking outliers, were carried out, together with conventional data exploratory analysis.

As every single test result (y_{ij}) is assumed to be the sum of a general mean (u), laboratory variation (b_i) and random error (e_{ij}) occurring in every test, the basic model in the present study can be written as:

$$y_{ij} = u + b_i + e_{ij}$$

where $i = 1 \ldots p$ laboratories, and $j = 1 \ldots n$ replications in each laboratory (in this trial $p = 14$ and $n = 3$).

An analysis of variance (ANOVA) was performed to estimate the interlaboratory performances and to determine variance components as given in Table 2. The repeatability value (r) and reproducibility value (R) were calculated[19] from the variance components of the mean squares of the ANOVA as follows:

$r = 2.8\ \sigma_r$ where $\sigma_r = \sqrt{\sigma_e^2}$ = repeatability standard deviation

$R = 2.8\ \sigma_R$ where $\sigma_R = \sqrt{\sigma_L^2 + \sigma_e^2}$ = reproducibility standard deviation

and

$$\sigma_L^2 = \frac{MS_L - \sigma_e^2}{n}$$

There was a difference between the ISO 5725 and the AMC model in the procedure for outlier checking. The ISO 5725 model identifies outliers, by the Cochran and Dixon tests, and advises their removal before calculating r and R. The AMC model, on the other hand, tests for outliers only to allow correction of spurious values and to identify outlying laboratories.

A major concern of the AMC is the possibility of underestimating the variability encountered in routine analyses by rejecting outliers, thereby reducing the variability of results. They contended that this was effectively providing variability parameters for perfect analytical conditions. In this trial, the tests for outliers were carried out following both models. The outliers identified by the Cochran and Dixon tests were,

Table 2 Analysis of variance (ANOVA) to estimate interlaboratory performance and to determine variance components

Source of variation	Degrees of freedom	Mean square	Variance components of mean squares
Between laboratories	$p-1$	MS_L	$\sigma_e^2 + n\sigma_L^2$
Within laboratories	$p(n-1)$	MS_e	σ_e^2

however, not removed prior to data analysis, in order to produce realistic values of the precision parameters.

The other statistical procedure was to apply a least significant difference (LSD) test following an ANOVA, when the result was significant.

Interlaboratory Trial Results

The data submitted by participating laboratories for CCA in timber in the Rotorua trial were tested for normal distribution and skewness. All data were found to be normally distributed with homogeneous variance and no skewing.

Determination of Precision Parameters

Although a standard test method was supplied to all laboratories, the variety of methods used was expected. It is still of value, however, to determine the repeatability and reproducibility values. The r and R values were calculated from raw data, following ISO 5725.[19]

The mean, r and R values for CCA for the Rotorua trial are given in Table 3. The repeatability and reproducibility values are used to test the difference between two analytical results from the same laboratory and from different laboratories, respectively. The ranges in r values were 0.0104–0.0497% for Cu, 0.0190–0.0600% for Cr and 0.0121–0.0418% for As. As a check on these values, there are 252 possible differences between individual results in one laboratory (14 laboratories × 6 concentrations × 3 possible differences per cell) for Cu and Cr, and 248 for As (which has two cells with a member missing). There are 21 (Cu), 19 (Cr) and 16 (As) differences exceeding the r values [or 8.3% (Cu), 7.5% (Cr) and 6.5% (As) of differences], which is a result compatible with a 95% probability level.

The R values range from 0.0121 to 0.0663% for Cu, 0.0389 to 0.1951% for Cr and 0.0342 to 0.1298% for As. The 95% critical difference (Cr D_{95}) can be used to compare laboratories performing analyses[19] (Table 3). Of the 546 differences between the means of each laboratory (14 laboratory means have 91 possible differences per concentration and there are six concentrations), 29 for Cu, 19 for Cr and 49 for As exceed the critical difference values for the respective concentrations (or 5.3% for Cu, 3.5% for Cr and 9.0% for As). This again is consistent with a 95% probability level.

The r and R values in Table 3 can be used in comparisons within and between laboratories. It is probable that in a collaborative trial (where a method is specified) lower r and R values would result than in a co-operative trial (where there is no method specified).

The raw data for the Queensland trial were also normally distributed with homogeneous variance and no skewness. The mean, r and R values for CCA are presented in Table 4. The ranges in r values were 0.0011–0.0159% for Cu, 0.0066–0.0252% for Cr and 0.0095–0.0265% for As. As a check on these r values, there are 54 possible differences between individual results in each laboratory (9 laboratories × 6 concentrations × 1 possible difference per cell) for each element. Therefore, there are one (Cu), two (Cr) and three (As) [or 1.9% (Cu), 3.7% (Cr) and 5.6% (As)] differences exceeding the corresponding r value, which is compatible with a 95% probability level for r. The R values span the ranges 0.0148–0.0454% (Cu), 0.0136–0.0512% (Cr) and 0.0267–0.0876% (As). Using the 95% critical differences (Table 4) to compare laboratories, of the 216 possible differences between the means of each cell for each laboratory (9 laboratories have 36 possible differences per concentration), eight (Cu), nine (Cr) and eight (As) [or 3.7% (Cu), 4.2% (Cr) and 3.7% (As)] exceed the appropriate 95% critical difference. This is consistent with a 95% probability level for R.

The values of r and R for both the Rotorua and Queensland trials are plotted in Fig. 1. The equations expressing r and R as a function of concentration are presented in Table 5. Although the concentration range for the Queensland data is about half that for the Rotorua trial, the r and R values are very similar functions of concentration except for Cr and As, where the Queensland r values are not linear functions of concentration. The values of r and R are similar for both trials, suggesting that laboratories participating in both trials have the same extreme

Table 3 Values for repeatability and reproducibility (g of metal per 100 g of wood) for Rotorua data

	Mean	σ_r	r_{95}	RSD(%)	σ_R	R_{95}	Cr D_{95}*
Cu	0.048	0.0039	0.0109	8.1	0.0043	0.0121	0.0081
	0.094	0.0037	0.0104	3.9	0.0053	0.0148	0.0121
	0.189	0.0097	0.0271	5.1	0.0110	0.0307	0.0213
	0.272	0.0078	0.0219	2.9	0.0144	0.0404	0.0362
	0.357	0.0097	0.0271	2.7	0.0198	0.0556	0.0510
	0.440	0.0177	0.0497	4.0	0.0237	0.0663	0.0524
Cr	0.087	0.0108	0.0302	12.4	0.0139	0.0389	0.0301
	0.177	0.0068	0.0190	3.8	0.0169	0.0472	0.0446
	0.349	0.0163	0.0457	4.7	0.0294	0.0822	0.0732
	0.507	0.0106	0.0297	2.1	0.0453	0.1267	0.1244
	0.666	0.0187	0.0523	2.8	0.0633	0.1773	0.1721
	0.824	0.0214	0.0600	2.6	0.0697	0.1951	0.1888
As	0.056	0.0055	0.0155	9.8	0.0150	0.0419	0.0399
	0.119	0.0043	0.0121	3.6	0.0122	0.0342	0.0327
	0.231	0.0082	0.0225	3.5	0.0167	0.0468	0.0430
	0.339	0.0118	0.0332	3.5	0.0333	0.0934	0.0894
	0.449	0.0149	0.0418	3.3	0.0357	0.1001	0.0941
	0.553	0.0124	0.0346	3.3	0.0464	0.1298	0.1267
As†	0.056	0.0037	0.0103	6.6	0.0147	0.0413	—
	0.116	0.0043	0.0122	3.7	0.0084	0.0236	—
	0.228	0.0081	0.0226	3.6	0.0135	0.0379	—
	0.331	0.0121	0.0339	3.7	0.0178	0.0498	—
	0.443	0.0148	0.0415	3.3	0.0299	0.0838	—
	0.542	0.0177	0.0328	3.3	0.0182	0.0509	—

* Cr $D_{95}(|Y_1 - Y_2|) = \sqrt{R^2 - \frac{2r^2}{3}}$ (Reference 19).

† Excluding Laboratory O data.

Table 4 Values of repeatability and reproducibility (g of metal per 100 g of wood) for Queensland data

	Mean	σ_r	r_{95}	RSD (%)	σ_R	R_{95}	Cr D_{95}*
Cu	0.031	0.0024	0.0066	7.7	0.0053	0.0148	0.0140
	0.037	0.0004	0.0011	1.1	0.0063	0.0176	0.0175
	0.090	0.0044	0.0124	4.9	0.0075	0.0209	0.0189
	0.135	0.0034	0.0096	2.5	0.0065	0.0183	0.0170
	0.175	0.0057	0.0159	3.3	0.0151	0.0424	0.0409
	0.223	0.0051	0.0142	2.3	0.0162	0.0454	0.0442
Cr	0.056	0.0042	0.0118	7.5	0.0049	0.0136	0.0108
	0.049	0.0024	0.0066	4.9	0.0089	0.0251	0.0246
	0.152	0.0037	0.0102	2.4	0.0082	0.0230	0.0218
	0.224	0.0049	0.0138	2.2	0.0094	0.0263	0.0244
	0.258	0.0090	0.0252	3.5	0.0178	0.0498	0.0465
	0.379	0.0040	0.0111	1.1	0.0183	0.0512	0.0506
As	0.062	0.0034	0.0095	5.5	0.0095	0.0267	0.0259
	0.028	0.0034	0.0096	12.1	0.0109	0.0304	0.0296
	0.183	0.0059	0.0166	3.2	0.0166	0.0465	0.0450
	0.272	0.0031	0.0087	1.1	0.0220	0.0617	0.0614
	0.296	0.0095	0.0265	3.2	0.0313	0.0876	0.0856
	0.250	0.0048	0.0133	1.9	0.0209	0.0584	0.0576

* Cr D_{95} ($|Y_1 - Y_2|$) = $\sqrt{R^2 - \frac{r^2}{2}}$ (Reference 19).

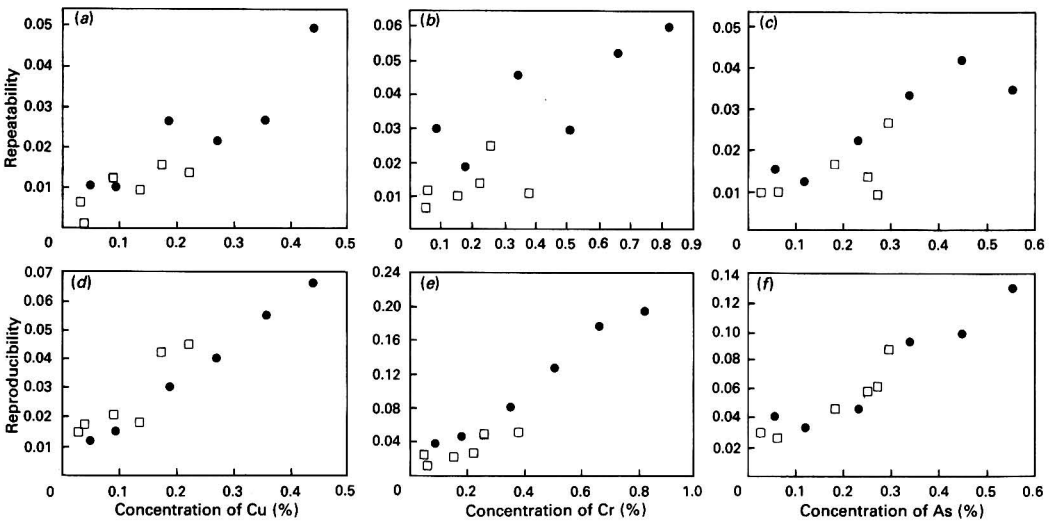

Fig. 1 Plots of repeatability and reproducibility for Cu, Cr and As as a function of concentration for both the Rotorua and Queensland data (●, Rotorua data; and □, Queensland data)

Table 5 Repeatability and reproducibility, parameters from the Rotorua and Queensland trials, as a function of concentration

	Rotorua	Coefficient of determination*	t_{slope}†	Queensland	Coefficient of determination*	t_{slope}†
Cu	r = 0.08185 × concentration + 0.00510	81.2	A	r = 0.05777 × concentration + 0.00332	66.7	A
	R = 0.14297 × concentration + 0.00329	99.5	B	R = 0.15747 × concentration + 0.00842	79.4	A
Cr	r = 0.04499 × concentration + 0.01991	66.9	A		—	ns
	R = 0.23014 × concentration + 0.01112	98.3	B	R = 0.10418 × concentration + 0.01209	74.2	A
As	r = 0.05514 × concentration + 0.01056	81.3	A		—	ns
	R = 0.19366 × concentration + 0.01798	91.9	B	R = 0.18395 × concentration + 0.01845	85.0	B

* Coefficient of determination (per cent. of variation due to regression; is the square of the correlation coefficient).
† t-Test for slope difference from zero: A = significant at the 5% level; B = significant at the 1% level; ns = not significant.

value ranges and similar sizes of errors within laboratories and between laboratories. According to the AMC[23] the variation between batches is usually of a comparable size to σ_e and σ_L (which can be calculated from r and R values) and, therefore, the maximum error for any further analytical determination, as estimated by σ_e and σ_L, would be an underestimation of the true error. An advantage of using σ_e and σ_L values as opposed to r and R values is that the source of the components of error in measurement can be directly seen.

When ANOVA was applied to both the Rotorua and Queensland raw data (Rotorua data not including target values) for CCA, there were only three instances (all Cu)

where ANOVA was not significant at the 1% level. When considering, firstly, the Rotorua data for all concentrations combined, the LSD tests for CCA all show differences in the means of each laboratory (Table 6). The methods used for analysis (Table 1) can be divided into one group of six laboratories (C, D, I, J, M and P) and two groups of two laboratories (A, B and H, N), the remainder of the methods being used by only one laboratory. The method used by the largest group of laboratories (the supplied method) has not been applied with uniform results. As such, the data from this method have not been analysed separately. For the method used by Laboratories A and I, the results for Cr were significantly different, but not for Cu and As. The use of the BS 5666 Method[13] (Laboratories H and N) achieved similar results for each element.

From the above observations it appears that the BS 5666 method[13] is possibly the most precise (a greater number of laboratories using this method would be necessary to confirm this). This method: (1) is a leaching method rather than a digestion method; (2) employs a temperature of 75 °C, which is less than that required for digestion; and (3) uses a leaching time of 30 min in contrast to many digestions where heating can be prolonged; a further point that may be relevant is the addition of sodium sulphate to the solutions of leachates and standards for atomic absorption analysis. These points could be responsible for ensuring more control over sample preparation and analysis and hence improved precision. The greater variation of data obtained with the other methods offers an opportunity for improvement in precision.

The LSD tests for CCA for the Queensland data, with all concentrations combined (Table 7), also suggest different performance by different methods.

Table 6 Least significant difference test (5% level) for CCA on Rotorua raw data. Means with a bar in the same column are not significantly different

	Laboratory	Mean	
Cu	B	0.250	
	C	0.243	
	D	0.242	
	F	0.239	
	O	0.239	
	N	0.238	
	M	0.238	
	H	0.237	
	J	0.229	
	L	0.225	
	I	0.224	
	E	0.223	
	P	0.221	
	A	0.220	
			LSD = 0.010
Cr	J	0.495	
	M	0.489	
	I	0.471	
	O	0.459	
	L	0.458	
	C	0.449	
	D	0.427	
	F	0.424	
	N	0.416	
	H	0.411	
	A	0.410	
	B	0.405	
	P	0.397	
	E	0.380	
			LSD = 0.032
As	O	0.358	
	D	0.300	
	F	0.298	
	I	0.295	
	N	0.295	
	A	0.294	
	J	0.291	
	H	0.289	
	L	0.284	
	P	0.282	
	M	0.280	
	C	0.276	
	E	0.273	
	B	0.262	
			LSD = 0.022

Determination of Statistical Outliers

Stragglers and outliers in both data sets were identified following recommendations of both ISO 5725 and the AMC. When using the Cochran and Dixon tests (ISO method), several stragglers and outliers were found (Table 8) (stragglers and outliers are data points significant at the 5 and 1% levels, respectively). Also included in Table 8 are points lying more than three standard deviations from the mean of each concentration. From the results of these three tests, it is clear that coincidence of straggler/outlier identification was unusual. None of the stragglers/outliers could be tagged as spurious as could have occurred from transcription errors or an order of magnitude error. For ANOVA stragglers were retained, while outliers were discarded and were replaced with values generated from a missing value estimation. Statistical outliers found for the Queensland data were from Laboratory D (Cu), lowest concentration (0.040% of Cu), and Laboratory E (Cr), second lowest concentration (0.070% of Cr).

In using Cochran's test, based on variance ratio, only the signficant concentration level is identified. The variance for each cell is then inspected and the cell with the highest variance is flagged as the outlier. The offending member of the cell is then found by inspection. This identification can sometimes be ambiguous and often Cochran's test needs to be re-applied.

Table 7 Least significant difference test (5% level) for CCA in the Queensland samples. Means with a bar in the same column are not significantly different

	Laboratory	Mean	
Cu	I	0.125	
	G	0.123	
	D	0.118	
	A	0.117	
	C	0.117	
	F	0.114	
	H	0.111	
	E	0.109	
	B	0.102	
			LSD = 0.009
Cr	G	0.199	
	C	0.194	
	F	0.188	
	H	0.187	
	I	0.186	
	A	0.184	
	D	0.183	
	E	0.183	
	B	0.172	
			LSD = 0.011
As	G	0.218	
	F	0.187	
	I	0.186	
	A	0.185	
	H	0.183	
	D	0.181	
	E	0.173	
	C	0.167	
	B	0.158	
			LSD = 0.013

Following the AMC approach,[23,24] values of α_i and α_{ij} statistics were calculated; α_i values greater than 2.5 identify a laboratory with data significantly different to the grand mean. The α_{ij} value is a within-concentration cell statistic, indicating variability within a laboratory. Values of α_i and α_{ij} greater than 2.5 suggest a systematic laboratory bias or a discrepant replication, respectively (Table 9). For As, the α_i values were significant for three of the six concentrations for Laboratory O; Laboratory O was therefore tagged as being an outlying laboratory, and the As data were re-analysed without the Laboratory O data. Twelve values of α_{ij} for Cu and Cr were greater than 2.5 as were nine for As; without Laboratory O data for As, six values were greater than 2.5.

When α_i and α_{ij} for the Queensland data were calculated, one α_i was greater than 2.5, namely, 2.57 (Cu, Laboratory B; mean concentration 0.223% of Cu). The significant α_{ij} values were 2.99 (Cu, Laboratory D; 0.030 and 0.040% of Cu; lowest concentration cell) and 2.99 (Cr, Laboratory E; 0.060 and 0.070% of Cr; second lowest concentration cell). No α_i and α_{ij} values for As were greater than 2.5.

Again there was a lack of agreement between outliers that resulted from tests following either the ISO 5725 or the AMC method. The AMC calculation was more sensitive in that for the Rotorua data it picked 33 points as outliers compared with 16 points for the ISO method.

Comparison With Nominal Target Values

Comparison of the laboratory analyses with the nominal target values was assessed in two ways. Firstly, the recovery of metal from the samples was considered. Secondly, with outliers identified by the Cochran and Dixon mean $\pm 3\sigma$ tests removed, ANOVA was carried out. Least significant difference tests were performed when ANOVA was significant.

By using the mean values obtained from determinations of each concentration level, it was found that 95% (Cu), 100% (Cr) and 93% (As) [or 91% (As) if Laboratory O data were excluded] of the nominal target concentrations were recovered (Table 10).

The LSD tests were employed to compare the means obtained by each laboratory for each element. In Table 11, the LSD test is for combined concentration data. Of the six laboratories, three were significantly above and three significantly below the nominal target values for Cr. For As, only one laboratory was above the nominal target value with eight below, while for Cu, no laboratories were above the nominal

Table 8 Identification of stragglers and outliers in the Rotorua data [ISO method (reference 19)]

	Mean	Cochran's test	Dixon's test	Mean $\pm 3\sigma$	Outlier
Cu	0.048	ns*	ns (M)	(0.032; M)†	M (0.032)
	0.094	ns	—	ns	—
	0.189	(A)‡	ns (A); (P)‡	(0.152; P)†	P (0.152)
	0.272	ns	—	ns	—
	0.357	(P)†	ns (P)	ns	P (0.312)
	0.440	(M)†	ns (M)	ns	M (0.383)
Cr	0.087	(M)†	ns (M)	(0.040, 0.036; M)†	M (0.040, 0.036)
	0.177	ns	—	ns	—
	0.349	ns	—	ns	—
	0.507	ns	—	ns	—
	0.666	(P)†	ns (P)	ns	P (0.562)
	0.824	(A)†	ns (A)	ns	A (0.724)
As	0.056	(O)†	ns (O)	ns	O (0.085)
	0.119	(C)†	ns (C)	ns	C (0.102)
	0.231	(C)†	ns (C)	(0.177; C)†	C (0.177)
	0.339	(C)†	ns (C)	(0.439, 0.446; O)†	C (0.277); O (0.439, 0.446)
	0.449	ns	—	ns	—
	0.553	(B)‡	(B)‡	(0.772, 0.713; O)†	O (0.712, 0.713)

* ns = not significant.
† Significant at 1% level.
‡ Significant at 5% level.

Table 9 Values of α_i and α_{ij} for CCA from the Rotorua data

	Cu		Cr		As		As (excluding Laboratory O data)	
Concentration level	α_i (Lab.)	α_{ij} (Lab.; concentration)	α_i (Lab.)	α_{ij} (Lab.; concentration)	α_i (Lab.)	α_{ij} (Lab.; concentration)	α_i (Lab.)	α_{ij} (Lab.; concentration)
1	2.62 (M)	2.72 (B; 0.043)	2.66 (M)	2.61 (M; 0.036)	2.87 (B)	3.63 (O; 0.085)	2.79 (B)	—
	—	2.62 (M; 0.032)	—	4.77 (M; 0.101)	—	3.34 (O; 0.053)		
2	—	2.75 (M; 0.101)	—	2.71 (M; 0.193)	2.80 (O)	3.51 (C; 0.102)	—	3.48 (C; 0.102)
	—	—	—	2.71 (M; 0.223)	—			
3	—	2.92 (A; 0.169)	—	2.98 (I; 0.331)	—	2.59 (C; 0.216)	2.62 (C)	2.63 (C; 0.216)
	—	2.92 (A; 0.215)	—	3.03 (P; 0.268)	—	3.24 (C; 0.177)	—	3.29 (C; 0.177)
	—	2.83 (P; 0.152)	—	—	—	2.74 (I; 0.193)	—	2.78 (I; 0.193)
4	—	2.87 (A; 0.267)	—	2.58 (J; 0.603)	3.23 (O)	2.52 (A; 0.381)	—	3.68 (C; 0.335)
	—	2.92 (B; 0.314)	—	—	—	3.76 (C; 0.335)	—	—
5	—	2.57 (P; 0.312)	—	2.54 (A; 0.654)	—	—	—	—
	—	3.50 (P; 0.360)	—	2.84 (A; 0.572)	—	—	—	—
	—	—	—	3.43 (P; 0.647)	—	—	—	—
6	—	4.17 (M; 0.502)	—	2.63 (A; 0.724)	3.41 (O)	3.21 (B; 0.552)	—	3.38 (B; 0.552)
	—	4.05 (M; 0.383)	—	4.51 (A; 0.849)	—	—	—	—

Table 10 Relative standard deviations, recovery of nominal target samples and 95% confidence limits (CL) for both trials (g of metal per 100 g of wood unless other units given)

	Rotorua					Queensland		
	Mean	RSD_R(%)	Nominal target	Recovery (%) (mean × 100/nominal target)	CL	Mean	RSD_R(%)	CL
Cu	0.048	9.0	0.050	96	0.046–0.049	0.031	17.1	0.028–0.033
	0.094	5.5	0.099	96	0.093–0.096	0.037	17.0	0.034–0.040
	0.189	5.8	0.196	98	0.185–0.192	0.090	8.3	0.086–0.093
	0.272	5.2	0.291	94	0.267–0.276	0.135	4.8	0.132–0.139
	0.357	5.4	0.384	93	0.351–0.363	0.175	8.6	0.167–0.182
	0.440	5.3	0.476	93	0.433–0.448	0.223	7.3	0.215–0.231
				95 ± 2				
Cr	0.087	15.9	0.088	102	0.082–0.091	0.056	8.8	0.053–0.058
	0.177	9.3	0.174	102	0.172–0.182	0.049	18.2	0.045–0.053
	0.349	8.3	0.345	101	0.340–0.358	0.152	5.4	0.148–0.156
	0.507	8.7	0.512	99	0.494–0.521	0.224	4.2	0.219–0.228
	0.666	9.3	0.675	99	0.647–0.686	0.258	6.9	0.249–0.266
	0.824	8.3	0.835	99	0.803–0.845	0.379	4.8	0.370–0.388
				100 ± 2				
As	0.056	26.1	0.063	89	0.052–0.061	0.062	15.3	0.057–0.067
	0.119	10.0	0.125	96	0.115–0.123	0.028	38.9	0.023–0.034
	0.231	7.1	0.247	94	0.226–0.236	0.183	9.1	0.175–0.191
	0.339	9.6	0.366	93	0.329–0.349	0.272	8.1	0.261–0.283
	0.449	7.8	0.483	93	0.438–0.460	0.296	10.6	0.281–0.312
	0.553	8.2	0.598	93	0.539–0.567	0.250	8.4	0.240–0.260
				93 ± 2				
As*	0.056	23.4	0.063	89	0.052–0.060			
	0.116	7.1	0.125	93	0.114–0.119			
	0.228	5.9	0.247	92	0.224–0.233			
	0.331	5.3	0.366	90	0.326–0.337			
	0.443	6.6	0.483	92	0.434–0.453			
	0.542	3.3	0.598	91	0.536–0.548			
				91 ± 1				

* Excluding Laboratory O data.

target value and eight laboratories were below. This spread of results is consistent with the recoveries of metal obtained from the participating laboratories.

Quality Assurance

The ranges of the results as a percentage of the grand mean for Cu, Cr and As are 12.8, 26.4 and 32% for the Rotorua data and 20.3, 14.0 and 32.9% for the Queensland data, respectively; if Laboratory O is omitted for the Rotorua As data, the range is 13.2% and similarly without Laboratory G for Queensland As data, the range is 15.8%. These ranges are very broad. Both trials appear to have produced, overall, comparable performances for As, but the Rotorua data are less spread for Cu whereas the Queensland Cr data are less variable. This behaviour is also seen in the RSD (Table 10). The 95% confidence limits (Table 10) for both data sets summarize the most likely range for the concentrations of CCA in samples.

The information on variability found in this study has shown that there is room for improvement in analyses. One outlying laboratory was identified in addition to a large number of statistical outliers. Such outcomes detract from the confidence of analysis for CCA in timber treated with preservative. Uncertainties in analyses have ramifications in the timber industry both in terms of adequate preservative levels for timber protection and in the commercial operations of preservative companies and timber-treatment sites. The most satisfactory conclusion from this study would be an increased awareness of the need for laboratories to operate quality management and quality assurance programmes.

The precision parameters produced in this work should be used by analysts in preservative analysis as minimum guidelines to acceptable precision. The accuracy of analyses for CCA in timber is one area that remains a major concern. Only nominal target values were available in the Rotorua study. The mean values produced, averaged over all laboratories, provide some information on concentrations and therefore recovery of metal in the samples. There is a lack of certified reference materials in the area of timber treated with preservative generally. Such materials would facilitate an evaluation of the accuracy of different analytical methods and allow bias of various methods to be addressed directly.

Recommendations on quality programmes by timber-preservation laboratories have been presented previously.[25,26] The same general principles can be applied in the analytical procedures for the determination of CCA in timber.

Conclusion

The ISO 5725 and AMC methods have been discussed with reference to analysing analytical data from two interlaboratory trials for the determination of CCA in timber. A proposed method of analysis was supplied to laboratories in the Rotorua trial and was employed by a minority of laboratories. Results from this method did not appear to suggest improvements in precision that may have been expected from the operation of a standardized method. In the Queensland trial there was no proposed method. Values of repeatability and reproducibility for the two trials were calculated and compared and statistical outliers were identified. Comparisons of the experimental data with nominal target values for CCA in the Rotorua trial showed good agreement for Cr, but less agreement for Cu and As. Finally, the continued application of quality assurance programmes is

Table 11 Least significant difference test (5% level) for CCA—data for all concentrations combined—Rotorua data. Means with a bar in the same column are not signficantly different

	Laboratory	Mean	
Cu	B	0.250	
	Nominal target	0.249	
	M	0.243	
	C	0.243	
	D	0.242	
	F	0.239	
	O	0.239	
	N	0.238	
	H	0.237	
	J	0.229	
	L	0.225	
	P	0.225	
	I	0.224	
	E	0.223	
	A	0.220	
			LSD = 0.011
Cr	M	0.496	
	J	0.495	
	I	0.471	
	O	0.459	
	L	0.458	
	C	0.449	
	Nominal target	0.438	
	D	0.427	
	F	0.424	
	N	0.416	
	A	0.414	
	H	0.411	
	B	0.405	
	P	0.399	
	E	0.380	
			LSD = 0.029
As	O	0.351	
	Nominal target	0.314	
	D	0.300	
	F	0.298	
	I	0.295	
	N	0.295	
	A	0.294	
	J	0.291	
	H	0.289	
	L	0.284	
	P	0.282	
	C	0.281	
	M	0.280	
	E	0.273	
	B	0.262	
			LSD = 0.020

of prime importance in continually improving the analytical performance of the participating laboratories.

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Comparison of Microwave and Conventional Extraction Techniques for the Determination of Metals in Soil, Sediment and Sludge Samples by Atomic Spectrometry

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A description is given of an extraction method for the determination of metals in sediment and soil samples using *aqua regia* [HCl–HNO_3 (3 + 1)] and a microwave oven. The elements Cd, Cr, Cu, Fe, Mn, Pb and Zn were measured in the solutions by means of atomic absorption spectrometry (flame or electrothermal) and inductively coupled plasma atomic emission spectrometry. The results for the analysis of six reference materials after microwave *aqua regia* extraction showed close agreement with the stated values. A comparison between microwave extraction and conventional reflux extraction for 30 samples showed that the former method gives the same or slightly higher results for the seven elements tested in sediments, soil and suspended matter.

Keywords: *Microwave extraction; reflux extraction; sediment; soil; atomic spectrometry*

Extraction is an important but generally very time-consuming stage in the determination of metals in sediment and soil samples by atomic spectrometry. Digestion methods are commonly used involving different mixtures of acids (HNO_3–HCl, HNO_3–H_2SO_4, HNO_3–$HClO_4$, *etc.*), occasionally in combination with HF in order to dissolve the silica matrix. For instance, in accordance with Netherlands NEN and German DIN standards,[1,2] metals in soil and sludge are extracted by heating the samples with *aqua regia* [HCl–HNO_3 (3 + 1)] in flasks fitted with reflux condensers. These methods, however, have several drawbacks. If open systems are used, there is a risk of atmospheric contamination and loss of volatile elements.

In order to avoid these problems, wet digestion is often carried out in polytetrafluoroethylene (PTFE) bombs.[3,4] As only a small amount of material can be used for this purpose, the sample needs to possess a high degree of homogeneity. Moreover, the bombs are not fitted with valves, and only a limited amount of energy can therefore be supplied, which might influence the extraction process.

A recently developed alternative[5–7] is the wet extraction technique which makes use of the rapid and attractive method of microwave heating. Microwave extraction has previously been tested extensively for organic-rich plant material,[8,9] using certified reference materials and real samples, and the method had also been compared with conventional digestion methods.

van de Wall *et al.*[10] have shown that for certain aquatic sediments and reference materials, reflux extraction in accordance with the Dutch national standard NEN 6465[1] and microwave extraction produce comparable results. van Delft and Vos[11] compared five extraction methods for measuring mercury in soil. They found close agreement between the results after microwave extraction and those after extraction with HNO_3 in a closed PTFE bomb and with neutron activation analysis.

In this paper, a microwave extraction method is described prior to the measurement of seven metals in different types of sediment and soil. These samples were taken from salt marshes, aquatic sediments, suspended matter (obtained with the aid of a sludge centrifuge), arable land, grassland and woodland. The experiments were carried out by three laboratories (A, B and C). Use was made of reference materials from the Community Bureau of Reference (BCR) and International Atomic Energy Agency (IAEA) and of real samples which were also digested by the conventional reflux extraction method.

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Experimental

Instrumentation

All sediment and soil samples were decomposed in a microwave oven (Model MDS-81D, CEM, Indian Trail, NC, USA), equipped with a microwave power range from 0 to 100% (600 W). The microwave oven was fitted with a rotary table on which a maximum of 12 120 ml perfluoroalkoxy (PFA) digestion vessels can be placed. The vessels were fitted with a pressure-relief valve and were sealed in a capping station. The microwave oven allows both time and energy to be programmed in a maximum of three steps.

Conventional extraction was carried out in Pyrex tubes or in flasks fitted with reflux condensers.[1,2]

The elements Fe, Mn and Zn were measured by laboratory A with a Perkin-Elmer Model 2380 atomic absorption spectrometer fitted with an air–acetylene burner. The elements Cd, Cu, Cr, and Pb were measured with a Perkin-Elmer Model 3030 instrument fitted with a Zeeman-effect background correction system, an HGA-600 furnace and an AS-60 autosampler. For the measurement of Cd, Cu and Pb, pyrolytic graphite coated graphite tubes were used, fitted with a L'vov platform. The measurements of Cd and Pb were carried out in accordance with the stabilized temperature platform furnace concept.[12–14]

In laboratory B the elements were determined with a Perkin-Elmer Model 600 inductively coupled plasma (ICP) atomic emission spectrometer with measurements for each element being carried out at two wavelengths.

Laboratory C carried out the Cd, Cu, Pb and Zn measurements in an air–acetylene flame, by using a Perkin-Elmer Model 5000 atomic absorption spectrometer. Cadmium was

measured following complexation with ammonium pyrrolidin-1-yldithioformate [ammonium pyrrolidinedithiocarbamate (APDC)] and extraction with isobutyl methyl ketone (IBMK). The elements Cr, Fe and Mn were measured with a Perkin-Elmer Model 6000 ICP instrument with measurements for each element again being carried out at two wavelengths.

In all instances, standardization was carried out by using a calibration graph. The standard solutions contained 12 ml of 37% HCl and 4 ml of 65% HNO_3 per 100 ml. Recoveries of standard solutions added to the samples prior to analysis were between 95 and 105%.

Table 1 gives the optimum instrumental conditions for the measurements.

Reagents

All chemicals were of analytical-reagent grade. Standard solutions of Cd, Cr, Cu, Fe, Mn, Pb and Zn were prepared from Merck Titrisol ampoules or BDH standard solutions (1000 mg l^{-1}). Nitric acid (65%) and hydrochloric acid (37%) were used for both microwave and conventional extraction.

For Cd and Pb, when measured with the Perkin-Elmer 3030 instrument, a chemical modifier was used consisting of 25 g of $NH_4H_2PO_4$ and 1.2 g of $Mg(NO_3)_2$ per litre.

For the Cd measurements in the flame, the element was first complexed with 1% m/v APDC solution at a pH of 7 and extracted with IBMK saturated with water.[15]

Table 1 Instrumental conditions for the determination of Cd, Cr, Cu, Fe, Mn, Pb and Zn

Graphite furnace conditions—

Element*	Wavelength/nm	Slit-width/nm	Charring temperature/°C	Cooling temperature/°C	Atomization temperature/°C
Cd	228.8	0.7	1100	—	1450
Cr	357.9	0.7	1350	—	2300
Cu	324.8	0.7	1200	30	2250
Pb	283.3	0.7	1150	—	1550

Flame conditions—

Element†	Wavelength/nm	Slit-width/nm
Cd	228.8	0.7
Cu	324.7	0.7
Fe	248.3	0.2
Mn	279.5	0.2
Pb	283.3	0.7
Zn	213.9	0.7

ICP conditions—

Plasma flow	15 l min^{-1}
Auxiliary flow	0.3–0.5 l min^{-1}
Sample uptake	1 ml min^{-1}
R.f. power	1250 W
Viewing height	15 mm
Laboratory B	'Crossflow' nebulizer, 180 kPa
Laboratory C	'V-groove' nebulizer, 170 kPa

Element	Wavelength/nm
Cd	214.438; 228.802
Cr	205.552; 267.716
Cu	223.008; 327.396
Fe	238.204; 239.562
Mn	257.610; 259.370
Pb	405.783; 220.353
Zn	202.548; 213.856

* Zeeman-effect background correction and pyrolytic graphite coated graphite tubes fitted with a L'vov platform were used.

† An air–acetylene flame and deuterium background correction were used.

Standards

Validation of the methods described here was performed by using four Certified Reference Materials (CRMs) from the Community Bureau of Reference (BCR) and two reference materials from the International Atomic Energy Agency (IAEA): BCR CRM 141 Soil-calcareous loam; BCR CRM 142 Soil-light sandy; BCR CRM 143 Sewage sludge-soil; BCR CRM 145 Sewage sludge; IAEA CRM SL-1 Lake Sediment; and IAEA CRM SOIL 7 Soil.

The BCR certificates report two sets of values: total trace metal content and the *aqua regia* soluble fractions. Throughout this study the *aqua regia* soluble values are used. The *aqua regia* values are not classified as 'certified'. The IAEA certificates report only the total trace metal content.

Procedures

Microwave dissolution

A 500 or 1000 mg portion of the (freeze or oven) dried and ground material was transferred into the 120 ml PFA vessels, and 16 ml of *aqua regia* (12 ml of 37% HCl and 4 ml of 65% HNO_3) were added. Before the vessels were sealed, they were first shaken until all the CO_2 from the $CaCO_3$ had escaped. During this study the following microwave programme was used: step 1, 30% power for 1 min; step 2, 80% power for 4 min; and step 3, 100% power for 60 min.

After the samples had been cooled, the PFA vessels were opened, the decomposition products were transferred into 50 or 100 ml polypropylene calibrated flasks and the solutions made up to volume with de-mineralized water. After homogenization, the solutions were filtered. Blanks were treated in the same way as the samples.

Conventional reflux extraction

A 2000 mg portion of the (freeze or oven) dried and ground material was transferred into a Pyrex tube, and 16 ml of *aqua regia* were added. Before placing a reflux condenser on the tube, it was first shaken until all the CO_2 from the $CaCO_3$ had escaped. The tube was then heated for 2 h, reflux cooling being used. Afterwards the coolers were rinsed, the decomposition products were transferred into 100 ml calibrated flasks and the solutions made up to volume with de-mineralized water. After homogenization, the solutions were filtered. Blanks were treated in the same way as the samples.

Results and Discussion

Optimization of the Microwave Extraction Method

Aqua regia was used as a decomposition reagent for both the microwave and conventional digestion procedures. For reflux extraction, 80–100% of the metals in soils and sewage sludges was dissolved with *aqua regia* compared with total digestion.[16,17] An optimum microwave oven programme was developed for the microwave extraction method. In order to increase the pressure in the PFA vessels gradually, a microwave programme consisting of three stages was used. In the third stage, the power supplied is set at 100%, which then permits optimization of the decomposition time.

As regards the extraction of a sludge sample from the river Maas, the duration of the final stage was varied by laboratory B from 10 to 100 min, with intermediate steps of 10 min. The results of these experiments[10] showed that after 60 min no further significant changes took place for the seven elements studied here. On this basis the following microwave programme was chosen: 1 min at 30% power, 4 min at 80% and 60 min at 100%.

When optimizing the microwave programme, the maximum pressure in the PFA vessels should not rise above 830 kPa otherwise the pressure-relief valve will vent and material might be lost from the vessels. When carrying out the various

microwave dissolutions with this microwave programme, the valves did not open. It should be noted that an optimum microwave programme should be developed for each type of microwave oven and for each type of matrix.

Accuracy of the Microwave Extraction Method

In order to ascertain the accuracy of the optimized microwave extraction method, laboratories A, B and C each digested two different CRMs, from the IAEA (CRMs SOIL 7 and SL-1) and the BCR (CRMs 141 and 142, and 143 and 145). Each laboratory measured Cd, Cr, Cu, Fe, Mn, Pb and Zn in the digested solutions. The results generally showed close agreement with the stated values for all seven elements in the reference materials (Table 2). In addition, laboratories B and C each extracted two reference materials using the conventional reflux method. These results showed that there were no significant differences between this method and the results after microwave extraction for Cd, Cu, Mn, Pb and Zn. For BCR CRMs 141 and 142 (laboratory C), significantly higher values were found for Cr and Fe after microwave extraction (Table 3). No explanation could be given for the high Pb content found in CRM 141 after microwave extraction.

In each series of 12 microwave extractions two blanks were included; these received the same treatment as the samples and were used to calculate standard deviations (SDs) and

Table 2 Results of the analysis of IAEA and BCR CRMs after microwave extraction with *aqua regia*. Results for Cd, Cr, Cu, Mn, Pb and Zn expressed in μg g^{-1}; results for Fe expressed in mg g^{-1}

Element	IAEA CRM SL-1*	This work, laboratory A†	IAEA CRM SOIL 7‡	This work, laboratory A†
Cd	0.26 ± 0.05	0.25 ± 0.02	1.3 (1.1/2.7)	1.22 ± 0.03
Cr	104 ± 9	107 ± 10	60 (49/74)	51.4 ± 1.8
Cu	30.0 ± 5.6	27.0 ± 1.0	11 (9/13)	9.6 ± 0.4
Fe	67.4 ± 1.7	76.3 ± 1.2	25.7 (25.2/26.3)	25.0 ± 0.2
Mn	3400 ± 160	3770 ± 80	631 (604/650)	697 ± 9
Pb	37.7 ± 7.4	38.5 ± 3.3	60 (55/71)	62.7 ± 3.0
Zn	223 ± 10	227 ± 19	104 (101/113)	104 ± 2.2
Element	BCR CRM 143§	This work, laboratory, B†	BCR CRM 145§	This work, laboratory B†
Cd	31.5 ± 2.0	30.8 ± 0.7	16.8 ± 1.6	15.9 ± 2.1
Cr	208 ± 20	192 ± 7	85.2 ± 16.3	69.6 ± 3.3
Cu	236 ± 13	226 ± 8	416 ± 24	399 ± 19
Fe	26.3 ± 0.7	25.6 ± 0.6	no data	7.5 ± 0.3
Mn	935 ± 100	940 ± 22	220 ± 15	223 ± 9
Pb	1317 ± 55	1309 ± 34	332 ± 22	311 ± 15
Zn	1301 ± 60	1234 ± 26	2772 ± 209	2608 ± 96
Element	BCR CRM 141§	This work, laboratory C†	BCR CRM 142§	This work, laboratory C†
Cd	0.30 ± 0.13	0.31 ± 0.01	0.22 ± 0.10	0.22 ± 0.02
Cr	53 ± 9	57.1 ± 3.0	44.4 ± 5.4	48.6 ± 2.8
Cu	31.2 ± 2.3	33.1 ± 0.5	25.3 ± 2.0	26.6 ± 0.4
Fe	23.7 ± 1.7	24.5 ± 0.4	17.5 ± 0.4	18.5 ± 0.2
Mn	512 ± 63	507 ± 8	527 ± 35	550 ± 14
Pb	26.3 ± 5.8	35.4 ± 1.6	30.9 ± 6.7	33.9 ± 2.3
Zn	70 ± 11	76.8 ± 1.6	79.6 ± 11.7	85.6 ± 1.2

* Mean and confidence limit of the mean ($p = 0.05$).
† Averages ± standard deviations ($n = 10$).
‡ Median and confidence interval of the median ($p = 0.05$).
§ *Aqua regia* results, mean ± standard deviation.

Table 3 Average values of metals in BCR CRMs, measured after reflux extraction (method A) or microwave extraction (method B). Results for Fe expressed in mg g^{-1}, for all other elements in μg g^{-1} ($n = 10$)

	BCR CRM 141 ± SD		BCR CRM 142 ± SD	
Element	Method A	Method B	Method A	Method B
Cd	0.33 ± 0.04	0.31 ± 0.01	0.19 ± 0.02	0.22 ± 0.02
Cr	49.8 ± 0.6	57.1 ± 3.0	40.7 ± 0.8	48.6 ± 2.8
Cu	32.3 ± 0.4	33.1 ± 0.5	25.9 ± 0.4	26.6 ± 0.4
Fe	23.6 ± 0.2	24.5 ± 0.4	17.9 ± 0.2	18.5 ± 0.2
Mn	496 ± 4	507 ± 8	539 ± 5	550 ± 14
Pb	25.3 ± 0.9	35.4 ± 1.6	33.2 ± 2.0	33.9 ± 2.3
Zn	74.2 ± 0.6	76.8 ± 1.6	83.7 ± 0.7	85.6 ± 1.2
	BCR CRM 143		BCR CRM 145	
Element	Method A	Method B	Method A	Method B
Cd	30.9 ± 0.5	30.8 ± 0.7	15.8 ± 0.5	15.9 ± 2.1
Cr	195 ± 2	192 ± 7	68.8 ± 3.7	69.6 ± 3.3
Cu	224 ± 3	226 ± 8	394 ± 12	399 ± 19
Fe	26.2 ± 0.7	25.6 ± 0.6	8.1 ± 1.3	7.5 ± 0.3
Mn	937 ± 8	940 ± 22	241 ± 8	223 ± 9
Pb	1266 ± 20	1309 ± 34	316 ± 11	311 ± 15
Zn	1231 ± 17	1234 ± 26	2642 ± 101	2608 ± 96

detection limits. A comparison of the blanks used in both extraction methods showed that the detection limits are the same or lower if microwave extraction is used.

The principal advantage of the proposed method is that extraction by means of a microwave oven is considerably faster than conventional reflux extraction (1 *versus* 3 h). The microwave oven also has a practical benefit: after digestion, the PFA vessels need only be rinsed with de-mineralized water before being re-used, whereas the glassware used in the conventional method must be warmed with dilute nitric acid before being re-used.

Interlaboratory Calibration of Microwave Extraction Using a Sludge Sample From the River Maas

The above experiments show that all three laboratories found close agreement between the stated values and results for the seven elements after microwave extraction. As each laboratory analysed different reference materials, an interlaboratory calibration was carried out with the aid of a sludge sample from the river Maas. Each laboratory performed three independent measurements of seven elements in the sample after extraction with *aqua regia* in the microwave oven.

The results of this experiment are summarized in Table 4. With the exception of Cr, the relative standard deviations (RSDs) are always lower than 10%. This implies that after microwave extraction the three laboratories produced comparable results for the seven elements.

Table 4 Results of an intercalibration study carried out with a sludge sample from the river Maas, after microwave dissolution. Results for Fe expressed in mg g^{-1}, for all other elements in μg g^{-1} ($n = 3$ for each laboratory)

		Reproducibility		Repeatability	
Element	Average	SD	RSD(%)	SD	RSD(%)
Cd	42.9	1.6	3.7	1.1	2.5
Cr	174.0	20.9	12.0	9.4	5.4
Cu	175.9	8.4	4.8	6.4	3.6
Fe	44.2	3.8	8.6	0.9	2.0
Mn	2091	162	7.7	58	2.8
Pb	496	21	4.3	8.2	1.7
Zn	2867	172	6.0	61	2.1

Comparison Between Microwave and Conventional Reflux Extraction Using Real Samples

In order to compare the results after microwave extraction with those after conventional reflux extraction for real samples, both extraction methods were applied to real samples selected from various types of sediment and soil samples from different locations in the Netherlands.

Laboratory A analysed soil samples from a salt marsh, laboratory B examined aquatic sludge and suspended matter samples obtained with the aid of a sludge centrifuge, while laboratory C analysed samples from grassland, arable land and woodland.

Table 5 shows the results of regression analysis for method A (reflux extraction) *versus* method B (microwave extraction) over the concentration range studied. The slopes and intercepts are close to 1 and 0, respectively, showing an excellent agreement between the two methods. The results show that for Cd and Cr no significant differences were found between the results after conventional reflux extraction and those after microwave extraction.

In general, no significant differences were found for Cu, Mn, Pb and Zn. In instances where differences were found between the two methods, the measurements after microwave extraction generally gave slightly higher values, which indicates a better yield.

Conclusion

Microwave extraction with *aqua regia* offers a good alternative to conventional reflux extraction for use in the determination

Table 5 Regression analysis of results for metals in 39 different soil and sediment samples after reflux extraction (method A) and microwave extraction (method B). Results for Fe expressed in mg g^{-1}, for all other elements in μg g^{-1}

Element	Method A	Method B	r*	a†	b‡	Range
Laboratory A: results for ten salt marsh samples—						
Cd	4.63	4.62	0.91	0.99	0.04	1.6–8.1
Cr	—	—	—	—	—	—
Cu	39.9	43.3	0.99	1.03	1.18	18.0–65.4
Fe	31.7	29.8	1.00	0.94	0.11	20.5–45.1
Mn	989	1051	1.00	1.01	51.37	613–1438
Pb	70.8	74.5	0.97	0.94	7.55	33.0–132.0
Zn	268	281	0.99	1.00	13.83	150–413
Laboratory B: results for nine aquatic sediments and suspended matter—						
Cd	104	103	1.00	0.99	−0.23	0.5–680
Cr	69.6	70.4	1.00	1.07	−3.65	6.7–190
Cu	195	198	1.00	1.02	−0.11	2.8–1083
Fe	26.8	27.7	1.00	1.05	−0.34	2.9–61.7
Mn	2365	2354	1.00	0.99	−23.05	112–11280
Pb	197	187	1.00	0.99	−0.34	4.4–618
Zn	1411	1427	1.00	1.01	−0.61	47–4496
Laboratory C: results for 20 soil samples—						
Cd	0.36	0.35	0.99	1.02	−0.02	0.02–1.47
Cr	—	—	—	—	—	—
Cu	15.1	15.0	0.99	0.92	1.12	0.5–49.0
Fe	—	—	—	—	—	—
Mn	—	—	—	—	—	—
Pb	24.7	27.4	0.99	1.09	0.52	9.5–88.2
Zn	41.2	41.4	1.00	1.00	0.05	4.2–95.2

* r = Correlation coefficient.
† a = Slope.
‡ b = y-axis intercept.

of Cd, Cr, Cu, Fe, Mn, Pb and Zn in sediment, sludge and soil samples. Microwave extraction involves rapid extraction in closed PFA vessels, with little risk of outside contamination. The results obtained after microwave extraction for seven elements in six reference materials (from the IAEA and BCR) showed close agreement with the stated values. The results after reflux extraction also demonstrated that there were no significant differences between this method and microwave extraction. By using the microwave oven and reflux extraction methods, 30 sediment and soil samples were dissolved. The results after microwave extraction of the seven elements were equal to or slightly higher than those obtained after reflux extraction.

An interlaboratory calibration between three laboratories was carried out with a sludge sample from the river Maas. After extraction in the microwave oven, the seven elements were measured; the RSD was between 3.7 and 8.6%, except for Cr, for which it was 12.0%.

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Sequential Atomic Absorption Spectrometric Determination of Chloride and Iodide in a Flow System Using an On-line Preconcentration Technique

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A flow injection method is described for the sequential determination of chloride and iodide in a mixture of the two. The chloride–iodide mixture is precipitated from solution by silver nitrate and the precipitated silver chloride is dissolved by ammonia solution to determine chloride after which the precipitated silver iodide is dissolved by potassium cyanide solution to determine iodide. The method allows the analysis of about 15 samples h^{-1} and mixtures with different chloride : iodide ratios can be analysed at the μmol dm^{-3} level.

Keywords: *Sequential determination; chloride and iodide determination; atomic absorption spectrometry; preconcentration*

The determination of anions is a significant problem in a variety of industries and numerous techniques have therefore been developed both for the determination of individual anions and for total anionic content.

Chloride has been determined indirectly using atomic absorption spectrometry (AAS) by precipitating it with silver nitrate and then either determining the remaining silver or the silver content in the precipitate after its dissolution by ammonia.[1] Flow injection (FI) with different detection methods such as spectrophotometry,[2–5] potentiometry[6,7] and AAS[8] has been used for the determination of chloride. The determination of iodide has been achieved by a number of methods; the most widely used is that based on the catalytic effect of iodide on the Ce^{IV}–As^{III} system.[9,10] This method has also been applied to the determination of iodide in pharmaceutical and food samples using a modular stopped-flow system.[11] The catalytic effect of iodide on the chlorpromazine –bromate reaction[12] and on the destruction of the thiocyanate ion by the nitrite ion[13] has been utilized for the determination of iodide.

Owing to the similarities in the chemical properties of the halide ions, the analysis of their mixtures is of particular interest and several reactions have therefore been developed that allow their sequential determination. These methods include sequential oxidation of the halide ions[14] or their conversion into organic halides followed by detection of the products by a suitable method such as gas chromatography[15] or by potentiometric titration with silver or mercury nitrate.[16]

Flow injection is a technique that has greatly increased the throughput of samples and the application of this technique to the simultaneous determination of a range of analytes is expanding rapidly.[17–19] Martinez-Jimenez *et al.*[20] have reported a method for the determination of chloride and iodide in a mixture of the two using FI-AAS. In this method the sample mixture is injected into a silver nitrate solution and the precipitates formed are retained on a stainless-steel filter, so that the total anion content can be determined from the decrease in the AAS signal of silver. The silver chloride precipitate is then dissolved by passing ammonia solution through the filter and the chloride content can be determined.

Chloride has been determined[21] using reversed FI-AAS by precipitating it with silver nitrate and then dissolving the precipitate, after a period of time, with a suitable dissolving agent. As the silver signal is proportional to the halide ion content in the sample, the latter can be determined. In this paper, the same method is applied to the sequential determination of chloride and iodide based on the solubility of silver chloride and the insolubility of silver iodide in ammonia solution. The mixture of chloride and iodide is first precipitated from solution by silver nitrate for a given period of time to allow for preconcentration; ammonia solution is then passed which dissolves the precipitated silver chloride only, after which cyanide solution is passed which dissolves the silver iodide precipitate. The chloride content is determined from the first AAS signal of silver and the iodide content from the second.

Experimental

Reagents and Solutions

All chemicals were of analytical-reagent grade. Solutions were prepared in distilled, de-ionized water. Standard solutions of silver ($AgNO_3$) were prepared by appropriate dilution of a 1000 ppm stock standard solution obtained from Merck. A standard iodide solution (1 g l^{-1}) was prepared by dissolving potassium iodide (Merck) (dried at 105 °C for 2 h) in de-ionized water. Working solutions were prepared by suitable dilution with de-ionized water. A standard chloride solution was prepared in the same way as for the iodide solution except that sodium chloride [Merck (formerly BDH)] was used instead of sodium iodide. Potassium cyanide was obtained from Merck and 30% ammonia solution was purchased from Fluka.

Apparatus

A Perkin-Elmer 372 atomic absorption spectrometer equipped with a silver hollow cathode lamp (4 mA) and a strip-chart recorder was used for the FI measurements. The wavelength was adjusted to 328.1 nm, the acetylene and air flow-rates were set to 1 and 8 l min^{-1}, respectively, and the slit-width was 2 nm. Teflon tubing of 1 mm i.d. from Beckman Altex was used in the flow system. Three Rheodyne loop injection valves were used to introduce either the washing or the dissolving solution into the precipitating loop. The length of the mixing coil was 5 cm and the i.d. 1 mm. The precipitating loop consisted of a Tygon tube (7 cm × 2.8 mm i.d.) filled with Pyrex glass beads (1.9 mm in diameter) (Thomas Scientific) which was connected vertically to the injection valve and to the nebulizer of the atomic absorption spectrometer *via* a Teflon tube (Fig. 1). The void volume of the precipitating loop was found to be 85 μl. A four-channel peristaltic pump was used to draw the cation and anion solutions into the precipitating loop and then to waste, and the negative pressure of the nebulizer was used to draw the washing and dissolving solutions through the precipitating loop to the nebulizer of the atomic absorption spectrometer. The flow-rate of the dissolving solution was coarsely con-

trolled by adjusting the nebulizer of the spectrometer. The experimental conditions of the system are listed in Table 1.

Procedure

Standard solutions of silver nitrate and a mixture of chloride and iodide are passed through the flow system using the manifold shown in Fig. 1. The two solutions mix in the mixing coil and are then pumped to the precipitating loop. Precipitation is allowed to proceed for 2 min under the conditions listed in Table 1 and the excess of silver nitrate is pumped to waste. The selecting valve 1 (injector II) first allows a stream of the washing solution (1×10^{-5} mol dm^{-3} HNO_3) to pass to the precipitating loop for 32 s until zero response is obtained, then ammonia is allowed to pass which dissolves the precipitated silver chloride and carries it to the nebulizer to be determined. Selecting valve 2 (injector III) then allows a cyanide solution to pass to the precipitating loop; this solution dissolves the precipitate of silver iodide and carries it to the nebulizer. The first AAS signal obtained is proportional to the silver concentration, which is in turn proportional to the chloride concentration, and the second signal is proportional to the iodide concentration. The concentration of the silver ion is 1000 ppm. The time required for washing the precipitate is 32 s. Dissolution of the precipitated silver chloride and silver iodide requires 32 and 22 s, respectively, at a concentration of 5×10^{-5} mol dm^{-3} each of chloride and iodide.

Results and Discussion

The determination of chloride using a continuous precipitation unit in a reversed FI system gives accurate, reproducible and rapid results.[21] The same technique can be applied to the sequential determination of chloride and iodide by precipitating these ions with silver nitrate for a given period of time, followed by dissolution of the silver chloride in ammonia and then dissolution of the silver iodide in cyanide solution. In order to obtain the most sensitive and reproducible results, several chemical and FI variables such as the concentration of the reagents, their flow-rates and the length of the mixing coil have to be optimized.

The effect of the silver ion concentration on the response was studied and it was found that the sensitivity increases with an increase in the silver ion concentration up to 800 ppm for iodide and 500 ppm for chloride as shown in Fig. 2. In order to ensure complete precipitation of chloride and iodide, a concentration of 1000 ppm of silver was used.

The concentration of the dissolving agents (ammonia and cyanide) was optimized. For complete dissolution of the precipitates, 0.45 mol dm^{-3} ammonia and 0.05 mol dm^{-3} cyanide solutions are required. A separate experiment indicated that silver iodide is insoluble in 0.45 mol dm^{-3} ammonia in the range studied (2–120 μmol dm^{-3}).

The effect of the flow-rate of the analyte and silver ion solution on the response was studied and it was found that the height of the signal increases linearly with an increase in the flow-rate (Fig. 3). This is to be expected as a higher flow-rate means that more reagents can be mixed and hence can produce more precipitate. A flow-rate of 1.2 ml min^{-1} was chosen to give sufficient sensitivity and reasonable reagent consumption. The signal is also affected by the flow-rates of the dissolving agents in that it increases with an increase in their flow-rates; however, flow-rates of 4.2 and 4.5 ml min^{-1} for the ammonia and cyanide solutions, respectively, gave the most reproducible results.

The effect of the length of the mixing coil on the response was studied at a constant flow-rate. A change in the length of

Table 1 Experimental conditions for the sequential determination of chloride and iodide

Hollow cathode lamp	Silver
Lamp current	4 mA
Wavelength	328.1 nm
Acetylene flow-rate	1 l min^{-1}
Air flow-rate	8 l min^{-1}
Slit-width	2 nm

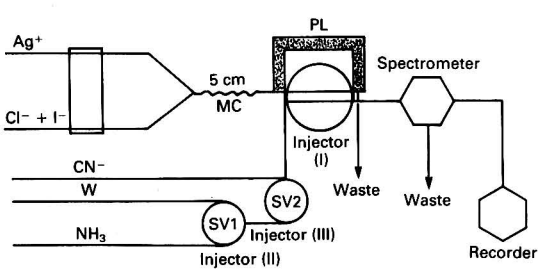

Fig. 1 Manifold used for the sequential determination of chloride and iodide: SV, selecting valve; W, washing stream; MC, mixing coil; and PL, precipitating loop

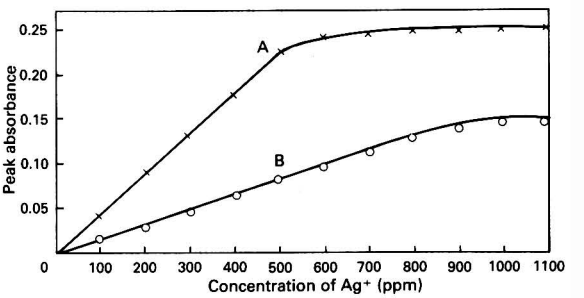

Fig. 2 Effect of silver ion concentration on the response of A, chloride and B, iodide

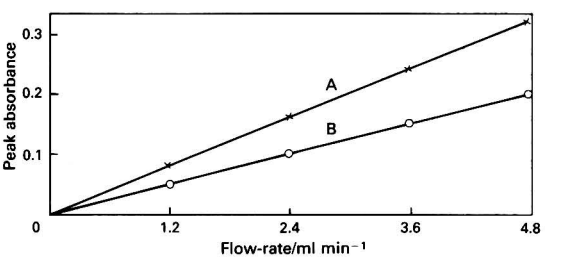

Fig. 3 Effect of flow-rate on the determination of A, 4×10^{-5} mol dm^{-3} chloride; and B, 4×10^{-5} mol dm^{-3} iodide in a mixture of the two

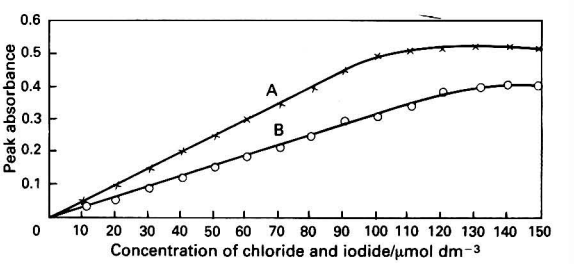

Fig. 4 Calibration graphs for A, chloride and B, iodide determination

Table 2 Analysis of different chloride–iodide mixtures

Amount added/ μmol dm^{-3}		Amount found/ μmol dm^{-3}		Amount added/ μmol dm^{-3}		Amount found/ μmol dm^{-3}	
Iodide	Chloride	Iodide	Chloride	Iodide	Chloride	Iodide	Chloride
120	2	120	2.1	40	60	40	61
120	5	119	4.9	30	70	29	71
120	10	118	11	30	60	31	60
110	10	109	9.8	30	80	30	79
100	10	98	9.7	20	90	19	92
100	20	100	9.6	20	100	21	97
90	30	89	31	10	100	9.6	97
80	50	80	49	5	100	4.9	101
60	50	61	50	2	100	2.1	99
50	50	52	50				

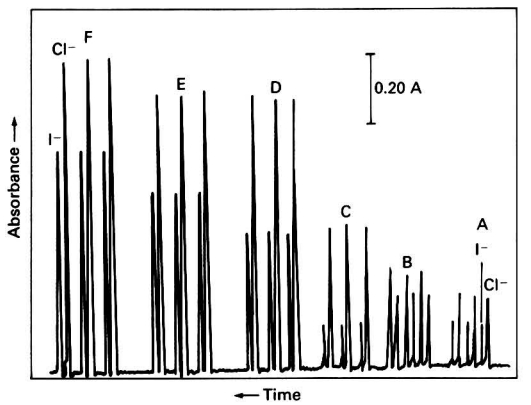

Fig. 5 Response of different chloride–iodide mixtures: A, 2×10^{-5} mol dm^{-3} each of Cl^- and I^-; B, 2×10^{-5} mol dm^{-3} Cl^- + 4×10^{-5} mol dm^{-3} I^-; C, 4×10^{-5} mol dm^{-3} Cl^- + 2×10^{-5} mol dm^{-3} I^-; D, 8×10^{-5} mol dm^{-3} Cl^- + 6×10^{-5} mol dm^{-3} I^-; E, 8×10^{-5} mol dm^{-3} each of Cl^- and I^-; and F, 9×10^{-5} mol dm^{-3} Cl^- + 1×10^{-4} mol dm^{-3} I^-. The chloride peak appears to the right of the iodide peak in each instance

the coil had no significant effect on the signal height, which indicates that the precipitation and dissolution reactions are completed inside the precipitating loop.

Under the optimum conditions described above, two calibration graphs were obtained, one for chloride and the other for iodide (Fig. 4). The linear working range is 2–100 μmol dm^{-3} chloride and 5–120 μmol dm^{-3} iodide. The upper linear limit is believed to be due to saturation of the precipitating loop. The lower detection limit, taken as that concentration which gives a signal three times the base line noise, is 2 μmol dm^{-3} chloride and 5 μmol dm^{-3} iodide. Fig. 5 shows signals for chloride and iodide standards. It can be seen that the signals are reproducible and the peak width is 30 s for chloride and 22 s for iodide, which means that a sampling frequency of about 15 h^{-1} can be achieved by using this method.

In order to test the applicability of the method, samples containing different chloride : iodide ratios were prepared and their contents analysed (Table 2). Mixtures with chloride : iodide ratios ranging from 1 : 60 to 50 : 1 can be analysed, with a relative standard deviation (RSD) of less than 3% for a series of eight samples in each determination.

Effect of Interferences

The interference from foreign ions in the system was studied with a solution containing 5×10^{-5} mol dm^{-3} each of chloride and iodide. The tolerance limits were taken as the largest amounts yielding an error of less than 5% in the peak absorbance. The results (Table 3) indicate that the most significant interferences are caused by anions that form precipitates with silver such as bromide, thiocyanate and cyanide, if they are present at a concentration which is twice that of the chloride–iodide mixture.

Table 3 Effect of foreign anions on the sequential determination of 5×10^{-5} mol dm^{-3} each of chloride and iodide in a mixture of the two

Ion added	Ratio of amount of foreign ion : chloride–iodide
Acetate, formate, NO_2^-	
F^-, PO_4^{3-}, ClO_4^-	50
SO_4^{2-}, CO_3^{2-}, CrO_4^{2-}	20
$C_2O_4^{2-}$	7
CN^-, Br^-, SCN^-	2

Conclusion

The simultaneous determination of chloride and iodide with an FI system at a sampling rate of about 15 samples h^{-1} and with an RSD of less than 3% is possible after precipitation of these ions with silver nitrate and the sequential dissolution of the precipitates with ammonia and cyanide solutions. It is possible to detect as little as 2 μmol dm^{-3} chloride and 5 μmol dm^{-3} iodide in a mixture of the two. Compared with a previously reported method,[20] the proposed method is simpler, and it is not necessary to change the precipitating loop as it is cleaned by the dissolving agents, which allows a higher sampling frequency.

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Determination of Urea in Serum by Using Naturally Immobilized Urease in a Flow Injection Conductimetric System

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A flow injection method was developed, aimed at the determination of urea in human serum. The system makes use of the naturally immobilized urease present in *Canavalia ensiformis* DC (jack bean). A column is filled with small pieces of this bean, and the sample (50 μl) containing urea passes through it carried by a 1% NaCl solution. On leaving the column the stream is merged with an alkaline reagent (0.5 mol dm^{-3} NaOH; 0.5% disodium dihydrogen ethylenediaminetetraacetate). The ammonium ions, arising from the enzymatic reaction that occurs inside the column, are changed into the molecular form, which permeates a polytetrafluoroethylene membrane and is received in a de-ionized water acceptor stream. The ammonia ionizes causing an increase in the conductance, which is proportional to the urea content of the sample. About 40 samples can be processed in 1 h with negligible carry-over and with a relative standard deviation of 1% or less. The results are in agreement with those obtained by a standard spectrophotometric method.

Keywords: *Serum urea determination; naturally immobilized urease; flow injection*

Several papers describing artificial urease-immobilization processes and the construction of urea sensors have been published.[1–4] Most of them were aimed at the development of potentiometric sensors to be used in batch procedures for the determination of urea. Artificial enzyme immobilization has also been pointed out to be one of the most suitable approaches to automating biochemical reactions in flow injection (FI) systems.[5,6] However, it has been demonstrated that, sometimes, it is possible to make use of the raw material containing the enzyme, naturally immobilized inside vegetable cells, in the construction of a biosensor. Wang and Lin[7] described a biosensor that was developed, from the natural occurrence of polyphenol oxidase in banana, to provide the enzyme source in the determination of dopamine. Plant tissues have also been used for constructing electrochemical sensors for glutamate,[8] phosphate and fluoride[9] and tyrosine.[10] Meal, obtained from the jack bean (*Canavalis ensiformis* DC), was used in the construction of a potentiometric biosensor for urea,[11] and good results were obtained in terms of stability and detection limits.

The use of naturally immobilized enzymes is very attractive as no immobilization process is required. However, the enzyme should exhibit sufficient activity, and the conditions under which the determination is made should allow a rapid transport of both the substratum to the inside of the cell and of the products to be detected outside of the cell membrane. The diffusion through the cell membrane is the rate-limiting factor for an FI system that is based on the use of natural immobilization.

This paper describes an FI system developed for the determination of urea in human serum by using a methodology that employs the naturally immobilized urease present in the jack bean. The ammonium ion originating in the enzymic hydrolysis of urea is detected by using the conductimetric methodology previously described.[12]

Experimental

Apparatus

The same instruments, conductimetric flow cell and polytetrafluoroethylene membrane previously described were used.[12] The diffusion cell was modified and had its dimensions enlarged to contain a shallow groove 0.5 mm deep, 4 mm wide and 10 cm long.

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The FI conductimetric manifold used for the determination of urea is outlined in Fig. 1. Column C_1 was filled with a mixed-bed resin, to effect a final purification of the de-ionized water, and was constructed as previously described.[12] Polyethylene tubing (0.8 mm i.d.) was used throughout. All the experiments were performed at ambient laboratory temperature (near 25 °C).

Column C_2, in Fig. 1, was made from a Tygon tube (2.5 mm i.d.) and filled with small pieces of bean. These pieces were directly cut from a grain of the bean, from which the skin had been removed, and had the form of small cubes with edges nearly 2 mm long. Two small plugs of glass wool were used to retain the bean pieces inside the column. A 5 cm long column contained between 15 and 20 pieces, with an average total mass of 0.13 g. The column was conditioned by passing a 1% NaCl solution (flow-rate: 1.0 ml min^{-1}) for 15 min. When not in use the column should be kept empty and stored in a refrigerator.

For comparison purposes an FI manifold was assembled to work with the free urease reagent solution. This system is the same as that shown in Fig. 1, but has the bean column replaced by a 50 cm reaction coil made from a polyethylene tube (0.8 mm i.d).

Reagents

Standard solutions of urea were prepared daily by suitable dilution of a 1000 μg ml^{-1} stock standard solution. Carbonate-free sodium hydroxide solutions were made by dilution of approximately 12 mol dm^{-3} sodium hydroxide prepared with

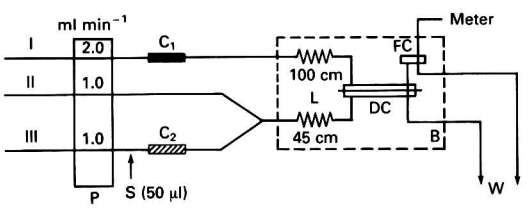

Fig. 1 Flow injection manifold for the determination of urea in serum using naturally immobilized urease and conductimetric determination. P, Peristaltic pump; S, sample; C_1, mixed bed ion-exchange column; C_2, bean tissue column; L, thermal equilibration coil; B, water-isolated bath; DC, diffusion cell; FC, conductimetric flow cell; and W, fluid discharge. I, De-ionized water; II, alkaline reagent: 0.5 mol dm^{-3} NaOH, 0.5% Na_2EDTA; and III, sample carrier fluid

freshly boiled de-ionized water. Disodium dihydrogen ethylenediaminetetraacetate (Na_2EDTA) (0.05%) was used in the reagent solution.

Free urease solutions were prepared by dissolving 0.30 g of the Merck product (83.35 nkat mg^{-1}) in 5 ml of water and adjusting the volume to 500 ml with 1% NaCl solution. Portions (20 ml) of this solution were placed in 100 ml calibrated flasks and the volume of each was adjusted with a suitable 0.02 mol dm^{-3} tris(hydroxymethyl)amino methane (Tris)–HCl buffer containing 1% of NaCl. The pH of these solutions was measured by a standard pH-measurement procedure using a calibrated glass electrode. All other Tris–HCl buffer solutions were 0.02 mol dm^{-3} and contained 1% of NaCl.

Standard solutions of urea, containing various amounts of the urease inhibitors F^- and HSO_3^-, were prepared from stock 0.100 mol dm^{-3} NaF and $NaHSO_3$ solutions. Analytical-reagent grade solutions and freshly prepared de-ionized water were used throughout.

Serum Samples

Human serum samples were obtained from the Clinical Hospital of the State University of Campinas by a single centrifugation of the whole blood. All samples were analysed for urea in the hospital laboratory and by the proposed FI method on the same day. For the proposed method, 100 µl of serum were diluted to 100 ml with water in a calibrated flask.

Results and Discussion

Preliminary experimental data were obtained using a 5 cm column constructed as described under Experimental. The reagent solution was 0.5 mol dm^{-3} NaOH containing 0.5% m/v of EDTA. If no salt is present in the carrier stream the rate of urea hydrolysis is very low and barely detectable. Adding a salt such as NaCl or KCl to the carrier causes a marked increase in ammonium ion production and a decrease in the sample washing-out time. Fig. 2 shows the dependence of the FI peak height on the concentration of NaCl in the carrier stream. The same behaviour and quantitative increase in ammonia production was observed for KCl.

Additional experiments were carried out on the evaluation of the effect of the column length and on the reproducibility among columns. Fig. 3 shows how the length of the column affects the hydrolysis of the standard urea solutions. The results for urea are compared with those obtained for standard ammonia solutions prepared to contain an equivalent amount of nitrogen. The comparison shows that the hydrolysis yields are about 70 and 95% for a 3 and a 5 cm long column, respectively. Use of longer columns also increases the wash time interval, thereby reducing the sample-processing capability of the system.

Five columns (each 5 cm long) were prepared as described under Experimental, and the conductimetric signals obtained for each, using standard urea solutions in the concentration range 1–5 µg ml^{-1}, were compared. A maximum relative difference of 5% was observed among signal peak heights.

The effect of the concentration of sodium hydroxide in the reagent stream was investigated. The results showed that the signals for a standard solution containing 1–5 µg ml^{-1} of urea were slightly affected by changing the hydroxide concentration from 0.1 to 1.0 mol dm^{-3}.

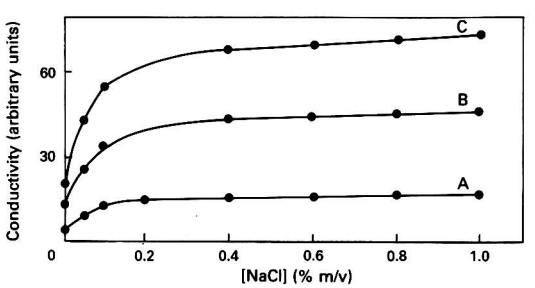

Fig. 2 Effect of the concentration of salt in the carrier stream on the analytical signal. Urea concentration: A, 1.0; B, 3.0; and C, 5.0 µg ml^{-1}

Effect of pH and Inhibitors

By using a 3 cm long column of beans, the effect of changing the pH of the carrier stream and of two urease inhibitors (F^- and HSO_3^-) was investigated. The results were compared with those obtained by using a solution of free urease as carrier in the manifold described under Experimental. The system employing free urease reagent showed a sensitivity that was about one-tenth of that employing the bean column. Therefore, the comparison was made with solutions that contained 3 µg ml^{-1} of urea for the column system and 30 µg ml^{-1} for that employing the free urease. No effort was made to optimize the free urease system as only relative results were required.

Fig. 4 shows how the pH of the carrier stream affects the peak height for both the systems. It can be observed that the free enzyme is more affected by the activity of the hydrogen ion in solution. The pH was previously reported as a critical parameter for a potentiometric biosensor constructed with use of a meal of jack bean[11] and for an artificially immobilized-urease potentiometric sensor.[13]

Fig. 5 shows the effect of the presence of F^- and HSO_3^- in the sample solution on both free and naturally immobilized-enzyme FI systems. In the absence of pH control the effect on the free enzyme is critical while the naturally immobilized enzyme is only slightly affected. The total effect on the inhibition of the enzyme activity comes from the presence of

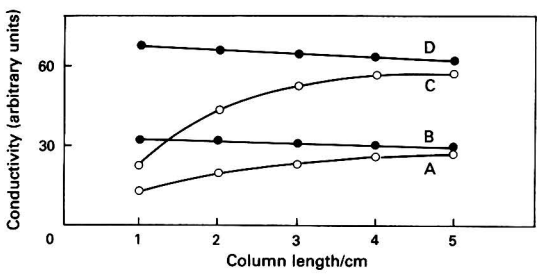

Fig. 3 Effect of the column length on the production of ammonium. ●, Ammonium standard solutions; and ○, urea standard solutions. Nitrogen concentration of the NH_4Cl and urea solutions: A and B, 1.0; and C and D, 2.0 µg ml^{-1}. Carrier solution, 1% NaCl

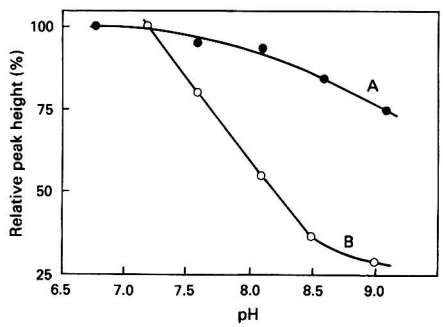

Fig. 4 Effect of the pH of the carrier solution on the analytical signal: A, using naturally immobilized urease (urea concentration of the test solution, 3 µg ml^{-1}); and B, using a solution of free urease (urea concentration, 30 µg ml^{-1})

the inhibitor itself and from the change it causes in the pH of the sample solution. It is worthwhile pointing out that the ratio of inhibitor to substratum is ten times greater when the bean column is employed as the urea concentration is ten times lower than that used in the free urease experiment. If a Tris–HCl buffer of pH 7.5 is used the inhibitor effect is cancelled for the naturally immobilized-enzyme system while it is still present in the free-enzyme system.

Although the response mechanism for the plant or animal tissue biosensors has not yet been well established[14] the results obtained in this work suggest that the cell integrity is maintained for the pieces of bean. The presence of a salt such as NaCl or KCl, frequently neglected when biosensor methodologies are developed, is essential to promote the rapid transport of the substratum to the inside of the cell where the enzyme-catalysed reaction occurs. The cell membrane acts as a selective filter, which is not permeable to some substances such as the inhibitors reported here, hence making the method less prone to interference. Also, the pH of the sample does not represent a critical parameter when the bean column is used as the urease source. To an extent, this is also a consequence of the fact that the reaction will occur inside the cell where the pH should be appropriate for the enzyme action. Furthermore, the conductimetric FI methodology described here does not require the enzymic reaction to occur at the same pH as that at which the detection is made, as is required for potentiometric sensors used in batch procedures.

Conditions for Determination of Urea in Serum

Based on the above results, the conditions for the determination of urea in serum were selected. A 3 cm long column was chosen along with a carrier solution containing 1% m/v of NaCl and a reagent solution, 0.5 mol dm^{-3} in NaOH, containing 0.5% m/v of Na_2EDTA. No buffer solution need be used owing to the high sample dilution employed. A sample volume of 50 μl was injected. A calibration run followed by signals obtained for some samples is shown in Fig. 6. Calibration runs obtained under these conditions showed a linear dependence of the peak height of the conductimetric signal in relation to the urea concentration in the range 1–10 μg ml^{-1}, with a typical correlation coefficient of 0.9997. The relative standard deviation for the analysis of ten replicates of a standard solution containing 3.0 μg ml^{-1} of urea was found to be 0.8%. About 40 samples could be processed in 1 h.

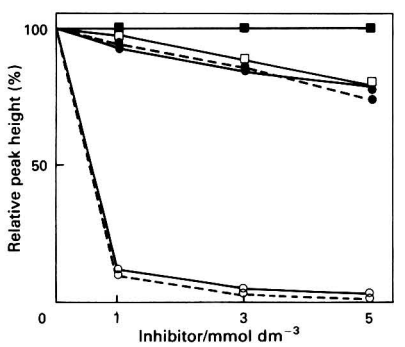

Fig. 5 Effect of the presence of F^- and HSO_3^- in the urea sample solution on the analytical signal. ● and ■, Naturally immobilized urease; and ○ and □, free enzyme reagent solution. Solid line, F^- added to the sample; and broken line, HSO_3^- added to the sample. Square symbols used when the pH was kept at 7.5 using Tris–HCl buffer. Urea concentrations were 3 μg ml^{-1} for the naturally immobilized urease and 30 μg ml^{-1} for the free enzyme solution system, respectively. All carrier solutions contained 1% NaCl

Column Lifetime

A 3 cm long column, prepared as described under Experimental, was fitted in the FI manifold and its long-term performance was evaluated for 10 h, injecting about 350 standard solutions containing 2 or 5 μg ml^{-1} of urea. The results show that, after this period, the activity of the column is reduced by about 10% in relation to its initial value. The rate of change in the column activity is slow. Therefore, periodical re-calibration can ensure good accuracy. In routine determinations a calibration involving use of three standard urea solutions was repeated every 30 min. The same column could be used to perform up to 1000 determinations, although it was found preferable to replace it every day in view of the low cost and ease of construction.

Accuracy of the Proposed Method

Sixty-five samples of human serum were analysed by the proposed method and by a spectrophotometric–enzymic method based on the reaction of the ammonium ion, produced in the hydrolysis of urea, with 2-oxoglutarate and reduced nicotinamide adenine dinucleotide (NADH) in the presence of glutamate dehydrogenase.[15] The decrease in the NADH concentration was monitored in the ultraviolet region. The method was performed in a Cobas Mira automatic analyser. Fig. 7 shows a comparison between the results obtained by the two methods. Least-squares statistical results show that the correlation between the two methods can be expressed as:

$$c_p = (0.47 \pm 0.31) + (0.9942 \pm 0.0237)\, c_s$$

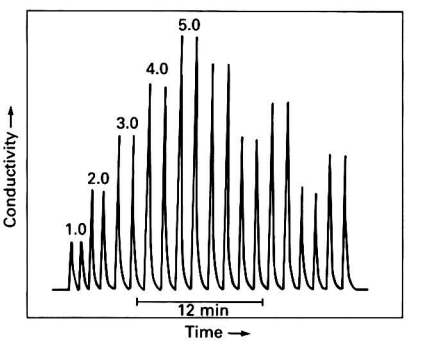

Fig. 6 Typical calibration run for serum urea determination using the naturally immobilized enzyme FI system. The signals for five standard solutions and for the five samples, introduced in duplicate, are shown. The numbers above the peaks are the urea standard solution concentrations in μg ml^{-1}

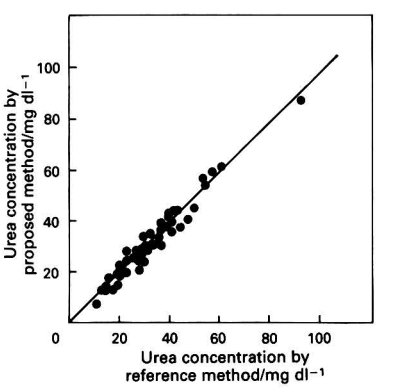

Fig. 7 Correlation of the results for 65 serum urea concentrations found by the proposed method and by a standard spectrophotometric method

where c_p is the urea concentration (mg dl^{-1}) found by the proposed method and c_s is the urea concentration (mg dl^{-1}) found by the reference spectrophotometric method. The correlation coefficient is 0.987 and the error of the estimate is ± 2.4 mg dl^{-1}. These results lead to the conclusion that the proposed method compares well with the conventional spectrophotometric method. The sensitivity of the conductimetric method permits a high sample dilution that helps in overcoming matrix effects. The method also presents advantages in relation to the cost and demonstrates that the naturally immobilized enzyme can be used to replace artificial immobilization in FI reactors when the raw material presents sufficient activity.

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Nitrate Ion-selective Electrodes Based on Quaternary Phosphonium Salts in Plasticized Poly(vinyl chloride) and Influence of Membrane Homogeneity on Their Performance*

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Nitrate ion-selective electrodes based on quaternary phosphonium salts in plasticized poly(vinyl chloride) (PVC) were found to be superior in stability and sensitivity to those based on quaternary ammonium salts in the same matrix and to Orion 93-07 electrodes. The difference in the characteristics of the electrodes tested was attributed to the different extent of homogeneity existing in the sensing membrane. Scanning electron microscopy revealed that quaternary phosphonium salts were homogeneously dispersed in the sensing membrane because of some specific interaction with the plasticized PVC. On the other hand, the other sensors tested were segregated to various extents. This specific interaction of quaternary phosphonium salts with the plasticized PVC, resulting in a homogeneous sensing membrane, was considered to be the cause for the improved characteristics of the nitrate electrodes. In line with this, additional rules for constructing ion-selective electrodes in non-porous polymer membranes are proposed.

Keywords: *Nitrate ion-selective electrode; quaternary phosphonium salt; membrane homogeneity; scanning electron microscopy; poly(vinyl chloride) membrane*

The development of ion-selective electrodes (ISEs) for the determination of nitrate began with the introduction of liquid-membrane electrodes, which utilized a porous membrane, holding a mixture of sensor and solvent. Sensors that have been commonly used included quaternary ammonium salts (QAS), triphenylmethane dyes, Fe^{2+}– or Ni^{2+}–*o*-phenanthroline complexes and quaternary phosphonium salts (QPS). The commercially available electrodes of this type, however, suffered from known drawbacks, namely, short-term stability and relatively poor selectivity and sensitivity. One of the major contributions to the design of ISEs was the introduction of a non-porous poly(vinyl chloride) (PVC) matrix, into which the sensor was embedded. This technique was first introduced for the construction of calcium ion-selective electrodes and subsequently extended to nitrate ISEs.[1–5]

Nielsen and Hansen[6] conducted a thorough study concerning the optimum combination of sensor–plasticizer–PVC for constructing nitrate ISEs based on QAS, which they found to be superior in stability and sensitivity to earlier electrodes. These workers considered that the electrode characteristics depended primarily on the distribution coefficient, q, of the sensor between the aqueous and the membrane phase. By using thermodynamic relationships and the concept of solubility parameter, d, they were able to relate q to the solubility parameters of water (d_{aq}), sensor (d_s), membrane matrix (d_m) and the volume fractions of sensor (V_s) and membrane matrix (V_m), through the equation:

$$q = \exp\{V_s V_m[(d_s - d_{aq})^2 - (d_s - d_m)^2]\}(RT)^{-1} \quad (1)$$

where, R = universal gas constant and T = temperature in K.

Equation (1) helped Nielsen and Hansen[6] to establish certain rules for selecting the membrane constituents in order to obtain optimum electrode characteristics. The derivation of eqn. (1), however, apart from the equilibrium condition imposed, rests also on two inherent assumptions: (*i*) the sensor maintains its monomeric form in both the aqueous and the membrane phase; and (*ii*) the sensing membrane constitutes a true solution (no separation of phases). However, it is difficult to ascertain in practice whether these two assumptions are met when the sensor is embedded in the plasticized PVC. A smooth, transparent membrane is a good indication of homogeneity. Homogeneity, a prerequisite for constructing good quality ISEs, is considered to be a measure of the compatibility of the sensor with the PVC matrix and the extent to which the sensing membrane approximates to a true solution.

During experiments in this laboratory aimed at constructing ISEs based on QAS and Fe^{2+}– or Ni^{2+}–*o*-phenanthroline complexes in a PVC matrix, it was observed that the smooth and transparent appearance of the sensing membrane was not an infallible proof of its homogeneity. Indeed, the sensors were not homogeneously dispersed in the non-porous medium as preliminary observations with an optical microscope revealed. This led us to believe that Nielsen and Hansen,[6] who also used QAS in plasticized PVC, had overlooked this fact and, therefore, their attempt to interpret the experimental results by means of eqn. (1) is open to question. It was also felt that a more detailed examination of the homogeneity achieved in the sensing membrane was needed and that further improvement of nitrate ISEs was still feasible by choosing other sensors, such as QPS, presumably more compatible with PVC.

In view of the above, the objectives of this study were: (*i*) to develop nitrate ion-selective electrodes based on QPS in a PVC matrix and to examine the degree of homogeneity achieved, when the sensor is embedded in this matrix, by means of optical and scanning electron microscopy; (*ii*) to check the characteristics and the performance of these electrodes in the determination of nitrate and compare them with well known electrodes; and (*iii*) to formulate simplified rules, if possible, for selecting the membrane constituents for optimum electrode characteristics.

* Part of the PhD Thesis of M. G. Mitrakas.

Experimental

Membrane Material

Sensors. Tetradodecylammonium bromide (TDDAB) and tributylhexadecylphosphonium bromide (TBHDPB) were Fluka purum-grade reagents. Tetraoctylphosphonium iodide (TOPI), tetradodecylphosphonium iodide (TDDPI) and tetrahexadecylphosphonium iodide (THDPI) were synthesized as described by Feshchenko *et al.*,[7] and TDDAB was purified by successive recrystallization.[8] All QPS were purified by recrystallization from ethanol–diethyl ether, followed by preparative thin-layer chromatography with chloroform (Silica gel 60 F_{254}, R_F 0.2) and recrystallization from chloroform–diethyl ether. The purities of QPS were checked by elemental analysis and melting point determination with differential scanning calorimetry (DSC) and verified by mass spectrometry.

Plasticizers. All plasticizers were of analytical-reagent grade. The plasticizers used were dibutyl phthalate (DBP), dioctyl phthalate (DOP) and trixylyl phosphate (TXP).

PVC. Breon S 110/10 (BP Chemicals) was used.

Membrane and Electrode Preparation

For QAS, the procedure of Nielsen and Hansen[6] was followed, with the sensing membrane containing 29% PVC.

For QPS, the amount of PVC was increased to 35% in order to render the membrane more rigid and durable as it was observed that dissolution of these sensors to a membrane containing 29% PVC made it softer. The membrane was prepared by dissolving 0.35 g of PVC and the appropriate amounts of plasticizer and sensor (see Table 1), making a total of 1 g, in 20 ml of tetrahydrofuran (THF). This solution was poured into a 55 mm i.d. flat Petri dish and left at room temperature for the slow evaporation of the solvent. The resulting membrane was conditioned for 48 h in a 0.1 mol dm^{-3} KNO_3 solution in order to replace iodide or bromide with nitrate. Therefore, all sensors are denoted with a suffix N henceforth in order to represent the nitrate in the anionic form. From this membrane (0.30 mm thick) a 10 mm diameter disc was cut and glued to the end of a PVC tube, with 4% m/m PVC in THF. The inner reference electrode was an Ag–AgCl wire (Metrohm, 6.0711.000) immersed in a solution of KNO_3–KCl (each 1×10^{-2} mol dm^{-3}).

The Orion 93-07 nitrate ISE was provided commercially.

Photomicrographs

The appropriate amounts of PVC, plasticizer and sensor, making a total of 0.5 g, were dissolved in 50 ml of THF. This solution was poured into a 90 mm i.d. flat Petri dish, which contained a piece of silicon foil ($5 \times 5 \times 0.5$ mm) and a glass plate ($20 \times 20 \times 1$ mm), and left at room temperature for the slow evaporation of the solvent. After conditioning, the membrane attached to the silicon foil was spattered with gold and photographed with a Jeol JSM 840A scanning electron microscope, while the membrane attached to the glass plate was photographed with an Axioma Zeiss optical microscope. Membranes consisting of PVC and the plasticizer only (without sensor) were also prepared in the same way and examined under the scanning electron microscope (see under Results and Discussion).

Photographs of the sensing membrane of the commercially available Orion 93-07 nitrate ISE were taken only with the optical microscope.

Apparatus

Measurements of the e.m.f. were performed with an Orion 901 microprocessor ionalyser, and pH measurements with a Metrohm E 520 pH meter. An Orion 90-02 double-junction external-reference electrode with Orion 90-00-02 and 0.04

Table 1 Response characteristics of nitrate ISEs based on quaternary phosphonium salts in a membrane containing 35% PVC, as influenced by the composition of the sensing membrane. The same characteristics of well known nitrate ISEs are also shown

					pNO_3		
					Lower linear limit	Detection limit	
Plasticizer	Sensor (%)	d_m/(cal $cm^{-3})^{1/2}$*	$E°$†/ mV	Slope/mV $decade^{-1}$		IUPAC‡	Stat.§
DBP	TBHDPN (8)	9.4	97	57.3	3.95	4.70	5.25
DBP	TBHDPN (15)	9.4	94	58.5	4.05	4.75	5.46
DBP	TBHDPN (20)	9.4	94	58.5	4.00	4.75	5.32
DOP	TBHDPN (8)	9.1	96	56.8	3.90	4.70	5.20
DBP	TOPN (4)	9.4	95	57.6	4.30	5.15	5.80
DBP	TOPN (7)	9.4	96	57.9	4.38	5.18	5.90
DBP	TOPN (10)	9.4	97	58.1	4.46	5.25	5.93
DBP	TOPN (12)	9.4	97	58.5	4.46	5.25	5.92
DBP	TOPN (15)	9.4	98	58.6	4.42	5.22	6.03
DOP	TOPN (14)	9.1	95	58.8	4.35	5.16	5.85
TXP	TOPN (14)	9.8	97	57.1	4.36	5.13	5.85
DBP	TDDPN (1)	9.4	98	56.9	4.46	5.36	5.95
DBP	TDDPN (3)	9.4	97	57.2	4.80	5.72	6.30
DBP	TDDPN (4)	9.4	95	55.8	4.70	5.60	6.28
DBP	TDDPN (6)	9.4	94	55.0	4.50	5.50	6.10
DOP	TDDPN (3)	9.1	97	57.2	4.63	5.50	6.20
DOP	TDDPN (4)	9.1	94	56.8	4.45	5.45	6.15
TXP	TDDPN (4)	9.8	97	56.4	4.45	5.58	6.18
DBP	THDPN (2)	9.4	102	56.3	4.70	5.67	6.35
DBP	TDDAN (4)	9.4	98	56.2	4.45	5.45	6.10
DBP	TDDAN (3)	9.4	85	56.0	4.30	5.25	5.72
DOP	TDDAN (4)	9.1	88	56.0	4.30	5.35	5.80
	Orion 93-07			76	56.0	4.40	5.15

* cal = calorie, 1 cal = 4.184 joules.

† The theoretical value of $E°$ at 20 °C and pNO_3 of 2.06 is 96 mV.

‡ Calculated according to IUPAC definition.[11]

§ Calculated statistically.[12]

mol dm^{-3} ammonium acetate inner and outer filling solutions, respectively, and a Metrohm E 402 saturated calomel electrode (SCE), were used.

Measurement Techniques

The characteristics of the electrodes (Table 1) in standard potassium nitrate solutions were determined. The solutions were not adjusted to identical ionic strength, and the concentrations were converted into activities by means of the Debye–Hückel equation. Electrode lifetimes and nitrate content of waters, soil and plant extracts were assessed in an ionic strength adjustor (ISA) solution. The ISA solution was composed of lead acetate (0.02 mol dm^{-3}), PbO (0.01 mol dm^{-3}), potassium acetate (0.02 mol dm^{-3}) and sulphamic acid (0.01 mol dm^{-3}),[9] and is denoted as ISA-Pb henceforth.

pNO_3 response

The e.m.f. was measured in solutions thermostated at 20 °C, under constant stirring, against the double-junction reference electrode. All slopes were calculated by means of regression analysis on the linear part of the calibration graph. The equation of the non-linear part of the calibration graph was computed by Marquardt[10] non-linear regression analysis. The lower linear limit was determined as the common point of the linear and non-linear calibration graphs.

Potential–pH curves

These were obtained manually, point-wise over the pH range 3–11.

Selectivity coefficients

These were obtained by the fixed interference method,[11] where the concentration of the interfering anion (X) in all instances was fixed at 0.01 mol dm^{-3}. In practice, they were computed as the intercept between the linear calibration graph and the lower limit of detection in the solution used and expressed as $pk^{pot}_{NO_3,X} = -\log k^{pot}_{NO_3,X}$.

Detection limits

Nitrate detection limit, c_L, as defined by IUPAC,[11] was determined as the nitrate concentration which gave a difference of 18 mV in the potential calculated from the linear and non-linear calibration graphs. Statistically, for nitrate ISEs the response of which is not limited by the solubility of the sensor, the detection limit was calculated by the equation[12] $c_L = (10^{L/S} - 1)c_b$, where, $L = 2 \times 1.645\,(\sigma_A^2 + \sigma_B^2)^{1/2}$ for the 95% confidence level, S = the slope, c_b = the nitrate concentration of the blank (calculated) and σ_A, σ_B, the e.m.f. standard deviations, are calculated from ten replicates of standard 1×10^{-3} mol dm^{-3} NO_3^- and blank solution, respectively.

E° values

These were measured in a 1×10^{-2} mol dm^{-3} NO_3^- solution *versus* the SCE (20 °C), as the NO_3^- concentration in the internal reference solution of the electrodes was also 1×10^{-2} mol dm^{-3}.

Solubility parameters

The solubility parameters of QPS, calculated from the relationship $d = \rho\Sigma G/M_r$ (ρ = density of the material, G = molar attraction constant and M_r = relative molecular mass) as suggested by Small,[13] were found to be: $d_{TBHDPN} = 9.8$, $d_{TOPN} = 9.7$, $d_{TDDPN} = 9.5$ and $d_{THDPN} = 9.4$ (cal cm^{-3})$^{1/2}$. Those of QAS were taken from Nielsen and Hansen[6] and those of PVC and plasticizers from Brydson:[14] $d_{PVC} = 9.5$, diethyl phthalate, $d_{DEP} = 9.9$; dibutyl phthalate, $d_{DBP} = 9.4$; dioctyl phthalate, $d_{DOP} = 8.9$; and trixylyl phosphate, $d_{TXP} = 9.9$.

The solubility parameter of the resulting membrane (d_m) was calculated from the equation:

$$d_m = w_{pvc} \times d_{pvc} + w_{plast.} \times d_{plast.} \quad (2)$$

where w refers to the percentage mass and the subscript 'plast' refers to the plasticizer.

Electrode lifetimes

These were measured by storing the electrodes in 100 ml of a mixture (1 + 1) of 1×10^{-3} mol dm^{-3} KNO_3 and ISA-Pb solutions under constant stirring. The mixture was renewed twice a week, and the calibration graph assessed at appropriate intervals with measurement of the slope and the detection limit.

Nitrate determination

All the solutions used were of analytical-reagent grade. Nitrate in water, soil and aqueous extracts of plants was determined by the electrodes using ISA-Pb[9] in a 1 + 1 mixture. Nitrate was also determined by ion chromatography[15] to serve as an independent method for comparison.

Results and Discussion

Characteristics of Nitrate ISEs Based on QPS

The optimum membrane composition (PVC–plasticizer–sensor), experimentally assessed, was: (*a*) 35 + 50 + 15–17 TBHDPN; (*b*) 35 + 51 +13–15 TOPN; (*c*) 35 + 61 + 3–4 TDDPN; and (*d*) 35 + 64 + 2 THDPN; where the plasticizer could be DBP, DOP or TXP. This optimum membrane composition was established by a large set of experiments involving various combinations, representative examples of which are shown in Table 1. Diethyl phthalate, a commonly used plasticizer with a relative molecular mass (M_r) of 222.2, was rejected because it dissolved significantly in water.

All electrodes with TBHDPN, TOPN and TDDPN as sensors showed no potential–pH dependency within the pH range 3–11. When THDPN was used as a sensor, however, the electrodes showed such a dependency above pH 6. This was attributed to insufficient purification of this sensor (melting point 93 °C) as verified by DSC, which showed the presence of an impurity with a melting point of 90 °C. None of the cations NH_4^+, K^+, Na^+, Ca^{2+}, Mg^{2+}, Al^{3+} and Pb^{2+} interfered. The response time was less than 1 min in pure nitrate solutions whose concentration was lower than 1×10^{-5} mol dm^{-3} NO_3^- and less than 20 s in concentrations higher than 1×10^{-5} mol dm^{-3} NO_3^-, and it was inversely related to the volume fraction of the sensor, V_s. The detection limit decreased (Table 1) in the sequence TBHDPN > TOPN > TDDPN > THDPN as expected, as the M_r of the sensors increased progressively. The selectivity coefficients of nitrate ISEs

Table 2 Selectivity coefficients of nitrate ISEs based on quaternary phosphonium salts in comparison with well known electrodes. They were measured by the fixed interference method and are expressed as $pk^{pot}_{NO_3,X}$

Interfering anion*	15% TBHDPN–DBP	15% TOPN–DBP	4% TDDPN–DBP	4% TDDAN–DBP	Orion 93-07
I^-	−1.20	−1.15	−1.15	−1.15	−1.15
Br^-	0.90	0.92	0.94	0.93	0.88
NO_2^-	1.25	1.35	1.35	1.20	1.30
Cl^-	1.80	2.10	2.25	2.30	2.25
F^-	2.20	3.00	3.35	3.25	2.70
HCO_3^-	2.20	2.80	3.25	3.40	—
$H_2PO_4^-$	2.20	3.30	3.70	3.70	2.80
SO_4^{2-}	2.20	2.70	3.20	3.30	2.80
CH_3COO^-	2.30	3.00	3.50	3.20	2.70

* 1×10^{-2} mol dm^{-3}.

based on QPS are presented in Table 2. It can be seen that, for interfering anions usually present in water, soil and plant extracts, these selectivity coefficients are comparable to those exhibited by the well known electrodes.

Comparison of Nitrate ISEs Based on QPS With Well Known Electrodes

Effect of d_s

The determined solubility parameters of QPS (R_4P^+) were similar to the corresponding QAS (R_4N^+) with the same chain length of R. This is attributed to the very bulky R substituents, which decrease the contribution of the central atoms (P or N) to the cohesive energy density of the molecule to a minimum. Consequently, any difference in performance observed (and discussed below) between the electrodes based on QPS and their QAS counterparts could not be attributed to differences in the value of d_s alone.

Effect of V_s *and* d_{plast}

The electrode characteristics should be influenced by the value of V_s and the type of plasticizer (as d_{plast} influences the value of d_m) as predicted by eqn. (1). However, for nitrate ISEs based on QPS, the experimental results (Table 1) showed that the type of plasticizer and the value of V_s (within the range used in this study) did not significantly influence the slope, linearity or detection limit, while in all instances the asymmetry potential was less than 3 mV. On the other hand, these factors had some effect on the electrodes based on QAS as was also reported by Nielsen and Hansen.[6] This difference in behaviour between the two groups of sensors and the apparent non-conformance to eqn. (1) of the electrodes based on QPS is elaborated in the next section.

Lifetime studies

Nitrate ISEs based on 4% of TDDPN–DBP and 3% of TDDPN–DOP exhibited an almost constant slope (Fig. 1) and detection limit (Fig. 2) for a period of 18 weeks, whereas this period for 15% TOPN–DBP was 13 weeks. It should be noted that the term lifetime implies the time up to which the electrode slope does not decrease by more than 2 mV. Beyond that time the electrode slope, and its characteristics in general, changed at such a rate that the precision and accuracy of the measurements were influenced. Consequently, it can be stated that the lifetime of the electrodes based on TOPN and TDDPN were 16 and 20 weeks, respectively. On the other hand, the slope and the detection limit (Figs. 1 and 2) of Orion 93-07 and TDDAN nitrate ISEs were continuously changing, giving lifetimes of 5 and 9 weeks, respectively. The lifetime of the latter electrode was in agreement with that reported by Wright and Bailey,[16] while the 16 week lifetime for the TOPN electrode far exceeded the 2 week lifetime of the corresponding ammonium-based electrode (TOAN) reported by the same workers.

Dispersion of the Sensors into the Membrane Matrix

The dispersion of the sensors into the membrane matrix was observed with both optical and scanning electron microscopy. This was necessary in order to explain the above mentioned improved characteristics of the electrodes based on QPS, on the one hand, and the apparent non-conformance of our experimental results to eqn. (1), on the other. With optical microscopy (Fig. 3) the sensors appear as black spots on a grey background (membrane matrix), while with scanning electron microscopy (Fig. 4) the sensors appear white on a grey–black background.

Optical microscopy revealed that the sensing membranes in the Orion 93-07 and 3% TDDAN–DBP electrodes were not homogeneous [Fig. 3(*a*) and (*b*)]. Light photographs of the Orion electrode were considered to be sufficient evidence for the existence of membrane heterogeneity, and scanning electron microscopy was judged not to be necessary. However, for the other QAS electrodes (4% TDDAN–DBP and 4% TDDAN–DOP), scanning electron microscopy was considered necessary to verify the membrane heterogeneity [Fig. 4(*a*) and (*b*)]. The term heterogeneity is used in the sense that two or more separate phases exist.

The asymmetry potential, E^{AS}, observed in certain of the electrodes was the first point that could be accounted for by means of the photographs taken; E° includes e.m.f. contributions from the internal reference electrode, inner phase boundary diffusion potentials and membrane asymmetry. The membrane asymmetry, owing to its heterogeneity, explains mainly: (*i*) the E^{AS} of −20 mV (Table 1) of the Orion 93-07 ISE [Fig. 3(*a*)]; and (*ii*) the dependence of E° and other electrode parameters on V_s, and on the type of plasticizer for TDDAN electrodes, found in this study and reported by Nielsen and Hansen[6] (see Table 1 and Figs. 3(*b*), and 4(*a*) and (*b*)]. Nielsen and Hansen[6] interpreted their results in terms of eqn. (1), believing that the observed differences in behaviour of the various electrodes tested reflected variations in the parameters V_s and d_m, which in turn influenced the value of the distribution coefficient, q. However, the findings of the

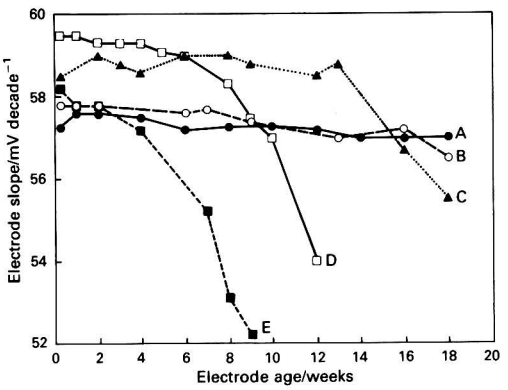


Fig. 1 Electrode slopes with ISA–Pb obtained during the lifetime studies of the nitrate ISEs, stored in a mixture of 1×10^{-3} mol dm^{-3} NO_3^- and ISA–Pb solutions (1 + 1). A, 4% TDDPN–DBP; B, 3% TDDPN–DOP; C, 15% TOPN–DBP; D, 4% TDDAN–DBP; and E, Orion 93-07

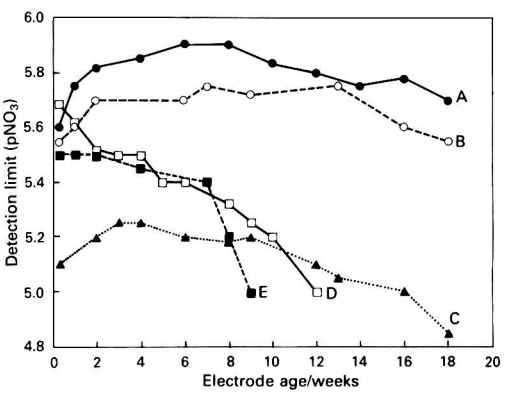


Fig. 2 Detection limits with ISA-Pb obtained during the lifetime studies of the nitrate ISEs, stored in a mixture of 1×10^{-3} mol dm^{-3} NO_3^- and ISA–Pb solutions (1 + 1). A, 4% TDDPN–DBP; B, 3% TDDPN–DOP; C, 15% TOPN–DBP; D, 4% TDDAN–DBP; and E, Orion 93-07

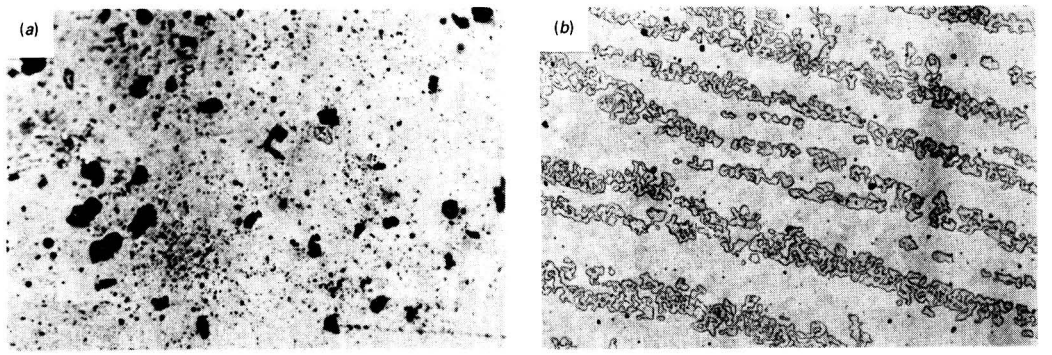


Fig. 3 Photomicrographs taken with an optical microscope (× 100 magnification) of two nitrate ISEs: (*a*) Orion 93–07; and (*b*) 3% TDDAN–DBP

present study suggested that the dependence of E° and other electrode parameters on V_s and d_m, when QAS were used as sensors, reflected the membrane heterogeneity rather than the variation in the values of V_s and d_m. Heterogeneity violates the theoretical assumptions on which eqn. (1) is based and in practical terms might cause differences in the electrical properties across the membrane and deterioration of its stability, owing to the incongruous dissolution of its components in the contacting solution (see also the following discussion on QPS). The 4% TDDAN–DBP ISE showed no asymmetry potential (Table 1; $E^{AS} = +2$ mV), in spite of its membrane heterogeneity, possibly because of the symmetrical distribution of the sensor in the matrix [Fig. 4(*a*)].

Optical microscopy was unable to reveal the extent of the dispersion of the sensor in the PVC matrix for QPS. Therefore, scanning electron microscopy was necessary in order to unravel the fine details of the whole structure and to distinguish the extent of disturbance, if any, of the PVC structure on addition of the sensor. The structure of the PVC matrix without the sensor (actual photographs not given) was almost the same as that shown in Fig. 4(*c*), (*d*) and (*e*) for the plasticizers DBP, DOP and TXP, respectively. It was, therefore, evident that, on addition of the sensor (QPS) and up to the point of the membrane saturation, no discrete phases were discernible and the structure of the PVC matrix was not significantly disturbed [Fig. 4(*c*), (*d*) and (*e*)]. In addition, no distinct particles were discernible and no contrast existed, even when using the ×4000 magnification power of the scanning electron microscope (where its resolution is 0.2 μm). Both facts implied the absence of localized accumulation of the sensor. All these findings clearly demonstrated that the sensing membrane was homogeneous in nature (no separation of the phases) and that QPS were more compatible with the plasticized PVC than the corresponding QAS and *o*-phenanthroline complexes [*cf.* Fig. 4(*c*) and (*d*) with Fig. 4(*a*) and (*b*) and Fig. 3(*a*)].

One can, therefore, postulate, with a reasonable degree of certainty, that the sensing membrane approximated to a true solution, *i.e.*, that the sensors (QPS) were dissolved in the plasticized PVC. The homogeneous dispersion of QPS in the plasticized PVC, irrespective of the type of plasticizer and the value of V_s used in this study, had the result that the electrode parameters were also independent of these factors as can be seen from the data of Table 1 and of Figs. 1 and 2. It also accounted for the following experimental observation: (*i*) the lower detection limit observed for the 4% TDDPN–DBP ISE compared with that of the 4% TDDAN–DBP electrode (Table 1 and Fig. 2); (*ii*) the longer lifetimes of QPS electrodes owing to the stronger retention of the sensor in the PVC matrix, as the major factor limiting the lifetime of a solvent–polymeric membrane is the loss of the sensor and/or the plasticizer in the solution analysed; and (*iii*) the presence of no asymmetry potential for the QPS-based electrodes, as the homogeneity of the sensing membrane ensures its symmetry and the congruous dissolution of its constituents into the contacting solution. It was evident, therefore, that when homogeneity of the sensing membrane was achieved, small differences in the value of d_m could not influence significantly the electrode parameters. This was also dictated by the mathematical form of eqn. (1). Also, a wide variation in the value of V_s up to the point of membrane saturation did not significantly change the electrode parameters, in disagreement with what eqn. (1) predicts. This non-conformance to eqn. (1) might be due to the fact that the state of equilibrium, mandatory for eqn. (1) to hold, is rarely, if ever, attained during the usually short periods of measurement. It seems, therefore, that, although eqn. (1) provides thermodynamic information as to which parameters influence the quality of nitrate ISEs, its practical usefulness is indeed open to question.

The isolated spots on Fig. 4(*c*) and (*d*) represent small undissolved particles of the sensor as the point of the membrane saturation is approached, a fact that was also verified at higher magnification. This explains the similar detection limits for 4% TDDAN–DBP and 4% TDDPN–DBP ISEs observed at the beginning of the lifetime studies (Fig. 2). When the point of saturation was exceeded (supersaturation) the initial membrane matrix structure was disturbed [Fig. 4(*f*)]. This super concentration of the sensor, depicted by the short white lines (contrast), had an adverse effect on the electrode parameters (see the detection limits of the 3 and 6% TDDPN–DBP ISEs given in Table 1) because of the membrane heterogeneity. It also explains the same detection limits observed for 4% TDDAN–DBP and 6% TDDPN–DBP ISEs (Table 1).

The higher compatibility and, in fact, dissolution of QPS into the plasticized PVC in comparison with the corresponding QAS and *o*-phenanthroline complexes deserves further attention. Plasticized PVC is an amorphous compound,[14] while all the sensors used possess crystalline properties at ambient temperature. Therefore, for dissolution of the sensor to occur, there must be hydrogen bonding or some other specific interaction, either with the plasticizer or with the supporting medium (PVC) or both. This specific interaction is necessary to overcome the tendency of crystalline compounds to form separate phases on solidification. Corroborating evidence for the existence of some specific interaction between the sensor and the supporting medium was obtained by separate experiments in which the dissolution of TDDAN and TDDPN in the plasticizers was examined. Neither sensor dissolved in the plasticizers alone. This observation coupled with the fact that TDDAN was segregated in the membrane matrix [Figs. 3(*b*) and 4(*b*)], whereas all QPS seemed to be dissolved, supported our belief that QPS must exhibit some specific interaction with the PVC, whereas QAS does not. As PVC is a weak proton–donor polymer,[14] one possible mechanism governing

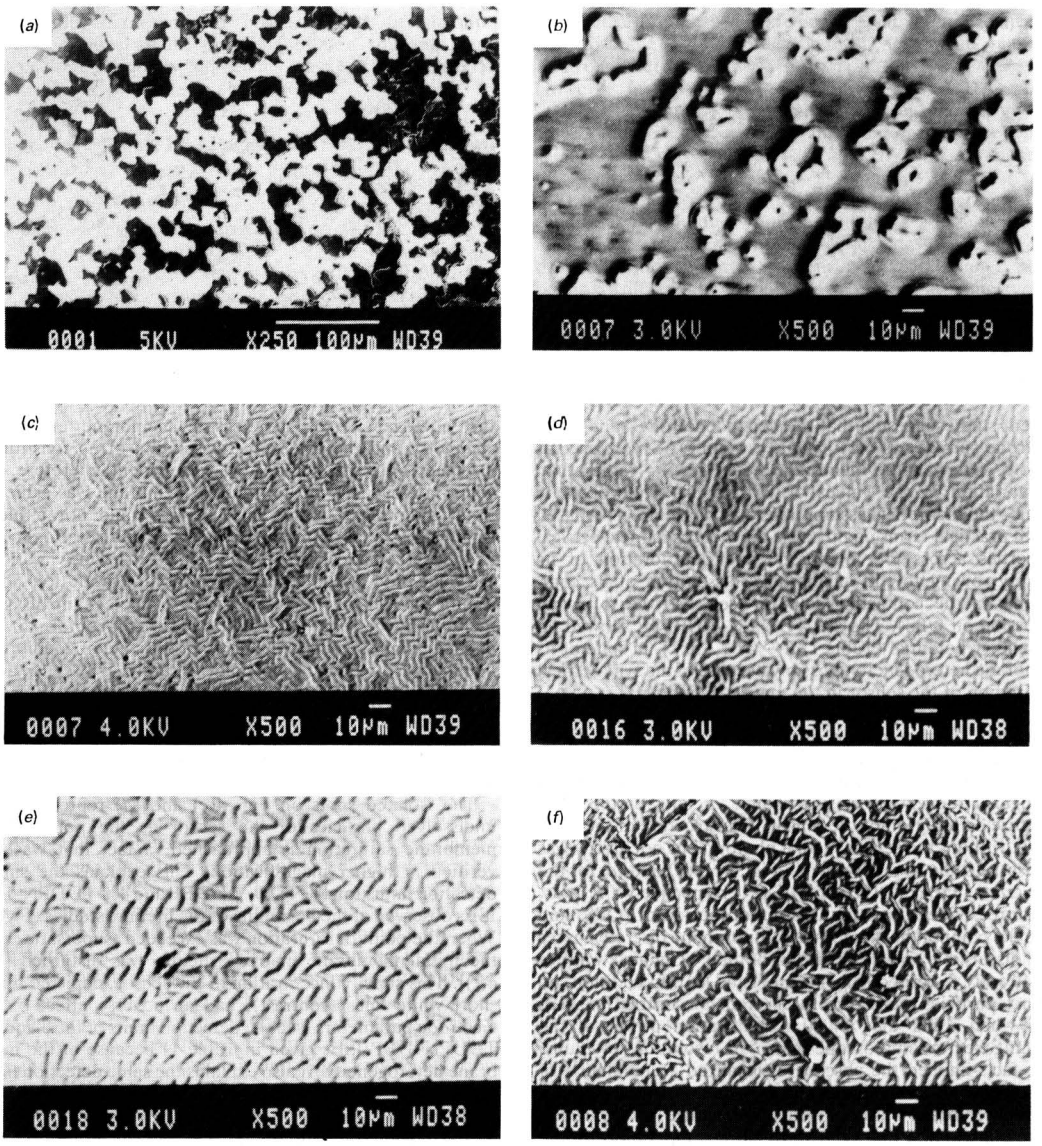
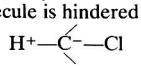


Fig. 4 Photomicrographs taken with an electron scanning microscope of various nitrate ISEs: (*a*) 4% TDDAN–DBP; (*b*) 4% TDDAN–DOP; (*c*) 4% TDDPN–DBP; (*d*), 4% TDDPN–DOP; (*e*) 14% TOPN–TXP; and (*f*) 6% TDDPN–DBP

QPS and QAS interaction with the PVC stems from the configuration of the QPS and QAS molecules and the distribution of the negative fractional charge, δ^-, in the polar bonds P–I, P–Br, N–I and N–Br. These bonds are considered because the sensors were dispersed in the matrix in the form of bromide or iodide salts. The molecule of the sensor consists of four organic chains at the apices of a tetrahedron, co-ordinated to one P or N atom at the centre. Bromide and iodide ions lie outside the organic tetrahedron and, considering the electronegativity values of P = 2.1, I = 2.5, Br = 2.8 and N = 3.0, it seems reasonable to postulate that, for the N–I or N–Br bond, the negative fractional charge δ^- is concentrated towards the central (N) within the tetrahedron and, hence for steric reasons, any interactions with the positive charge of the PVC molecule is hindered.



However, for the P–I or P–Br bond, δ^- is more concentrated towards the I^- and Br^- outside the tetrahedron. This external concentration of δ^- favours van der Waal's attraction forces with the PVC molecule, rendering the two compounds compatible with each other and preventing the formation of separate phases.

Applications

The nitrate ISEs based on QPS in a PVC matrix were used to determine nitrates in waters and in soil and plant extracts (Table 3). Nitrate values assessed with these electrodes were closely correlated with those obtained with 4% TDDAN–DBP, Orion 93-07 nitrate ISEs and ion chromatography, while the regression lines had a slope and an intercept not statistically different from 1 and 0, respectively, at the 5% probability level (Table 3). The precision of the measurements

Table 3 Determination of nitrate with nitrate ISEs based on quaternary phosphonium salts. The results are compared with those obtained with TDDAN and Orion 93-07 nitrate ISEs and the ion chromatography (IC) method

	NO_3^-/mg dm^{-3}					
Sample	15% TOPN–DBP (y_1)	4% TDDPN–DBP (y_2)	3% TDDPN–DOP (y_3)	4% TDDAN–DBP (y_4)	Orion 93-07 (y_5)	IC (x)
Tap water	2.9	2.8	2.9	2.5	2.7	2.6
Tap water	21.9	21.3	21.3	20.6	21.0	21.5
Tap water	42.1	40.6	42.2	41.2	41.5	42.5
Well water	55.5	55.8	56.7	56.7	56.0	57.8
Well water	69.0	69.4	71.0	69.7	69.8	70.1
Well water	107.3	104.9	105.3	103.8	104.9	109.5
Plant	159.1	157.9	158.5	155.2	156.6	157.1
Plant	210.1	211.5	213.0	210.5	209.2	214.1
Soil	92.5	89.4	92.1	88.5	88.4	90.4
Soil	76.9	79.0	78.7	78.4	79.0	78.9

Correlation coefficient (r)	Regression equation
0.9995*	$y_1 = 0.14 + 0.989x$
0.9997*	$y_2 = -0.58 + 0.993x$
0.9996*	$y_3 = -0.01 + 0.997x$
0.9999*	$y_2 = -0.24 + 1.009y_4$
0.9999*	$y_2 = -0.64 + 1.012y_5$

* Significant at the 5% probability level.

(σ_A) and blank values (σ_B) obtained with the electrodes were assessed from ten replicates of a standard solution of 100 mg dm^{-3} of NO_3^- and were found to be: (*i*) 15% TOPN–DBP or DOP, 0.60 ± 0.15, 1.30 ± 0.20; (*ii*) 4% TDDPN–DBP or DOP, 0.50 ± 0.10, 0.60 ± 0.20; (*iii*) 4% TDDPN–TXP, 0.60 ± 0.15, 0.70 ± 0.20; (*iv*) 4% TDDAN–DBP, 0.90 ± 0.15, 1.00 ± 0.20; and (*v*) Orion 93-07, 0.90 ± 0.15, 1.00 ± 0.20 mV.

Conclusions

The homogeneous dispersion of QPS in a PVC matrix resulted in nitrate ISEs, superior to earlier electrodes in both stability and sensitivity. It also helped in clarifying the following practical aspects for the development of nitrate ISEs based on non-porous PVC membranes: (*i*) the solubility parameter of PVC is 9.5 (cal cm^{-3})$^{1/2}$ and the solubility parameters of the plasticizers range from 8.5 to 10.5 (Brydson[14]) resulting in a membrane solubility parameter range of 9–10; consequently, according to the general rule that $d_s = d_m$, the solubility parameter of the sensor used in a PVC matrix must lie within the range 9–10; (*ii*) the point of membrane saturation with the sensor must not be exceeded; and (*iii*) the homogeneous dispersion of the sensor (a crystalline compound) in a plasticized PVC matrix is ensured when the sensor is either dissolved in the plasticizer or linked by some mechanism to the supporting medium (PVC). It is our opinion that the sensor must exhibit proton-acceptor properties and must, by necessity, be linked to the PVC.

In general, the choice of the membrane constituents (polymer and/or sensor) should be made in such a way that they should have the same solubility parameters and exhibit some specific interaction, favouring the homogeneity of the sensing membrane.

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Adsorptive Stripping Voltammetric Behaviour of Copper(II) at a Hanging Mercury Drop Electrode in the Presence of Excess of Imidazole

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In the presence of excess of imidazole (1.0×10^{-3} mol dm^{-3}), copper(II), at pH 8.5, adsorbs at a hanging mercury drop electrode to give two adsorptive stripping voltammetric peaks at -0.36 and -0.46 V. The peak at -0.36 V is only present at accumulation potentials more negative than -0.05 V *versus* Ag–AgCl: it increases in height as the accumulation potential becomes more negative up to and beyond -0.6 V, the voltammetric sweep being started at -0.20 V. This peak appears to be due to the adsorption of polymeric $[Cu^{II}(Im)_2]$ or its reduced copper(I) form. The peak at -0.46 V is only present at high imidazole concentrations (>5.0×10^{-4} mol dm^{-3}): the accumulation is uniform from 0.0 to -0.36 V but is negligible at potentials more negative than -0.46 V. This peak appears to be due to adsorption of $[Cu(Im)_4]^{2+}$. On cycling between 0.0 and -0.6 V this latter complex is converted into the polymeric complex and only the peak at -0.36 V remains. Copper(II) can be determined by using the peak at -0.46 V after accumulation at 0.0 V, or at -0.36 V after accumulation at -0.6 V. The latter method is more sensitive: the detection limit is about 2.0×10^{-9} mol dm^{-3} after accumulation for 3 min.

Keywords: *Adsorptive stripping voltammetry; copper(II); imidazole complex*

The imidazole ring, which is present in the amino acid histidine, functions as a ligand towards transition metal ions in a variety of biologically important molecules including the iron–haem system, vitamin B_{12} and its derivatives and several metalloproteins.[1] The imidazole nitrogen atoms of the histidine residues provide one of the primary means by which metal ions can be bound to proteins. The relationship between the structural property of the imidazole ring, its function in biological systems and its complexes with a number of transition metal ions have been reviewed.[2] Imidazole is amphoteric, being a moderately strong organic base capable of accepting a proton at N-3 (the pyridine-like nitrogen, pK_a = 7.1) and also a very weak acid capable of losing a proton from N-1 (the pyrrole-like nitrogen, pK_a = 14.3).

In neutral solutions the unprotonated imidazole molecule usually functions as a ligand by using the unshared pair of electrons on N-3. In sufficiently basic media the conjugate base of imidazole, Im^-, is formed and its complexes with dipositive metal ions are considered to have a stoichiometry $[M(Im)_2]$. These complexes have been considered to be polymeric, and are, in general, insoluble. The imidazole salt of copper(I) can be prepared also and it has been considered to have a polymeric bridge structure.[3] Li *et al.*[4] have investigated the polarographic behaviour of the copper(II) complex formed at high concentrations of imidazole in water–ethanol mixtures and showed that the complex is reduced in two steps giving two waves of approximately equal height, the first at -0.19 V and the second at -0.57 V *versus* a saturated calomel electrode (SCE). Both waves are due to a one-electron reduction. They also found the copper(II) and copper(I) complexes with the highest Im : Cu ratio to be $[Cu(Im)_4]^{2+}$ and $[Cu(Im)_2]^+$, respectively.

Recent work in this laboratory[5,6] has indicated that histidine can be determined at the nanomolar level at a hanging mercury drop electrode (HMDE) as its copper(II) complex, and that copper(II) can be accumulated rapidly and selectively at an HMDE modified by adsorption of a poly(L-histidine) film. In view of the biochemical importance of compounds containing the imidazole ring, and the affinity of this ring for coordinating metal ions,[7] a study has been made, and is reported here, of the adsorptive stripping voltammetric behaviour of copper(II) in the presence of excess of the parent imidazole molecule. Copper(II) can be determined by this means.

Experimental

Adsorptive stripping voltammetry was carried out by using a Metrohm 626 Polarecord with a 663 VA stand in conjunction with a multi-mode electrode in the HMDE mode. The three-electrode system was completed by means of a glassy carbon auxiliary electrode and an Ag–AgCl reference electrode.

All potentials given are relative to this Ag–AgCl electrode. A pulse amplitude of 50 mV was used with a scan rate of 10 mV s^{-1} and a forced pulse interval of 1 s. A Princeton Applied Research Model 174A polarographic analyser in conjunction with a VA 663 stand was used for cyclic voltammetry. The pH measurements were made with a Corning combined pH/reference electrode by using a Radiometer PHM 64 pH meter. Imidazole [ultraviolet(UV) spectroscopic grade, specially prepared for use in UV spectrophotometric beta-lactam assays] was obtained from Merck (formerly BDH) and the other chemicals were purchased from Sigma. All were used without further purification.

A 0.2 mol dm^{-3} solution of imidazole was prepared by dissolving 0.1361 g of imidazole in water acidified with 6 drops of 6 mol dm^{-3} HCl in a 10 ml calibrated flask.

Procedure

The general procedure used to obtain adsorptive stripping voltammograms was as follows. A 20 ml aliquot of 0.01 mol dm^{-3} hydrogen carbonate buffer solution was placed in a voltammetric cell and the required amounts of standard imidazole and copper(II) solutions were added. The stirrer was switched on and the solution was purged with nitrogen for 6 min. Subsequently, de-oxygenation was carried out for 15 s between adsorptive stripping cycles. After forming a new HMDE, accumulation was effected for 2 min at the required potential while stirring the solution. At the end of the accumulation period the stirrer was switched off, and, after 20 s had elapsed to allow the solution to become quiescent, a

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negative potential scan was initiated between the accumulation potential and −0.7 V. In the study of the influence of accumulation time, the adsorptive accumulation was carried out at potentials more negative than −0.4 V, and immediately after this step, the potential was changed to −0.1 V from where a negative potential scan was initiated.

Cyclic voltammetry was preceded by accumulation at 0.0, −0.1 or −0.6 V for 2 min. A scan rate of 50 mV s^{-1} was used. The initial sweep was to more negative potentials when accumulation was performed at 0.0 or −0.1 V. Accumulation at −0.6 V was followed by an anodic sweep to 0.0 V. Second and third scans were made on the same drop immediately after the first scan without further accumulation.

Direct current voltammetry was performed by using the HMDE and starting at 0.18 V with a scan rate of 2 mV s^{-1}.

Results and Discussion

The shapes of the differential-pulse stripping voltammograms and of the cyclic voltammograms obtained after accumulation in a 1.0×10^{-7} mol dm^{-3} solution of copper(II) in the presence of excess of imidazole were found to be dependent on the pH, accumulation potential, accumulation time, and copper(II) and imidazole concentrations. The influence of the pH on the differential-pulse stripping peak current of a 2.0×10^{-7} mol dm^{-3} solution of copper(II) in the presence of 1.0×10^{-3} mol dm^{-3} imidazole is summarized in Table 1.

At pH 4.5 (0.1 mol dm^{-3} acetate buffer) no significant adsorption of the complex at the electrode surface was observed. In neutral or basic media two different peaks, at −0.36 and −0.46 V *versus* Ag–AgCl, were observed and the heights of both of these decreased with increasing pH above 8.5. This effect can be attributed to the formation of hydroxo complexes with copper(II).[4] As the highest currents were obtained in 0.1 mol dm^{-3} hydrogen carbonate buffer (pH 8.5), this buffer was chosen for use in further studies. A very small shift (only a few mV) was observed in the peak potentials when the pH was varied from 7.0 to 10.5, showing that there is no loss or gain of protons in the reduction process.

The influence of the accumulation potential on the cyclic voltammograms of a 3.0×10^{-7} mol dm^{-3} solution of copper(II), at various imidazole concentrations, is shown in Fig. 1. The cyclic voltammograms obtained when accumulation was carried out at 0.0 V for 120 s [Fig. 1(*a*)], in the presence of 1.0×10^{-3} mol dm^{-3} imidazole, gave a single peak at −0.42 V in the cathodic scan. Two small, broad peaks at −0.40 and −0.35 V were observed in the subsequent anodic sweep. No peak was observed when the imidazole concentration was significantly lower. When accumulation was performed at −0.1 V, an increase in the peak current and a shift in the peak potential were observed when the imidazole concentration was increased from 1.0×10^{-6} to 1.0×10^{-3} mol dm^{-3}. A single peak at −0.17 V was observed in the presence of 1.0×10^{-6} mol dm^{-3} imidazole. At imidazole concentrations between 1.0×10^{-4} and 1.0×10^{-3} mol dm^{-3} [Fig. 1(*b*)], two peaks were observed in the cathodic scans: the first at about −0.36 V and the second at −0.46 V. Associated with them, peaks at −0.40 and −0.30 V were observed in the anodic scans. Small shifts in the peak potential of the first peak were observed with increasing imidazole concentration. Decreases in the height of the peak at −0.36 V and increases in the height of the peak at −0.46 V were observed with increasing imidazole concentration. At an imidazole concentration of

Table 1 Effect of pH on the peak current obtained for the differential-pulse adsorptive stripping voltammetry of the copper(II)–imidazole complex after accumulation at −0.1 V for 120 s. [Copper(II)] = 2.0×10^{-7} mol dm^{-3}; [imidazole] = 1.0×10^{-3} mol dm^{-3}

pH	Peak current at −0.46 V/nA
4.5	0
7.0	12.2
8.5	29.2
9.5	9.0
10.5	5.0

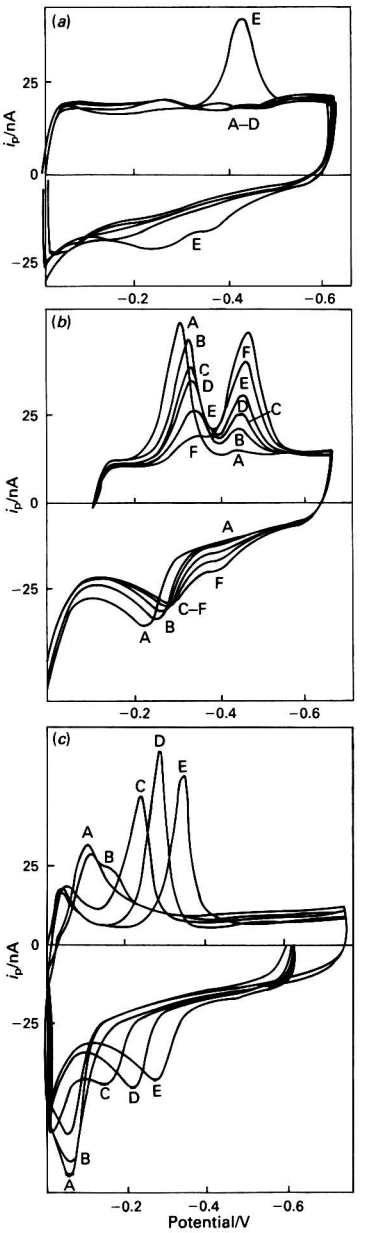

Fig. 1 Effect of the imidazole concentration and the accumulation potential on the cyclic voltammograms, obtained at an HMDE, for a 3×10^{-7} mol dm^{-3} copper(II) solution in 0.1 mol dm^{-3} hydrogen carbonate buffer at pH 8.5 with an accumulation time of 120 s. (*a*) Accumulation at 0.0 V; (*b*) accumulation at −0.1 V; and (*c*) accumulation at −0.6 V. Concentration of imidazole [Im]: A, 0; B, 1.0×10^{-6}; C, 1.0×10^{-5}; D, 1.0×10^{-4}; and E, 1.0×10^{-3} mol dm^{-3} for (*a*) and (*c*). For (*b*) A, 1.0×10^{-4}; B, 3.0×10^{-4}; C, 5.0×10^{-4}; D, 7.0×10^{-4}; E, 1.0×10^{-3}; and F, 1.5×10^{-3} mol dm^{-3}

2×10^{-3} mol dm^{-3}, only the peak at -0.46 V was present in the cyclic voltammogram.

The presence of a single peak in both the cathodic and anodic scans was observed in the voltammograms when accumulation was carried out at potentials more negative than -0.4 V and scanning from 0.0 V [Fig. 1(*c*)]. In this instance the difference in the peak potentials of the cathodic and anodic waves was found to be 60 mV and the value of the width at half-height observed was about 60 mV. This suggests a one-electron reduction.

The influence of the accumulation potential on the peak currents for a 3×10^{-7} mol dm^{-3} solution of copper(II) in the presence of 1.0×10^{-3} mol dm^{-3} imidazole is shown in Table 2. The potential of the peak at the less negative potential was shifted significantly in the negative direction with an increase in the imidazole concentration, as expected for complex formation (see Table 3).

Cyclic voltammograms of a 3×10^{-7} mol dm^{-3} solution of copper(II) in the presence of 1.0×10^{-3} mol dm^{-3} imidazole obtained by successive scans at the same drop are shown in Fig. 2. Initial accumulation was performed for 120 s. No further accumulation was carried out between the scans. When accumulation was performed at -0.1 V [Fig 2(*a*)], the two cathodic peaks at -0.36 and -0.46 V, and associated with them two anodic peaks at -0.40 and -0.30 V, were observed. A decrease in the height of the peak at -0.46 V and a simultaneous increase in the height of the peak at -0.36 V were observed with increasing scan number. An isosbestic point was obtained at -0.41 V. The appearance of two cathodic peaks appears to be due to accumulation of two different copper(II)–imidazole complexes at the electrode surface. The subsequent predominance of the peak at -0.36 V must be caused by the complex which is reduced at -0.46 V being converted into that responsible for the peak at -0.36 V. In fact, Nozaki *et al.*[8] observed four different copper(II) complexes in the copper(II)–4-methylimidazole system, the zone of predominance of each depending on the 4-methylimidazole concentration. Only the cathodic peak at -0.37 V and the anodic peak at -0.30 V were observed in the voltammograms when accumulation was carried out at -0.6 V [Fig. 2(*b*)]. A small increase in the peak current with successive scans suggests film formation on the electrode surface.

The influence of the addition of copper(II) on the cyclic voltammograms obtained for a 1.0×10^{-3} mol dm^{-3} solution of imidazole in 0.1 mol dm^{-3} hydrogen carbonate buffer (pH 8.5) is shown in Fig. 3. With accumulation at -0.1 V [Fig. 3(*a*)], a single peak at about -0.42 V was observed at copper(II) concentrations lower than 1.0×10^{-7} mol dm^{-3}. The height of this peak increased with increasing copper(II) concentration. At copper(II) concentrations higher than 1.0×10^{-7} mol dm^{-3}, a second peak was observed at -0.36 V.

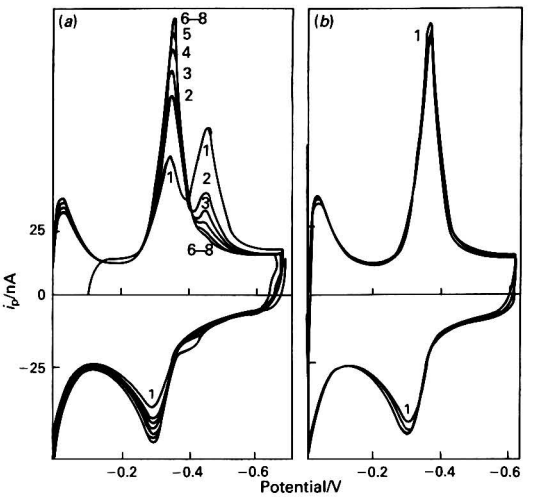

Fig. 2 Effect of successive scans on the cyclic voltammograms of a 3×10^{-7} mol dm^{-3} copper(II) solution in the presence of 1×10^{-3} mol dm^{-3} imidazole in 0.1 mol dm^{-3} hydrogen carbonate buffer at pH 8.5. Accumulation for 120 s at (*a*) -0.1 V and (*b*) -0.6 V. The numbers indicate scan number

Table 2 Influence of the accumulation potential (E_{acc}) on the peak currents (i_p) of a differential-pulse adsorptive stripping voltammogram of a 3×10^{-7} mol dm^{-3} solution of copper(II) in presence of 1.0×10^{-3} mol dm^{-3} imidazole in 0.1 mol dm^{-3} hydrogen carbonate buffer (pH 8.5). Accumulation time: 120 s

	i_p/nA	
E_{acc}/V	−0.36 V	−0.46 V
0.0	—	37.5
−0.05	5.0	37.5
−0.10	26.0	42.5
−0.15	37.0	44.0
−0.20	35.5	41.0
−0.30*	35.5	40.0
−0.33*	37.0	39.0
−0.37*	42.5	33.5
−0.40*	61.5	19.0
−0.42*	72.5	12.0
−0.45*	84.0	—
−0.50*	89.0	—

* Potential scanned from −0.20 V.

Table 3 Effect of the imidazole concentration on the cathodic peak potential (E_p) of the copper(II)–imidazole complex in 0.1 mol dm^{-3} hydrogen carbonate buffer (pH 8.5). [Copper(II)] = 3×10^{-7} mol dm^{-3}. Accumulation step, -0.6 V for 120 s

[Imidazole]/ mol dm^{-3}	E_p/V
1.0×10^{-6}	−0.18
1.0×10^{-5}	−0.22
1.0×10^{-4}	−0.27
1.0×10^{-3}	−0.36

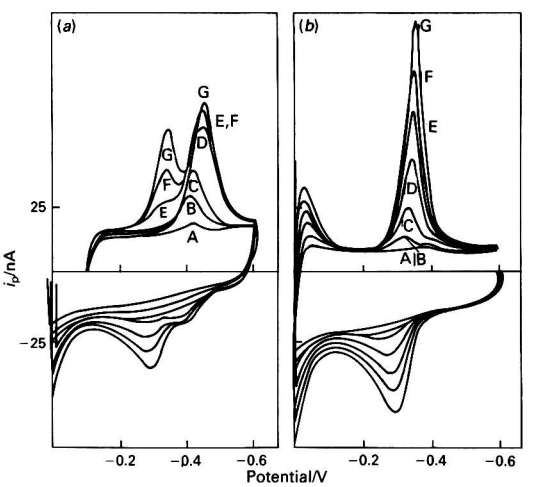

Fig. 3 Effect of the copper(II) concentration on the cyclic voltammograms at the HMDE, for a 1×10^{-3} mol dm^{-3} imidazole solution in hydrogen carbonate buffer at pH 8.5. Accumulation at (*a*) -0.1 V and (*b*) -0.6 V for 120 s. Copper(II) added: A, 0; B, 5×10^{-8}; C, 1×10^{-7}; D, 2×10^{-7}; E, 3×10^{-7}; F, 4×10^{-7}; and G, 5×10^{-7} mol dm^{-3}

The height of this peak increased with a further increase in the copper(II) concentration. When this peak was present in the voltammogram, only a small increase in the height of the peak at −0.42 V was observed with increasing copper(II) concentration. A similar behaviour was observed in the anodic scans and, in this instance, a peak at −0.30 V, associated with the cathodic peak at −0.36 V, predominated. These results agree with those obtained by increasing the imidazole concentration and suggest that the complexes responsible for the two peaks are strongly dependent on the ratio of copper(II):imidazole.

These experiments suggest that the complex undergoing reduction at −0.36 V is more stable and more easily formed at the electrode surface during the scan time.

The nature of these peaks is still not clear. One hypothesis for the cyclic voltammograms obtained is that the reduction observed at −0.46 V could be due to the reduction of a higher order complex of copper(II) present in the solution, possibly $[Cu(Im)_4]^{2+}$, according to the reaction

$$[Cu(Im)_4]^{2+}\ (ads) + e^- \rightarrow [Cu(Im)_2]^{+}(ads) + 2Im$$

with the subsequent formation of a polymeric copper(I)–imidazole complex at the electrode surface.

The peaks at −0.30 V (oxidation) and −0.36 V (reduction) would be due to the reduction or oxidation of the metal ion in the polymeric film formed at the electrode surface.

In fact, copper(I) was reported to react with imidazole at pH values higher than 4.5, forming $[Cu(Im)_2]^+$ which, at pH >6.5, polymerizes. In this instance imidazole acts as a bidentate ligand for copper(I).[9] The formation of a polymeric complex between copper(II) and imidazole containing two imidazole molecules per metal atom in aqueous hydrogen carbonate solution has also been described.[3]

The peak current, obtained when accumulation was performed at −0.6 V and the scan was started at 0.0 V, increased rectilinearly with the square root of the scan rate. This relationship suggests diffusional behaviour probably caused by multimolecular film formation.[10] A shift in the cathodic peak potential from −0.32 to −0.36 V was observed when the scan rate was varied from 10 to 100 mV s^{-1}. A shift from −0.30 to −0.26 V was observed for the anodic peak under the same conditions. Similar results were obtained for both peaks (*i.e.*, those at −0.36 and −0.42 V) when accumulation was carried out at −0.1 V. These results suggest that the system is irreversible to a small extent.

From the above results it is apparent that copper(II) can be determined by using an excess of imidazole as an accumulation reagent. When the accumulation was performed at −0.6 V, the height of the copper(II)–imidazole peak at −0.36 V, obtained by using a solution containing 6×10^{-8} mol dm^{-3} copper(II) and 1×10^{-3} mol dm^{-3} imidazole, showed a rectilinear relationship [correlation coefficient (r) = 0.998] with the accumulation time up to 6 min. Rectilinear calibration graphs were obtained for copper(II) in the presence of 1.0×10^{-3} mol dm^{-3} imidazole when accumulation was carried out at 0.0 or −0.6 V for 3 min. When accumulation was performed at 0.0 V for 3 min, a rectilinear calibration graph was obtained from 5×10^{-9} to 1×10^{-7} mol dm^{-3} copper(II) (r = 0.9998) with a slope of 2.84×10^8 nA mol^{-1}. At copper(II) concentrations higher than 1×10^{-7} mol dm^{-3}, deviation from linearity was observed probably owing to saturation of the electrode surface. Better sensitivity (5.2×10^8 nA mol^{-1}) and range [5×10^{-9}–1.5×10^{-7} mol dm^{-3} copper(II)] were obtained when accumulation was performed at −0.6 V. The limit of detection was 2×10^{-9} mol dm^{-3} for accumulation at −0.6 V for 3 min.

Several reagents have been suggested for the differential-pulse adsorptive stripping voltammetric determination of copper(II).[11,12] The limits of detection for determinations with catechol and 8-hydroxyquinoline are given as 3×10^{-10} and 1×10^{-10} mol dm^{-3}, respectively,[13] based on an accumulation time of 1 min from a stirred solution: the use of catechol has the disadvantage that its solutions are readily oxidized by air and the reagent must be freshly prepared.[11] The detection limit using imidazole does not appear to be as good.

Further studies are in progress on the adsorptive stripping voltammetric behaviour of a range of imidazole derivatives, including several of pharmaceutical importance. The determination of these compounds by using this technique will be investigated; those compounds with reducible groups, *e.g.*, the nitroimidazoles, might be determinable directly or as copper(II) complexes. The use of imidazoles to determine copper and other metal ions will be studied in greater detail in order to find the most suitable reagent(s) for these metals. It is also intended to study the adsorptive stripping voltammetry of imidazole derivatives of beta-lactam antibiotics.

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Simultaneous Determination of Acetylsalicylic and Salicylic Acids in Human Serum and Aspirin Formulations by Second-derivative Synchronous Fluorescence Spectrometry

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A second-derivative synchronous scanning spectrofluorimetric method for the simultaneous determination of acetylsalicylic acid (ASA) and salicylic acid (SA) is described. The method is based on the native fluorescence of both acids in a 1% acetic acid–chloroform solution. Both ASA and SA can be determined within the concentration ranges 0.2–70 and 0.03–10 μg ml^{-1}, respectively. The effect of each acid on the signal of the other has been studied in detail. Empirical equations have been used to overcome this effect, thus allowing the accurate determination of both acids in binary mixtures, without a separation step. The method has been applied to the determination of ASA and SA in blood serum and to the determination of SA impurities in aspirin formulations. Recoveries from sera spiked with both ASA (2.5–50 μg ml^{-1}) and SA (100–160 μg ml^{-1}) varied from 99.5 to 106.7% (mean = 102.6%) and from 93.0 to 98.0% (mean = 95.8%), respectively. Recoveries of SA from spiked aspirin solutions (0.25–1.5 mg g^{-1} of aspirin) varied from 98.0 to 102.0% (mean = 100.3%).

Keywords: *Second-derivative synchronous fluorescence spectrometry; acetylsalicylic acid; salicylic acid; human serum; aspirin formulation*

The large number of published methods for the determination of acetylsalicylic acid (ASA) and salicylic acid (SA) in biological fluids and pharmaceuticals, which make use of a large variety of analytical techniques, is not only indicative of the great interest in the determination of these compounds, but also of the problems encountered with their determination with regard to directness, specificity, sensitivity, simplicity, *etc.* Analytical methods, allowing the simultaneous determination of both acids and/or the determination of trace amounts of each compound in the presence of a large excess of the other, would be of great practical importance in pharmacokinetic investigations of ASA. They are also important in the quality control of aspirin formulations, considering the wide therapeutic spectrum and range of uses of SA and its derivatives.

From the published techniques used for the simultaneous determination of ASA and SA in a single sample, gas–liquid chromatography (GLC) is the most specific. However, chemical derivatization, such as silylation, is necessary in order to make these compounds suitable for GLC.[1] Derivatization is inherently time consuming and may complicate the analysis by partial hydrolysis of ASA to SA. Other chromatographic methods, such as thin-layer chromatography (TLC)[2] and high-performance liquid chromatography (HPLC),[3,4] have also been reported.

The development of a simple, specific, sensitive and inexpensive method, requiring only mild pre-treatment of the sample in order to avoid hydrolysis of ASA, as an alternative to chromatographic techniques, would be highly advantageous for the determination of ASA and SA in biological fluids and pharmaceuticals. Fluorescence spectrometry appears to be the most convenient instrumental technique for the development of such an analytical method and a considerable amount of research has been carried out in this area.

Salicylic acid has an intense fluorescence in various solvents and several trace analysis methods are based on its native fluorescence.[5,6] Acetylsalicylic acid can also be determined directly by use of its native fluorescence in acetic acid–chloroform solutions,[7] but more commonly, is measured after hydrolysis to SA. The sample is hydrolysed either directly[8] or after a separation step when the sample contains both acids.[6,9]

The fluorescence properties of ASA and SA are strongly solvent-dependent. In polar solvents (water, alcohols, *etc.*) the spectral properties of ASA and SA are almost identical.[10] In acetic acid–chloroform solutions the fluorescence maxima of ASA and SA are sufficiently resolved so as to be useful for the direct and simultaneous determination of both compounds. However, the much higher quantum efficiency of SA[7] and the high SA : ASA and ASA : SA ratios commonly encountered in biological fluids and pharmaceuticals, respectively, inevitably lead to spectral overlaps, hindering the simultaneous determination of these compounds by conventional fluorescence spectrometry (CFS).

Synchronous scanning[11] and derivative[12] fluorescence spectrometry are excellent improvements of CFS, and are particularly suitable for severely overlapping spectral bands, whereas their combination is, apparently, even more versatile. The basic principles, the main advantages and the analytical applications of these techniques have been reviewed.[13]

In the present paper, a method is described for the simultaneous determination of ASA and SA in a single sample by second-derivative synchronous (scanning) fluorescence spectrometry (SDSFS). A commercial fluorescence spectrometer was interfaced to a low-cost microcomputer and appropriate software was developed in order to implement and take full advantage of the SDSFS technique. The results obtained show that complete analysis of ASA–SA mixtures can be readily achieved by SDSFS, and small amounts of each acid can be determined reliably in the presence of large amounts of the other. The proposed method was applied to the determination of ASA and SA in human serum and in aspirin formulations with satisfactory results; in human serum ASA can be determined down to 2.5 μg ml^{-1} in the presence of a 40-fold excess of SA, whereas in aspirin formulations SA can be determined in the presence of up to a 1000-fold excess of ASA.

Experimental

Apparatus

A Model 512 fluorescence spectrometer (Perkin-Elmer, Norwalk, CT, USA), equipped with a 150 W arc xenon lamp

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and a magnetic stirrer under the cell holder, was used. All measurements were obtained in a standard 10 mm (path-length) quartz cell, thermostated at 25.0 ± 0.5 °C. Excitation and emission monochromators were locked together and scanned simultaneously with a constant difference $\Delta\lambda = \lambda_{em} - \lambda_{ex}$.

The digital readout unit of the spectrometer was interfaced to an Amstrad CPC-6128 microcomputer for spectral data acquisition, calculation of the spectrum derivatives and automatic evaluation and presentation of the signals. An electromagnetic relay controlled by the microcomputer was connected in parallel to the poles of the switch that activated the scanning of the monochromators.

The control program named F-MOD (Fluorimetric-MODule), was written in BASIC, except for a short data acquisition routine which was written in Z-80 machine language. The F-MOD program is a menu-driven, interactive program which allows the measurement of spectrum derivatives, thus considerably extending the analytical capabilities of the fluorescence spectrometer.

The spectrum is displayed on screen during the measurement, whereas after scanning, the stored spectrum is subjected to smoothing and differentiation using the Savitzky–Golay method.[14,15] The smoothed spectrum and its active derivative are displayed on a split screen. At the request of the operator, a cursor appears on both spectra, in order to facilitate the selection of the spectral ranges where the automatic evaluation of the signals will take place. Up to ten spectral ranges may be defined, hence, up to ten different signals can be evaluated simultaneously.

Reagents

Spectroscopic quality grade chloroform (Merck, Darmstadt, Germany) was used to make up 1% v/v acetic acid in chloroform. Henceforth this mixture will be referred to as 'mixed solvent'. Chemically pure ASA and SA (Fluka, Buchs, Switzerland) were used and stock solutions containing 4.00 and 2.00 mg ml^{-1} of the acids, respectively, were prepared in the mixed solvent. The purity of ASA and SA was tested using standard United States Pharmacopeia (USP) procedures.[16] The stock solution of SA was stable for at least 1 year at room temperature, whereas the stock solution of ASA was prepared daily. Standard working solutions were prepared by appropriate dilution of the stock solutions with mixed solvent.

Aqueous stock solutions of ASA and SA containing 1.00 and 0.5 mg ml^{-1} of the acids, respectively, were prepared daily in distilled, de-ionized water. These solutions were used for recovery experiments in albumin solutions and in normal serum (Ciba-Corning, Quality Control Serum).

A stock solution containing 100 mg ml^{-1} of bovine albumin (Sigma) was prepared in distilled, de-ionized water.

Procedures and Calculations

To measure the signal for ASA, ΔI^2_{ASA}, and SA, ΔI^2_{SA}, obtain the synchronous fluorescence spectra by scanning both monochromators simultaneously at a constant difference $\Delta\lambda$ = 60 nm (λ_{ex} = 240–320 nm), and $\Delta\lambda$ = 130 nm (λ_{ex} = 290–380 nm), respectively. (Hereafter, all wavelengths referring to synchronous spectra are taken as being equal to those of the corresponding excitation wavelengths.)

Evaluate ΔI^2_{ASA} within the spectral range 260–290 nm, and ΔI^2_{SA} within the spectral range 310–344 nm and calculate the slopes S_{ASA} and S_{SA} of the standard calibration graphs of ASA and SA using their standard working solutions (in the mixed solvent) covering the concentration ranges 0.2–60 and 0.05–10 μg ml^{-1}, respectively.

Serum samples

Place 0.50 ml of serum containing 1.25–25 μg of ASA and 50–80 μg of SA into a test-tube. Add 2.00 ml of the mixed solvent, sonicate the mixture for 1 min and centrifuge for 10 min at 1500*g*. Discard the upper layer and transfer 1.00 ml (0.50 ml for higher concentrations of SA) of the organic layer into the cuvette, add mixed solvent to a total volume of 2.00 ml and start the stirrer. Obtain ΔI^2_{SA} as described above.

Calculate the concentration of SA in the extract, c_{SA} (μg ml^{-1}), from the standard calibration graph (c_{SA} is required for the subsequent ASA determination). The total concentration of SA in the serum is calculated from a calibration graph obtained with control serum standards spiked with SA (concentration range 40–200 μg ml^{-1}) and treated similarly.

For the determination of ASA in serum the signal for the extract, ΔI^2_{ASA}, is obtained as described above. The concentration of ASA in the extract, c_{ASA} (μg ml^{-1}), is given by

$$c_{ASA} = \frac{\Delta I^2_{ASA}}{S_{ASA}(1.008 - 0.0173 c_{SA})} + 0.157 c_{SA} + 0.013 \quad (1)$$

The total concentration of ASA in serum, $c_{ASA(serum)}$ μg ml^{-1}), is given by

$$c_{ASA(serum)} = 1.05 \text{ df } c_{ASA} \quad (2)$$

where df is the actual dilution factor (8 or 16).

Aspirin formulations

Grind 20 aspirin tablets to a fine powder, transfer an accurately weighed amount of the powder (30–50 mg) into a 10 ml calibrated flask, dilute to the mark with the mixed solvent and allow any undissolved material to precipitate. Transfer 20 μl of the clear supernatant solution into a cuvette, add 2.00 ml of the mixed solvent and start the stirrer. Obtain ΔI^2_{ASA}, and find c_{ASA} (μg ml^{-1}), from the standard calibration graph. The percentage of ASA in aspirin is given by

$$\text{ASA (\%)} = 101\, c_{ASA}/W \quad (3)$$

where W is the amount of weighed aspirin (in mg).

For the determination of impurities of SA in aspirin, transfer an additional 480 μl volume of the aspirin solution into the same cuvette, obtain ΔI^2_{SA}, and calculate the corresponding concentration of SA, c_{SA} (μg ml^{-1}), from the standard calibration graph.

The percentage of SA in aspirin is then given by

$$\text{SA (\%)} = \frac{5\,(c_{SA}/F)}{W} \quad (4)$$

where F is a correction factor and its value depends on the actual concentration of ASA, typically 1.05 for this particular determination.

Results and Discussion

Evaluation of the Signal

In derivative fluorescence spectrometry, the signal, ΔI^n_X, of the analyte X, is evaluated by the following equation:

$$\Delta I^n_X = (I^n_{max} - I^n_{min})\ \lambda_1, \lambda_2 \quad (5)$$

where I is the relative fluorescence intensity, and I^n_{max} and I^n_{min} the maximum and minimum value of the nth order derivative of I ($I^n = d^n I/d\lambda^n$), within a relatively narrow spectral range bounded by the wavelengths λ_1 and λ_2, as shown in Fig. 1.

The F-MOD program can be used to evaluate automatically the analytical signal as defined by eqn. (5), provided that the operator has previously defined the spectral range (λ_1,λ_2) in which the minimum and maximum should occur. The nth order derivative of a simple spectral band (*e.g.*, a Gaussian peak) consists of n closely packed pairs of ΔI^n peaks of opposite sign, therefore the spectral range (λ_1,λ_2) must be sufficiently narrow and carefully defined in order to include only a single pair of peaks. In the present case (n = 2) the actual pair used is the most distant from the interfering

spectral band. This pair of peaks were found to produce results that were more reproducible and less dependent on the concentration of the interfering species.

As the spectrum and its derivatives are stored in the memory of the microcomputer as a sequence of discrete equidistant points, and because the peaks of the spectrum derivatives can occasionally be very sharp, it is possible that the local extreme points will be slightly different from the real extremes of the derivative. Assuming that the end of each peak is approximately parabolic, the real extremes of the derivative used in eqn. (5) were approximated by the extremes of the quadratic equations defined by the points $I^n_{X,m-1}$, $I^n_{X,m}$ and $I^n_{X,m+1}$, as depicted in Fig. 1. This treatment leads to signals that are significantly more reproducible than those obtained using simply the local extreme points.

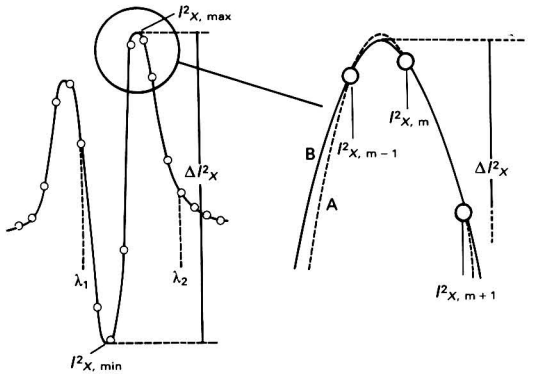

Fig. 1 Evaluation of the signal in SDSFS. Detail: fit of the three extreme points in a quadratic equation for a more precise evaluation of the analytical signal. A, actual derivative and B, quadratic equation

Comparison of Spectra

The fluorescence properties of ASA and SA in 1% acetic acid–chloroform solutions and the optimum experimental conditions for their measurement (concentration of acetic acid, quality of the organic solvent) have been reported previously.[7]

Despite the satisfactory resolution of the excitation and emission maxima of ASA (λ_{ex} = 284 nm, λ_{em} = 344 nm) and SA (λ_{ex} = 314 nm, λ_{em} = 444 nm), spectral overlaps may occur in their binary mixtures, when one of the components (especially SA) is present in a large excess. Conventional emission and synchronous spectra and their corresponding second derivatives obtained for different SA–ASA mixtures in the mixed solvent are shown in Fig. 2. Using a fixed excitation wavelength at 314 nm for measuring SA [Fig. 2(*a*), line A] and 284 nm for measuring ASA [Fig. 2(*b*), line A], the recorded fluorescence spectra consist of overlapping spectral bands and the specimen of interest exists as a small shoulder. Clearly, it is almost impossible to perform simultaneous determinations of

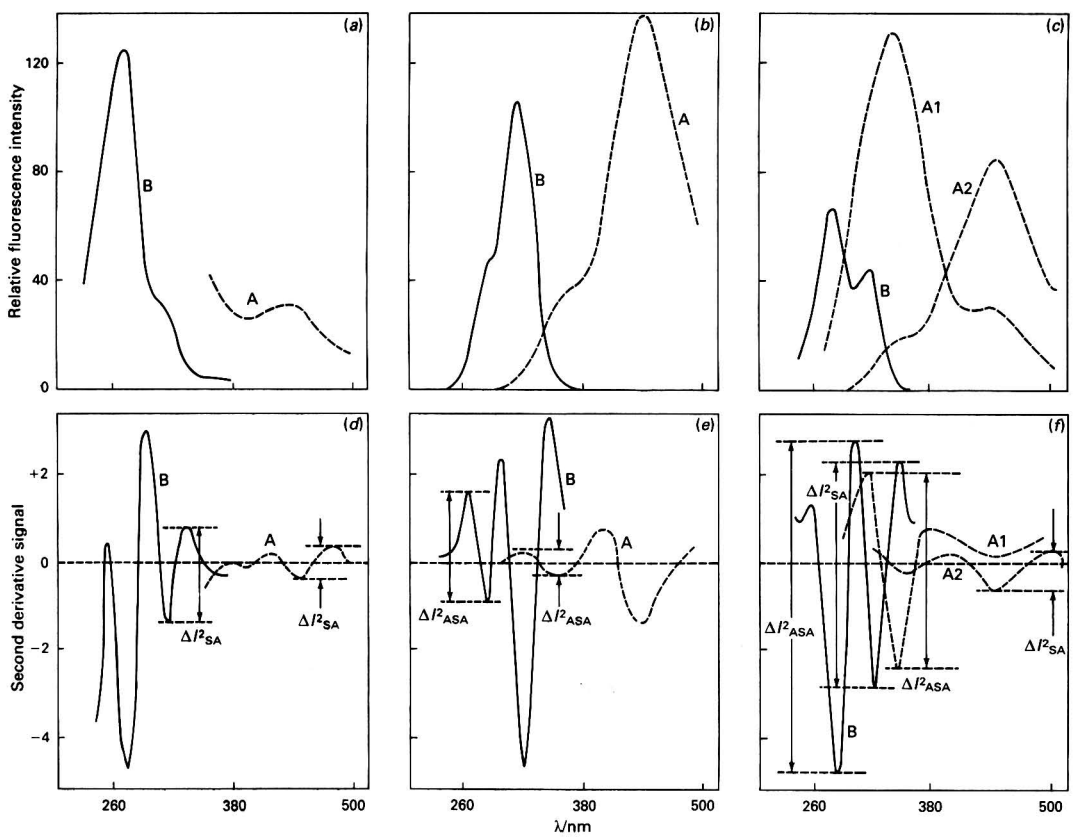

Fig. 2 A, Emission and B, synchronous spectra of (*a*), (*b*) and (*c*) ASA–SA mixture and (*d*), (*e*) and (*f*) their second-derivative spectra in 1% acetic acid–chloroform solution. (*a*) c_{ASA} = 200 μg ml^{-1}, c_{SA} = 0.30 μg ml^{-1}, (λ_{ex} = 314 nm, $\Delta\lambda$ = 130 nm, sensitivity = 10); (*b*) c_{ASA} = 2.0 μg ml^{-1}, c_{SA} = 16.0 μg ml^{-1}, (λ_{ex} = 284 nm, $\Delta\lambda$ = 60 nm, sensitivity = 3); (*c*) c_{ASA} = 50 μg ml^{-1}, c_{SA} = 6.0 μg ml^{-1}, ($\lambda_{ex,A1}$ = 284 nm, $\lambda_{ex,A2}$ = 314 nm, $\Delta\lambda$ = 90 nm, sensitivity = 1)

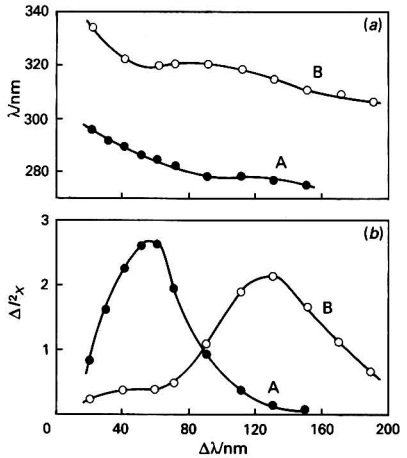

Fig. 3 Effects of Δλ on the second-derivative synchronous spectra of A, ASA and B, SA. (*a*) Effect of the acids on the wavelength corresponding to the minimum of second-derivative peaks, and (*b*) effect on the analytical signals

ASA and SA in mixtures of these compounds simply using their emission spectra. However, if a constant difference is maintained between the excitation and emission wavelengths (Δλ = 130 nm for SA and Δλ = 60 nm for ASA), and the second derivatives of the synchronous spectra are employed [Fig. 2(*d*) and (*e*), line B], well separated and measurable analytical signals are obtained, resulting in a significant improvement in both the selectivity and sensitivity.

The emission spectra of a mixture of SA–ASA at a mass ratio of 6:50 are shown in Fig. 2(*c*), (lines A1 and A2), each one obtained using the optimum excitation wavelength and the synchronous spectrum (line B). The corresponding second derivatives are shown in Fig. 2(*f*). It can be seen that, at this ratio, signals for both SA and ASA may be obtained by CFS. However, this determination requires two measurements, each one obtained using the optimum excitation wavelength for each component [Fig. 2(*f*), lines A1 and A2]. On the other hand, SDSFS allows the simultaneous measurement of both acids in a single scan with Δλ = 90 nm [Fig. 2(*f*), line B]. The observed decrease in sensitivity for both acids, when synchronous fluorescence spectrometry is employed (with Δλ not being optimum for both compounds) is compensated for by the increase of the signals, when the second derivative of the synchronous spectrum is employed. On the other hand, the second derivative of the conventional fluorescence spectra in all instances [Fig. 2(*d*) (line A), (*e*) (line A) and (*f*) (lines A1 and A2)] results in much poorer signals.

Selection of Δλ

The most important parameter in the simultaneous analysis of mixtures by synchronous fluorescence spectrometry is the selection of the optimum wavelength difference between excitation and emission monochromators (Δλ). For this reason a wide range of Δλ values (20–200 nm) was examined. The position of the minimum of the second-derivative spectrum and the signal for ASA and SA as functions of Δλ are shown in Fig. 3(*a*) and (*b*), respectively.

The best spectral resolution of the ASA–SA mixture is obtained at Δλ = 90 nm. However, at this Δλ each signal is about half that of the maximum for both components. The maximum signal for each component is obtained when Δλ is equal to the difference between the two peaks, λ_{em} and λ_{ex}, of their conventional emission and excitation spectra. Consequently, there are three choices in selecting the optimum Δλ. For measuring small amounts of ASA or SA in the presence of an excess of the other (mass ratio ASA : SA < 0.5 or ASA : SA > 100) the optimum Δλ is 60 nm for measuring ASA and 130 nm for measuring SA. When the ratio is 0.5 < ASA : SA < 100 a Δλ of 90 nm is optimum for their simultaneous determination.

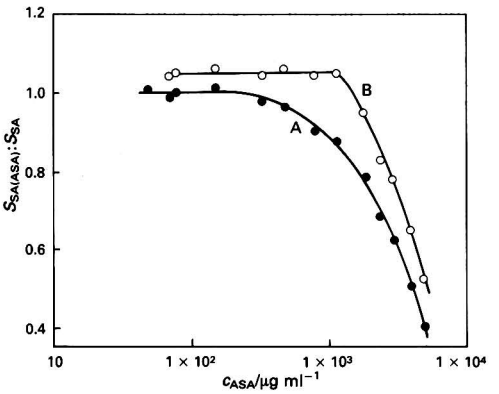

Fig. 4 Effect of the concentration of acetylsalicylic acid on the slope of the working curve of salicylic acid obtained by A, CFS and B, SDSFS

Other Instrumental Parameters

Selection of the other instrumental parameters is not critical because the differentiation is attained numerically and not electronically. A scan speed of 4 nm s^{-1} and a 'fast' response time were selected. The sampling rate was defined to be 1 point every 2 nm. For the calculation of the second-derivative spectra by the Savitzky–Golay method, a width of $2n + 1 = 9$ points (polynomial degree: 4, the corrected convoluting integers given in reference 15) gave a satisfactory signal to noise ratio.

General Analytical Characteristics

The linear concentration ranges were 0.2–70 and 0.03–10 μg ml^{-1} for ASA and SA, respectively. Pearson's correlation coefficients[19] (*r*) for the standard calibration graphs were 0.9995 (ASA) and 0.99992 (SA). The detection limits attained by SDSFS, defined as three times the standard deviation of the lowest concentration, were 0.040 and 0.010 μg ml^{-1} for ASA and SA, respectively. The detection limits attained by CFS, with the same instrument, were 0.24 and 0.010 μg ml^{-1} for ASA and SA, respectively.

To test the precision, three series of samples covering the range of interest for ASA (0.20, 9.00 and 50.0 μg ml^{-1}) and for SA (0.100, 1.50 and 10.0 μg ml^{-1}) were analysed, and the corresponding RSD (%) values ($n = 9$) were 7.2, 1.4 and 0.8 for ASA, and 4.5, 2.0 and 1.4 for SA, respectively.

Determination of ASA and SA in Binary Mixtures

In order to apply the SDSFS technique to the simultaneous determination of ASA and SA in binary mixtures, a detailed study on the influence of the excess of each acid on the signal of the other was performed.

The effect of the concentration of ASA on the slope of the calibration graph for SA, obtained by CFS and SDSFS (Δλ = 130 nm) is shown in Fig. 4.

The slope of the calibration graph for SA, in the presence of ASA, $S_{SA(ASA)}$, obtained by CFS, decreased considerably with increasing ASA concentration. The slope obtained using the SDSFS technique was increased initially by a mean value of 5.0%. This increase is attributed to the elevation of the small shoulder peak of SA by the large ASA peak. Within the

Table 1 Recovery data for ASA and SA in synthetic serum mixtures

Concentration added/ μg ml^{-1}		Concentration found/ μg ml^{-1}		Recovery (%)	
ASA	SA	ASA* ± SD	SA* ± SD	ASA	SA
50.0	160	52.3 ± 0.3	154 ± 2	104.6	96.2
40.0	160	39.8 ± 1.9	154 ± 4	99.5	96.2
15.0	120	16.0 ± 0.6	115 ± 3	106.7	95.8
5.0	100	5.1† ± 0.2	93 ± 1	102.0	93.0
2.5	100	2.5† ± 0.1	98 ± 1	100.0	98.0
			Mean	102.6	95.8

* Average of three measurements.
† Measurable after a known addition of ASA.

Table 2 Recovery of SA in aspirin formulations by CFS and SDSFS

Concentration of SA in aspirin/mg g^{-1}						
Added	Measured		Found*		Recovery (%)	
	CFS	SDSFS	CFS	SDSFS	CFS	SDSFS
0	0.24	0.02	—	—	—	—
0.25	0.51	0.27	0.27	0.25	108.0	100.0
0.50	0.79	0.51	0.55	0.49	110.0	98.0
1.00	1.35	1.04	1.11	1.02	111.0	102.0
1.50	1.84	1.54	1.60	1.52	106.7	101.3
				Mean	108.9	100.3

* After subtraction of blank.

Table 3 Comparison of USP,[16] Trinder,[18] and SDSFS methods for the determination of ASA and SA in aspirin tablets

	Aspirin sample					
	A	B	C	D	E	F
ASA (%) (USP method)	35.4	35.4	82.4	82.7	83.3	83.5
ASA (%) (SDSFS method)	38.4	39.3	84.4	82.8	81.0	82.3
SA (%) (Trinder method)	0.04	ND*	0.03	0.02	0.02	ND*
SA (%) (SDSFS method)	0.014	0.010	0.013	0.010	0.008	0.006

* ND = Not determined.

ASA concentration range 60–1200 μg ml^{-1}, $S_{SA(ASA)}$ remains constant and then decreases gradually with further increases in c_{ASA}. The decrease in the slope observed with both methods may be attributed to the quenching effect caused by the presence of large amounts of ASA. On the other hand, the overlapping of emission spectra which occurs for both compounds at high concentrations of ASA, inevitably leads to positive errors in the determination of SA by CFS. These errors are negligible when the SDSFS technique is used. Therefore, it is possible to measure SA in the presence of up to 1200 μg ml^{-1} of ASA, using the standard calibration graph for SA obtained by SDSFS, provided that a correction factor F equal to 1.05 is taken into consideration, eqn. (4).

The detection limit for SA in the presence of a large excess of ASA (230 μg ml^{-1}) by the SDSFS technique was 0.034 μg ml^{-1}.

The effect of the concentration of SA on the slope of the calibration graph for ASA, obtained by the SDSFS technique ($\Delta\lambda$ = 60 nm), is more complicated. Inner filtering occurs at a relatively small excess of SA, because of the strong overlap between the excitation spectrum of SA and the emission spectrum of ASA. It was found that the slope of the calibration graph for ASA in the presence of SA, $S_{ASA(SA)}$, decreased linearly with c_{SA}, up to 35 μg ml^{-1}, according to the equation

$$\frac{S_{ASA(SA)}}{S_{ASA}} = -0.017\,c_{SA} + 1.008 \quad (r = 0.990) \qquad (6)$$

The increase in the concentration of SA in an ASA–SA mixture leads to an apparent 'loss' of ASA because of the inner-filter effect. It was found that the hidden concentration of ASA, $c_{ASA,h}$ is linearly related to c_{SA}, according to the equation

$$c_{ASA,h} = 0.157\,c_{SA} + 0.01 \quad (r = 0.998) \qquad (7)$$

As can be seen from eqn. (7) the minimum detectable c_{ASA} in binary ASA–SA mixtures is about one sixth of that of c_{SA}. For ASA : SA < 0.15 the second-derivative signal of ASA is completely hidden, but c_{ASA} can still be determined after a known addition of ASA, corresponding to one sixth or more of the c_{SA} previously determined.

The concentration of ASA at a given c_{SA} can be calculated from eqn. (1). This equation takes into account the proportionality between ΔI^2_{ASA} and c_{ASA}, the corrections on the slope and the hidden concentration of ASA described by eqns. (6) and (7), respectively.

Measurements of ASA in synthetic ASA–SA mixtures, (c_{ASA} = 4–19 μg ml^{-1}, c_{SA} = 2–32 μg ml^{-1}) gave an average recovery of 98.4% (range: 86.1–111.9%).

Synthetic mixtures of ASA and SA in serum from normal adults and aspirin formulations were analysed for both ASA and SA by the described procedure.

Serum Samples

Serum samples or solutions containing albumin were extracted into the mixed solvent prior to measurement. The signals obtained for ASA and SA in serum were smaller than those obtained with pure aqueous standards, due to the binding of salicylates to serum proteins.[17] A detailed study of the effect

of the concentration of albumin on the slopes of ASA and SA working curves was carried out. It was found that S_{ASA} decreased by 5% in 45 g l^{-1} albumin solutions, and S_{SA} decreased linearly with the concentration of albumin, c_{alb} (g l^{-1}), up to 50 g l^{-1}, according to the equation

$$\frac{S_{SA(alb)}}{S_{SA}} = -0.104\,c_{alb} + 0.998 \quad (r = 0.992) \qquad (8)$$

where, $S_{SA(alb)}$ is the slope of the calibration graph for SA in the presence of albumin.

To overcome the small error due to the binding of ASA, a correction factor of 1.05 is simply introduced into eqn. (2). Owing to much stronger binding with proteins, SA in serum can be quantified by using: (*i*) the standard additions technique, (*ii*) a working curve obtained with SA solutions containing a similar albumin concentration or (*iii*) the standard calibration graph and applying the correction as dictated by eqn. (8). In this study the second approach was used.

Recovery data for ASA–SA synthetic mixtures, added to serum, are summarized in Table 1. The selected concentrations are typical for ASA–SA levels in rabbit plasma[3] during the first 60 min, after intravenous administration of 50 mg kg^{-1} of aspirin.

The absence of detectable blanks in salicylate-free serum indicates that the constituents of normal serum do not interfere with the fluorescence measurement.

Aspirin Formulations

The conventional method used to determine trace amounts of SA in aspirin formulations[7] by CFS does not account for errors caused by the presence of an excess of ASA as shown above. On the other hand the quantification of trace amounts of SA by SDSFS is not affected by the presence of up to 1200 μg ml^{-1} of ASA. Recovery results obtained by adding different amounts of SA to aspirin solutions measured by both techniques are shown in Table 2.

The determination of ASA in aspirin formulations by CFS and SDSFS techniques gave identical results. Different batches of aspirin tablets were analysed for ASA and SA by SDSFS. The results for both ASA and SA were compared with those obtained by the USP volumetric method[16] (for ASA) and the Trinder method[18] (for SA). Percentages of ASA obtained by the USP volumetric method for samples A and B (Table 3, aspirin for infants) are lower than those obtained by SDSFS. This difference may be attributed to the much larger content of these samples in natural and artificial sweeteners, colouring and flavouring compounds and other excipients. Percentages of SA obtained by the Trinder method[18] are considerably higher than those obtained by SDSFS. This is attributed to the partial hydrolysis of ASA and SA under the conditions of the Trinder reaction (Table 3).

The determination of ASA and SA is not affected by concomitant substances in aspirin formulations, such as cellulose, lactose, carbowax, magnesium stearate or saccharin.

Conclusions

The proposed method for the determination of ASA and SA in serum and aspirin formulations illustrates the advantages of the combined synchronous scanning and second-derivative fluorescence spectrometry techniques. Ratios of ASA:SA that cannot be measured with conventional fluorescence spectrometry can be readily quantified by the combined technique using the appropriate empirical equations, which contain non-instrument specific factors. Generally, binary mixtures of closely related compounds with overlapping emission–emission and emission–excitation spectra, may be considered as potent analytes, with this technique. Separation steps are not required, provided that a thorough investigation of the effect of each compound on the signal of the other has been previously carried out.

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Solvent Extraction of Trace Amounts of Fluoride Prior to, Mainly, Spectrometry—A Review: Recent Advances in the Alizarin Fluorine Blue Approach

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Methods for the solvent extraction of very small amounts of fluoride, usually prior to gas chromatography or absorptiometry, have been reviewed. Additionally the Alizarin Fluorine Blue–lanthanum procedure has been further developed in order to allow the determination of about 1 μg of fluoride in 500 cm³ of water with good precision. Although the method is time-consuming, it yields a detection limit for fluoride of 40 ng l^{-1} and a relative standard deviation (at 4 μg of fluoride) of 0.60% with the use of only very basic apparatus. The sensitivity (0–10 μg of fluoride in 500 cm³ of water) is 0.176 A μg^{-1} (using cells with a pathlength of 4 cm).

Keywords: *Alizarin Fluorine Blue; solvent extraction of fluoride; absorptiometry; trace analysis; review*

Introduction

For many years efforts have been made to improve the determination of trace amounts of fluoride by solvent extraction, utilizing the advantages of concentration, freedom from interferences and presentation, in a form suitable for instrumental analyses such as gas chromatography or mass spectrometry.

Use of Aryl Antimony(v) Salts

The first recorded extraction of fluoride was by Moffett *et al.*[1] in 1956. They showed that although the salt $(Ph_4Sb)_2SO_4$ was very soluble in water the halides were not and they extracted Ph_4SbF from 20 cm³ of weakly acidic water into carbon tetrachloride. The solvent was evaporated to determine the Ph_4SbF using gravimetry. This idea lay dormant until 1970 when Carmichael and Whitley[2] used it to extract about 100 μg of fluoride from 12 cm³ of water at a pH of approximately 3, using 1.6×10^{-3} mol dm^{-3} $(Ph_4Sb)_2SO_4$ and chloroform as the solvent, in a sub-stoichiometric ^{18}F isotope dilution experiment. In 1971, Aulagnier *et al.*[3] investigated the solvent-extraction chemistry of Ph_4Sb^+ in depth; they extracted 1×10^{-7}–1×10^{-5} mol dm^{-3} of fluoride into carbon tetrachloride using 1×10^{-4}–1×10^{-3} mol dm^{-3} Ph_4Sb^+ and found the formation constant of Ph_4SbF to be 3.6×10^3. In the same year Jean[4] allied the method with absorptiometry by extracting Ph_4SbF into hexane–chloroform (40 + 25) and measuring the absorbance at 264 nm. The molar absorptivity was 3.2×10^3 l mol^{-1} cm^{-1} and the calibration graph was linear for up to 50 μg of fluoride.

An alternative approach, developed by Chermette *et al.*,[5] was to extract fluoride with triphenylantimony(v) dichloride (Ph_3SbCl_2) at pH 2–6 into carbon tetrachloride or benzene. The detection limit was 1×10^{-9} mol dm^{-3} of fluoride and easy separation from a large excess of aluminium was achieved by masking with cyclohexane-1,2-diaminetetraacetic acid (CDTA). These workers describe their investigations clearly in a paper published in 1972.[6] The fundamental reaction is

$$Ph_3SbCl_2 + F^- + OH^- \rightleftharpoons Ph_3SbF(OH) + 2Cl^- \quad (1)$$

The formation constant of $Ph_3SbF(OH)$ is $10^{14.5}$. An alternative but much slower reaction of interest involves concurrent oxidation of triphenylantimony(III):

$$Ph_3Sb + H_2O_2 + F^- \rightleftharpoons Ph_3SbF(OH) + OH^-$$

Chermette *et al.*[6] regarded reaction (1) as being more complete and selective than reactions involving Ph_4Sb^+. The partition constant of $Ph_3SbF(OH)$ between water and benzene is 7000. Extraction from 20 cm³ of water into 20 cm³ of benzene is virtually complete over the pH range 3–6; at a lower pH, HF formation reduces extractability while at a higher pH, the formation of $Ph_3Sb(OH)_2$ competes for extraction. Reducing agents interfere by causing the reaction $Sb^V \rightarrow Sb^{III}$ to occur and some anions such as acetate and citrate co-extract with $Ph_3SbF(OH)$. However, phosphate and sulphate do not interfere, which is helpful in the analysis of water. Fluoride-complexing cations such as Al^{3+}, Zr^{IV} and Fe^{3+} are readily masked with CDTA. The method described above for the isolation and concentration of fluoride has been allied with the use of the fluoride electrode to give a powerful method.[7] In 1974, Chermette *et al.*[8] back-extracted fluoride from $Ph_3SbF(OH)$ in carbon tetrachloride into 0.1 mol dm^{-3} sodium hydroxide solution and completed the analysis using zirconium–Eriochrome Cyanine R bleach chemistry and measurement by absorptiometry. Measurement of fluoride concentrations of about 1×10^{-8} mol dm^{-3} was possible.

The most recent work (1985) along these lines was carried out by Dittrich *et al.*[9] They prepared $Ph_3Sb(OH)_2$ by liquid ion exchange between Ph_3SbBr_2 and sodium hydroxide and then used it to extract fluoride from 6 cm³ of 1×10^{-7}–1×10^{-4} mol dm^{-3} solution into *m*-xylene, isobutyl methyl ketone or pentyl acetate. The fluoride was stripped into a barium hydroxide solution containing aluminium and then subjected to graphite furnace spectroscopy using the molecular absorption of AlF. The detection limit was 0.3 ng. The use of $Ph_3Sb(OH)_2$ is only necessary when halide ions need to be absent from the stripping solution.

Use of Organosilanes

Fluoride reacts with silanols and chlorosilanes to give products that can be extracted into organic solvents. Bock and Semmler[10] carried out a comprehensive investigation of reactions of this type. High acidity (pH ≈ 0), high reagent concentrations (≈0.25 mol dm^{-3}) and long shaking times (10–60 min) are desirable. Typical reagents are $(CH_3)_3SiCl$, $(C_2H_5)_3SiOH$, $(CH_3)_2(C_6H_5)SiOH$ and $(C_6H_5)_2Si(OH)_2$; the solvents are diisopropyl ether, tetrachloroethylene, toluene and *m*-xylene. Fluoride may be back-extracted into aqueous alkali prior to absorptiometric or potentiometric[11] measurement but more usually the covalent fluorosilane, in particular $(CH_3)_3SiF$ and $(C_2H_5)_3SiF$, is determined by gas chromatography[10,12–20] or mass spectrometry.[10] As the extracted fluoro compound is not itself used as an optically absorbing entity, such reactions will not be considered further here.

The extraction of triphenyltin fluoride (Ph_3SnF) into chloroform has been used as the basis of a sub-stoichiometric radiochemical analysis.[21]

Use of Alizarin Fluorine Blue (AFB)–Lanthanoid Complexes

At a pH of 4.0–4.6, Alizarin Fluorine Blue (1,2-dihydroxyanthraquinon-3-ylmethylamine-*N,N*-diacetic acid), often known as Alizarin Complexan, forms a yellow solution, λ_{max} 423 nm. This strongly chelating agent forms a red complex $(AFB)_2La_2$, λ_{max} 500 nm, with lanthanum(III) or cerium(III) ions, and in the presence of fluoride a blue ternary complex $(AFB)_2La_2F$, λ_{max} 567 nm, is produced. Only in the presence of a large excess of fluoride does the common 'bleach' reaction $M{\cdot}Dye + nF^- \leftrightharpoons MF_n + Dye^{n-}$ take place. The complex $(AFB)_2La_2F$ forms the basis of a useful, sensitive and very selective one-phase absorptiometric method for fluoride, especially when dissolved in about 20% of acetone or other solvents.[22] In 1959, Freiser suggested that it might be possible to extract the ternary complex from the binary $(AFB)_2La_2$ complex selectively. This would be useful as, in the one-phase method, $(AFB)_2La_2$ produces a large blank absorbance. A brief initial investigation by Leonard and West[23] showed that indeed a solution of various amines in medium-sized alcohols (C_4–C_6) would selectively extract $(AFB)_2La_2F$ from a mildly acidic solution by ion-pair formation, and they tentatively suggested a dilute solution of tributylamine in pentanol although underestimating the size of the blank. In 1961, Johnson and Leonard[24] concluded that a 0.06% solution of tribenzylamine in pentanol–*sec*-butyl alcohol (30 + 70) was a good selective extractant. They determined 0.25–7 μg of fluoride in 150 cm^3 of water (0.002–0.05 ppm of fluoride) with a sensitivity of 0.13 A μg^{-1} of fluoride (4 cm cells, λ = 580 nm). The blank value was A = 0.4 *versus* ethanol. Phosphate, aluminium and iron(III) were the only common fairly serious interferents.

Solvent extraction methods for fluoride would appear to fall into two groups; those concerned with low fluoride masses in small volumes and those which deal with low fluoride concentrations in large volumes. The latter methods are very useful when used with large volumes of solutions from Willard–Winter H_2SiF_6 distillations or pyrohydrolytic HF separations. In 1963 Hall[25] published a method of the former type; he extracted 0.1–1 μg of fluoride from 2 ml of an aqueous solution into 0.03 mol dm^{-3} hydroxylamine hydrochloride in isobutyl alcohol. The extract was washed with cold water in order to reduce the blank absorbance to a very low value; even without washing, it was only 4% of the absorbance given by 1 μg of fluoride. Hall used a succinate buffer which was found to be preferable to acetate and added *tert*-butyl alcohol to the extraction system in order to reduce precipitation of the complex at the interface. The method was applied to fluoride obtained by an elegant small-scale HF diffusion from ashed vegetable matter.[26] Davies and Foreman[27] determined 0–10 μg of fluoride in plutonium material pyrohydrolysate (50 cm^3) by extracting $(AFB)_2La_2F$ into 10% *N*-propylaniline in butanol. At a wavelength of 650 nm the blank absorbance was low. Numerous other aromatic amines were examined, which, being less basic than aliphatic amines, caused a smaller increase in the pH of the aqueous phase. In 1968 Cox and Backer Dirks[28] revised Hall's small-scale method to determine fluoride in blood serum. They, like Davies and Foreman, favoured an *N*-aryl extractant (3% v/v aniline in isobutyl alcohol). Beer's law was obeyed at 570 nm over 0.1–1.2 μg of fluoride; precision was 2.3% relative standard deviation (RSD). Back-washing with water was not helpful. These last three papers criticized the high blank levels obtained using Leonard and West's tributylamine suggestion, however, this had never been proposed as a serious method. Aliphatic amines returned to favour with the work of Haarsma and Agterdenbos.[29] They operated on both scales extracting 0.1–1 μg of fluoride from 4 cm^3 of water and 5–25 μg of fluoride from 90 cm^3 of water as $(AFB)_2Ce_2F$ into 5% triethylamine in pentanol. The calibration graph was linear and the blank only 0.024 A although sensitivity appeared to be much the same as with the one-phase method. They also investigated the aqueous phase pH-change during extraction and proposed the addition of a pH 9 acetate buffer prior to extraction. Their small-scale method was simpler than that of Hall[25] or Cox and Backer Dirks[28] and twice as sensitive. Centrifuging sharpened the separations. In 1976 Marczenko and Lenarczyk[30] extracted $(AFB)_2La_2F$ from an alkaline solution (pH 9.8) with 3% dioctylamine in isobutyl alcohol. This was very selective towards the ternary complex; an alternative was to extract both complexes from mildly acidic solution and then strip out the binary complex with dilute ammonia solution or carbonate–hydrogen carbonate buffer.

Undoubtedly the most popular extractant for $(AFB)_2La_2F$ has been *N,N*-diethylaniline. This was proposed by Hirano *et al.* in 1966.[31] They extracted 0.5–10 μg of fluoride from 150 cm^3 of 6% acetone, pH 4.4 aqueous solution as $(AFB)_2La_2F$ with 5% *N,N*-diethylaniline in isopentyl alcohol. Absorbance measurements at 575 nm yielded an RSD for 5 μg of fluoride of 1.47% (12 determinations). The molar absorptivity of the complex was 2.35×10^4 l mol^{-1} cm^{-1} and the blank showed an absorbance of only 0.06 (1 cm cell). Hirano and co-workers later (1969)[32] applied this method to 50 cm^3 of pyrohydrolysate from tantalum, although, prior to measuring the absorbance of the organic layer, the extract was washed with acetate buffer. Likewise, in 1974,[33] 5–50 μg of fluoride were determined in the presence of up to 250 μg of aluminium by coprecipitation of the aluminium with iron(III) at a pH of 10 and then as described in reference 32. In 1981 Zhao and Liang[34,35] optimized the method by multiple factor orthogonal testing and applied it to 0.05–5 μg of fluoride obtained by acid distillation from pure beryllium; any traces of beryllium carried over were masked with acetylacetone. Recovery was 99.1%, RSD 5%. In 1986 Fuwa and co-workers[36,37] linked the method to an internal reflection capillary cell with a long pathlength. The extract was mixed with carbon disulphide and acetone (80 + 20) to give a refractive index of 1.57 (cell wall 1.474) and the absorbance was measured in a 4 m cell (i.d. 1 mm) at 620 nm. The recovery of 50 μg l^{-1} of fluoride from drinking water was 93–104% with a detection limit of 10 ng l^{-1}. The advantage of the long cell appears to have been subdued by taking only a small fraction (one hundredth) of the extract but the method could undoubtedly reach very low levels and is being actively pursued. Finally, concerning *N,N*-diethylaniline, Miyazaki and Bansho,[38] extracted up to 1.2 μg cm^{-3} of fluoride from 50 cm^3 of water into a 4% solution of the amine in hexanol. The extract was washed with a dilute acetate buffer (pH 4.4) then examined directly by inductively coupled plasma optical emission spectrometry using the lanthanum 333.75 nm line well away from the cyanogen band interference. The detection limit was 0.59 ng cm^{-3}; RSD 2.6%. Significantly, Miyazaki and Bansho[38] investigated several other amine–solvent pairs but returned to the original process of Hirano.[31] They reported no interference from sulphate or phosphate and gave a procedure for removing common heavy metals by ammonium pyrrolidin-1-yldithioformate (ammonium pyrrolidinedithiocarbamate) extraction at pH 4.4 into diisobutyl ketone.

Finally in this section, where fluoride is extracted as an ion pair with AFB, we reach the use of diphenylguanidine in various solvents. In 1976 Levkov and Novak[39] extracted 0.1 μg of fluoride from 5 cm^3 of water containing a little acetone at pH 4.5 as $(AFB)_2La_2F$ into chloroform–isobutyl alcohol (1 + 3) in the presence of 1.6×10^{-4} mol dm^{-3} diphenylguanidine hydrochloride. Good separation from the binary complex blank was achieved, extraction equilibrium was reached in 5 min and the absorbance of the extract remained steady for 5 h. Separation was aided by centrifugation; the RSD was 2.6% and a 500-fold excess of sulphate did not interfere. Rudenko *et al.*[40] patented a flotation method using toluene, benzene or carbon tetrachloride with the addition of ethanol or dimethylformamide to the separated extract in order to dissolve the precipitated ion pair. The diphenylguanidine

reagent (1%) was made up in acetic acid–acetone (5.5 + 94.5). The flotation technique improved the selectivity and large amounts of heavy metals were tolerated. The method was applied to industrial waste waters and aerosols.[41] Teng *et al.*[42] extracted up to 200 μg of fluoride from a solution with a pH of 5.2 using $(AFB)_2Ce_2$ and a 5% diphenylguanidine reagent. The solvent was methanol–carbon tetrachloride (1 + 1) and the absorbance was measured at 626 nm. The method was applied to corroded metal foils with the separation of the fluoride from iron and aluminium by alkaline and 8-hydroxyquinoline precipitation.

One curious example has arisen in which the $(AFB)_2La_2F$ complex is extracted without the intervention of a cationic pairer by using a salting-out process. Iyer *et al.*[43] extracted 2–12 μg of fluoride from 50 cm^3 of 20% methanol, 4% in sodium chloride and buffered with pH 4.6 succinate, into 10 cm^3 of benzyl alcohol.[43] The method is not as sensitive as some amine–alcohol methods but the blank absorbance at 615 nm is reasonable; 4 μg of fluoride gives 0.225 A with a blank of 0.125 A.

Other Systems

An interesting solvent extraction method not involving AFB directly was developed by Shkrobot and Tolmacheva.[44] Following isolation from interfering metals by alkaline precipitation, fluoride (0.1–12 ppm) was extracted from 1 mol dm^{-3} nitric acid containing 40% isopropyl alcohol (IPA) and 1% disodium tetraborate into diethyl ether as a BF_3·IPA complex. The extract was washed, then treated with sodium hydroxide to give a pH of 7–8 and heated to remove the solvents. The fluoride was then determined by conventional one-phase $[(AFB)_2La_2]$ absorptiometry at 630 nm.

Fluoride has been extracted with a few metal–dye systems not involving AFB. In 1962 Kakita and Goto[45] proposed a method for the extraction of trace amounts of tantalum involving triphenylmethane dyes, in particular Malachite Green (MG), and hydrofluoric acid. This was adapted by Dahl[46] as a fluoride method. A 5 cm^3 aliquot of a solution containing 50–200 μg of fluoride was treated with tantalum sulphate, tartrate and MG oxalate (pH 1.7–2.0), and $MG^+ \cdot TaF_6^-$ was extracted into 25 cm^3 of benzene. The absorbance was measured at 630 nm in a 5 cm cuvette; detection limit 5 μg cm^{-3}, RSD 2.4%. As with AFB methods, careful selection of solvents is necessary in order to minimize the blank value.

Aluminium in the presence of Xylenol Orange (XO), fluoride and trioctylamine (TOA) forms a 1 : 2 : 2 : 3 complex. Pyatnitskii and Pinaeva[47] used this reaction to extract 0.01–0.1 mmol dm^{-3} fluoride with 0.1 mol dm^{-3} TOA at pH 3.9 into chloroform with 95–98% recovery; λ_{max} = 550 nm, ε = 41 000 l mol^{-1} cm^{-1}. Aluminium can be determined using the same reaction.

Finally, two fluoride extractions not involving dyes are mentioned. Tantalum-182, a γ-emitter of $t_{\frac{1}{2}}$ = 115 d, is extracted from 6.5 mol dm^{-3} sulphuric acid as a fluoride complex into diisobutyl ketone.[48] The count rate of the extract is approximately proportional to the fluoride mass (up to 840 μg); detection limit, 40 μg of fluoride. The proposed reaction is

$$H_2TaF_7(aq) + 2R_2C{=}O \rightarrow [(R_2C{=}OH)_2{}^+TaF_7{}^{2-}](org)$$

Kletenik and Bykhovskaya[49] established that, in the presence of zirconium, thorium, titanium(IV) and tin(IV), fluoride can be extracted into a solution of bis(2-ethylhexyl) phosphate in such solvents as hexane, carbon tetrachloride, benzene and tributyl phosphate. The preferred extractant for use with mineral acid media (0.2 mol dm^{-3}) is a solution of zirconium bis(2-ethylhexyl) phosphate in hexane; Zr : F ratio 1 : 1, fluoride distribution ratio ≈ 20. Fluoride may be re-extracted into alkaline tributyl phosphate and acetone for conventional absorptiometry.

Examination of the Alizarin Fluorine Blue–Lanthanum–Fluoride Cation–Isobutyl Alcohol System

In early work[23] it was quickly recognized that $(AFB)_2La_2F$ and in some instances the reagent complex $(AFB)_2La_2$ can be extracted only into a limited range of higher alcohols which must, in addition, contain a proportion of a hydrophobic amine. The presence of the amine is essential for the successful extraction of the ternary complex and this would seem to indicate that an ion-association system is involved, *e.g.*, $[(AFB)_2La_2F]^- \cdot R_4N^+$. The most effective alcohols are of various isomeric forms from butanol to hexanol including cyclohexanol.[24] As is apparent from the review, numerous amines have been examined for use in conjunction with these alcohols. In some instances the amine proves too efficient and causes the extraction of the $(AFB)_2La_2$ reagent complex in addition to $(AFB)_2La_2F$, while in others the action of the amine is too weak to promote the full extraction of either complex. The whole matter is complicated by the fact that the extraction efficiency is highly dependent on the concentrations of the amine and buffer (see Fig. 6).

Johnson and Leonard[24] found that a very satisfactory combination of amine and alcohol, in terms of sensitivity, preferential extraction of the fluoride complex and clean separation, was a solution of tribenzylamine in a mixture of pentanol and *sec*-butyl alcohol. Further details of this approach together with all other related investigations are given in the earlier part of the present paper.

The fundamental aim of this work was to push the method to its lowest concentration limit for a high sample volume. We began by investigating the ability of a wide range of cations to extract an equimolar amount of fluoride, relative to AFB and lanthanum, from 500 cm^3 of water into isobutyl alcohol. Of the 44 cations briefly examined, 20, including all those previously suggested in the literature, were chosen for further study.

Experimental

Instrumentation

A Perkin-Elmer Lambda Nine ultraviolet/visible/near infrared spectrophotometer was used to produce the absorption spectra. Single wavelength absorbance measurements were carried out on a Pye Unicam SP6-550 spectrophotometer. The pH measurements were made using a Corning pH/Ion meter type 135 with a combination glass electrode (Russell pH, Auchtermuchty, Fife, UK).

Reagent Solutions

All aqueous solutions were prepared using doubly distilled water.

Alizarin Fluorine Blue, 5.0 × 10^{-4} mol dm^{-3}. Dissolve 0.2107 g of AFB dihydrate (Aldrich, Gillingham, Dorset, UK) in successive portions of a solution of 2.0 cm^3 of 1 mol dm^{-3} AnalaR ammonia solution in 40 cm^3 of water. Transfer the portions into a 1 l beaker. Dilute to about 750 cm^3 then reduce the pH to about 5 using dilute AnalaR or Aristar hydrochloric acid. Dilute to 1 l and store in a brown glass bottle.

Lanthanum nitrate, 7.5 × 10^{-4} *and* 5.0 × 10^{-4} mol dm^{-3}.

Sodium fluoride, 5.00 × 10^{-4} mol dm^{-3} *and* 1.00 μg cm^{-3} *of fluoride.*

Acetate buffer solution, 1 mol dm^{-3}, *pH* 4.5. Mix 60 cm^3 of AnalaR acetic acid with about 800 cm^3 of water, adjust the pH to 4.50 by the addition of 1 mol dm^{-3} AnalaR ammonia solution and dilute to 1 l.

Large cation solutions. Solutions of the compounds listed under Results were made up in 160 cm^3 of re-distilled isobutyl alcohol at concentrations such that the fluoride sensitivity was

good while the blank absorbance was small. Diphenylguanidine and tribenzylamine were recrystallized from ethanol.

Diphenylguanidine 2.0 × 10^{-4} mol dm^{-3} *in re-distilled isobutyl alcohol.*

Procedure

After investigation, the following procedure was devised for the determination of 0–1 μg of fluoride in 500 cm^3 of water.

Set a large water-bath at 20 °C. Pour 500 cm^3 of doubly distilled water into each of six 1 l cylindrical separating funnels, place the funnels in the water-bath and swirl the contents occasionally for about 20 min. To each funnel add 2.0 cm^3 of acetate buffer solution (pH 4.5, 1 mol dm^{-3}), 10.0 cm^3 of 5 × 10^{-4} mol dm^{-3} AFB, (sequentially) 0, 0.2, 0.4, 0.6, 0.8 and 1.0 cm^3 of 1.00 μg cm^{-3} standard fluoride solution, 60 cm^3 of re-distilled acetone and, with swirling, 10.0 cm^3 of 7.5 × 10^{-4} mol dm^{-3} lanthanum nitrate solution. Immediately add 96 cm^3 of a 2.0 × 10^{-4} mol dm^{-3} solution of diphenylguanidine in isobutyl alcohol and shake for 30 s. Shake each funnel for 30 s every 15 min for 4 h. Remove and clamp the funnels and allow the phases to separate for 30 min. Slowly run the lower aqueous phase to waste, stopping the flow when the interface is a little above the stopcock. Wait 5 min then bring the interface to the bottom of the stopcock bore. Dry the separator stem with a small plug of cotton wool and pass the organic phase into a dry 25 cm^3 calibrated flask. Rinse the region of the separating funnel which finally contained the organic phase with 1 cm^3 of ethanol and allow the rinsings to pass through into the calibrated flask. Remove the flask and make up to the mark with pure ethanol. Mix thoroughly and then read the absorbance at 580 nm against isobutyl alcohol, a method blank, or a blank derived from 520 cm^3 of water, 2 cm^3 of acetate buffer, 60 cm^3 of acetone and 96 cm^3 of extractant, in cells with a pathlength of 4 cm.

Results

The absorption spectra derived from 10 μg of fluoride, the blank and an excessively large [$(AFB)_2La_2$] blank obtained by extraction with 2 × 10^{-3} mol dm^{-3} diphenylguanidine are shown in Fig. 1. The $(AFB)_2La_2F$ produces an attractive royal blue extract peaking at 580 and 620 nm. Measurement at 580 nm gives good sensitivity but at 620 nm the already modest blank is further reduced. There is, in practice, little to choose between these two wavelengths.

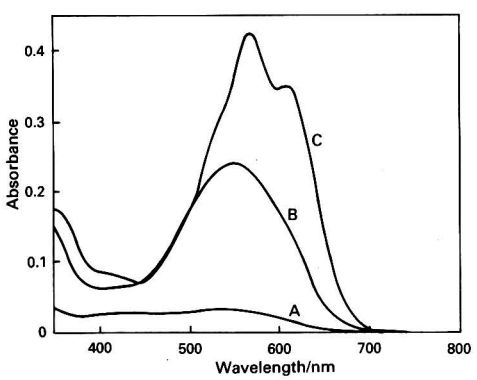

Fig. 1 Absorption spectra of extracts. A, Procedural blank; B, excessively large blank obained by extraction with 2 × 10^{-3} mol dm^{-3} diphenylguanidine; and C, spectrum derived from 10 μg of fluoride using the proposed procedure. Cell pathlength 1 cm; the reference cell contains a procedural blank without AFB and lanthanum

Performance

The calibration graph obtained for the range 0–1 μg of fluoride is shown in Fig. 2. It is almost linear and at 1 μg of fluoride shows a sensitivity of 0.017 A per 100 ng of fluoride in 500 cm^3 of water (4 cm cell). A set of blank values obtained using a method described earlier, identical except for the use of 5.0 × 10^{-4} mol dm^{-3} lanthanum nitrate gave, 0.0760, 0.0765, 0.0757, 0.0748, 0.0763 and 0.0769 (five readings of each). The standard deviation s = 0.00073 (at 620 nm this was 0.00081). At 580 nm, $3s$ = 0.0022 A. From the calibration graph (Fig. 2) $3s$ at the origin corresponds to 0.020 μg of fluoride. Hence, the $3s$ detection limit is 0.020 μg of fluoride in 500 cm^3 or 40 ng l^{-1} (2 × 10^{-9} mol dm^{-3}).

A calibration graph for 0–10 μg of fluoride is virtually linear and shows a sensitivity of 0.176 A μg^{-1} of fluoride per 500 cm^3 of water (4 cm cells). A linear regression computer analysis yielded further results: y-axis intercept, 0.093 A; correlation coefficient, 0.999944; residual sum of squares, 2.31 × 10^{-4} A; standard deviation of points about the line, 7.60 × 10^{-3} A; lowest determinable value, 0.159 μg of fluoride; and confidence limits (95%) for 4.01 μg of fluoride taken, 3.92–4.09 μg.

A repeatability study for 4 μg of fluoride (AFB : La, 1 : 1) gave: 0.6610, 0.6620, 0.6684, 0.6648, 0.6608, and 0.6586, each a mean of five readings; s = 0.0035, blank = 0.084. Hence, the actual fluoride absorbance = 0.663 − 0.084 = 0.579. Relative standard deviation (RSD) = (0.0035/0.579) × 100 = 0.60%. A reproducibility study on 4.0 μg of fluoride using the method described, taking readings (0.778, 0.779, 0.777, 0.781, 0.785, 0.787, 0.781, 0.786, 0.789 and 0.781 over 34 d gave: average, 0.782; standard deviation, 0.0041; and RSD, 0.53%.

The 95% confidence limit for a single result = 4.00 ± 0.06 μg of fluoride.

Effect of Experimental Variables

Choice of ion-pairing cation

Of the many tertiary and quaternary amines and other compound types studied, including all those mentioned in the literature, 20 were chosen for detailed study. Various solutions of these amines at different concentrations were prepared in isobutyl alcohol and used to extract a 1 : 1 : 1 AFB : La : F composition (10 cm^3 of 5 × 10^{-4} mol dm^{-3} NaF) under, approximately, the conditions given under Procedure but with no acetone present. A 'figure of merit' calculated from:

$$[A_{(AFB)_2La_2F} - A_{(AFB)_2La_2}]/A_{(AFB)_2La_2}$$

was obtained for all these compounds. Consideration of this value together with the numerator alone allowed the amines (or other cations) to be ranked in order of decreasing effectiveness as follows: diphenylguanidine; tribenzylamine; *N,N*-diethylaniline; imipramine hydrochloride; *N*-propylani-

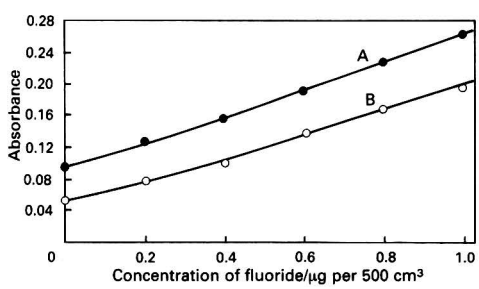

Fig. 2 Calibration graphs from 0 to 1 μg of fluoride. Wavelengths: A, 580 and B, 620 nm, cell pathlength 4 cm, with isobutyl alcohol in the reference cell

line; chlorpromazine hydrochloride; 1,1′-diheptyl-4,4′-bipyridinium dibromide; methyldioctylamine; dioctylamine; benzethonium chloride; cetylpyridinium chloride; triisooctylamine; dodecylethyldimethylammonium bromide; tripentylamine; aniline; tetrahexylammonium bromide; butyltriphenylphosphonium chloride; tetraphenylarsonium chloride; benzhydryltriphenylphosphonium chloride; 1-naphthylmethyltriphenylphosphonium chloride; triethylamine. Experience showed that, in general, hydrophobic amines, especially those with both aliphatic and aromatic character, proved to be the most successful as extractants. Some cations at higher concentrations, particularly surfactants, caused rather stable emulsions to form which prevented the clean separation of the layers. The best three compounds in the above list, all previously recommended by groups of workers, were subjected to close comparison. From experience, an equilibrium pH of 4.5 was used for all these compounds; this pH is high enough to give a large conditional formation constant for the ternary complex yet low enough to protonate the amines. For diethylaniline, which is a comparatively strong base and is used at a higher concentration than the other amines, the volume of 1 mol dm^{-3} HCl (about 0.45 cm^3) required to give an equilibrium aqueous pH of 4.5 was determined by a preliminary experiment. The concentration of a particular amine in isobutyl alcohol was, in each instance, adjusted to yield an approximately 10% loss of the maximum possible absorbance at 580 nm, judged to be that produced by 4×10^{-4} mol dm^{-3} diphenylguanidine, *i.e.*, 1.551. Figures of merit could thus be compared under these more equitable conditions. Absorbances > 1 were measured in 0.5 cm cells but recorded as for 1 cm cells. Results are shown in Table 1.

This study shows that there is little to choose between diphenylguanidine, tribenzylamine and diethylaniline. These three compounds were further compared by producing calibration graphs (no acetone, 1 : 1 ratio of AFB to La) over the ranges 0–1 and 0–5 μg of fluoride; results confirmed the ranking shown in Table 1 and supported the original conclusions of Levkov and Novak concerning diphenylguanidine.[39]

Kinetics

An earlier much used procedure in this work used no acetone and a 1 : 1 ratio of AFB to La. The time dependence of the complex formation and extraction for 4 μg of fluoride is shown by curve A in Fig. 3. Curve B shows the blank value response. Clearly the behaviour shown by A is far from ideal. It was remembered that in the original one-phase AFB method, the addition of acetone (15–20% v/v) greatly improved the kinetics although it degraded the spectroscopic performance. Hence, the influence of acetone on the extraction process was investigated. Curve C in Fig. 3 shows the result of adding acetone (12% v/v); the curve levels off after 4 h and even after 2 h, 88% of the final absorbance has been achieved. Less acetone causes the absorbance to rise steadily while more acetone causes a small absorbance decrease after 4 h. Because of the mutual miscibility of acetone with water and isobutyl alcohol, the volume of extractant required increased from 80 to 96 cm^3. The addition of acetone caused no marked increase in the blank. Later checking of the literature showed this idea to have been tried by Hirano *et al.*[31] in 1966 and by Levkov and Novak[39] in 1976.

Ratio of AFB to lanthanum

Unfortunately, a ratio of 1 : 1 was used for most of this study. Fig. 4 shows that not only does a 1 : 1.5 AFB to La ratio give a 24% increase in absorbance but a response plateau is also reached, all with very little increase in the blank. However, a plot of $[A_{(AFB)_2La_2F} - A_{(AFB)_2La_2}]/A_{(AFB)_2La_2}$ *versus* mole ratio does peak at 1 : 1.

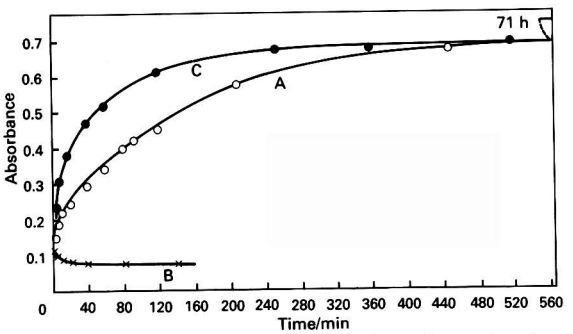

Fig. 3 Time dependence of complex formation and extraction. A, 4 μg of fluoride, ratio of AFB : La = 1 : 1, no acetone; B, blank behaviour under the same conditions; and C, 4 μg of fluoride with addition of 60 cm^3 of acetone. Wavelength 580 nm, cell pathlength 4 cm, with isobutyl alcohol in the reference cell

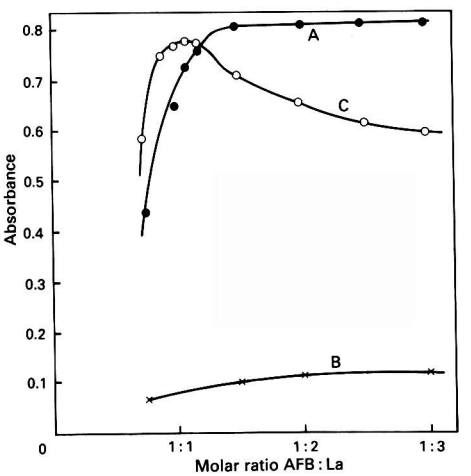

Fig. 4 Variation of ratio of AFB : La. A, 4 μg of fluoride; B, blank; and C, with *y*-axis $\{[A_{(AFB)_2La_2F} - A_{(AFB)_2La_2}]/A_{(AFB)_2La_2}\}/10$ (see text). Wavelength 580 nm, cell pathlength 4 cm, with isobutyl alcohol in the reference cell

Table 1 Comparison of selective extraction ability with $(AFB)_2La_2F$ of the best three amines at pH 4.5 using 1 cm cells

Amine	Concentration/ mol dm^{-3}	Absorbance at 580 nm			Figure of merit	Loss of $(AFB)_2La_2F$ (%)	Absorbance at 620 nm			Figure of merit
		$(AFB)_2La_2$	$(AFB)_2La_2F$	Difference			$(AFB)_2La_2$	$(AFB)_2La_2F$	Difference	
Diphenylguanidine	2.0×10^{-4}	0.018	1.484	1.466	81	4.3	0.010	1.242	1.232	123
	1.0×10^{-4}	0.014	1.360	1.346	96	12.3	0.008	1.124	1.116	140
Tribenzylamine	2.0×10^{-3}	0.062	1.460	1.398	23	5.9	0.028	1.218	1.190	43
	1.0×10^{-3}	0.031	1.434	1.403	45	7.5	0.014	1.200	1.186	85
	5.0×10^{-4}	0.020	1.390	1.370	69	10.4	0.010	1.158	1.148	115
N,*N*-Diethylaniline	1.0×10^{-2}	0.024	1.438	1.414	59	7.3	0.013	1.188	1.175	90
	8.8×10^{-3}	0.022	1.398	1.376	63	9.9	0.012	1.174	1.162	97

Influence of pH

Fig. 5 shows the effect of pH on the extraction of 4 μg of fluoride using a 1:1 AFB to La procedure. The apparent equilibrium aqueous phase pH of 4.7 can be achieved by using a buffer of pH 4.5 as in the procedure given. Increasing the buffer concentration reduces both the sensitivity and the blank value.

It is of interest that once the fluoride complex is formed it becomes, like the iron(II)–*o*-phenanthroline complex, inert to attack by H^+, far more so than the blank. Thus the absorbance of a 1:1:1 (AFB : La : F) complex took 16 h to fall from 1.86 to 0.82 at pH 2.7.

Concentration of diphenylguanidine

Fig. 6 shows the influence of diphenylguanidine concentration on the absorbance given by 4 μg of fluoride and the blank. Maximum sensitivity can be achieved at a concentration of $\approx 1 \times 10^{-3}$ mol dm^{-3} but the blank is high and in fact the term $[A_{(AFB)_2La_2F} - A_{(AFB)_2La_2}]/A_{(AFB)_2La_2}$ peaks at the recommended value of 2×10^{-4} mol dm^{-3}. Over the range of the experiment the pH of the aqueous phase increases slightly (from 4.65 to 4.8); this has a negligible influence (Fig. 5).

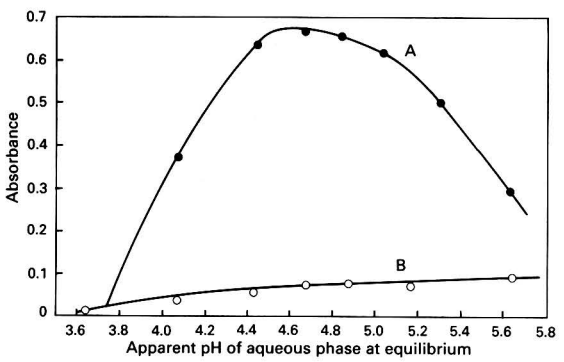

Fig. 5 Influence of the pH of the aqueous phase at equilibrium. Procedure as described except that 10.0 cm^3 of 5×10^{-4} mol dm^{-3} of lanthanum nitrate and 1 mol dm^{-3} of HCl and NH_3 were used. A, 4 μg of fluoride; B, blank. Wavelength 580 nm, cell pathlength 4 cm, with isobutyl alcohol in the reference cell

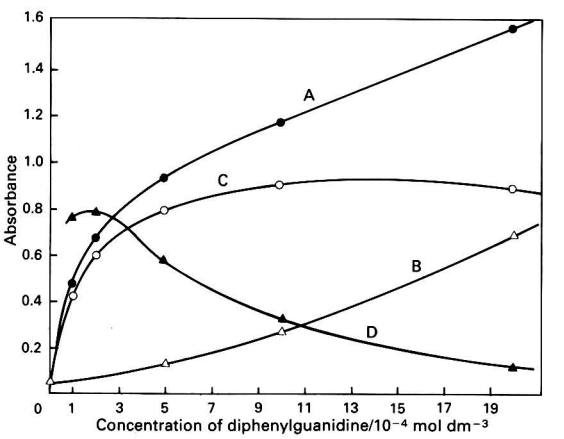

Fig. 6 Effect of concentration of diphenylguanidine. A, 4 μg of fluoride; B, blank; C, difference between A and B; and D with *y*-axis $\{[A_{(AFB)_2La_2F} - A_{(AFB)_2La_2}]/A_{(ABF)_2La_2}\}/10$. Wavelength 580 nm, cell pathlength 4 cm, with isobutyl alcohol in the reference cell

Interferences

Like the one-phase AFB fluoride method, this solvent extraction procedure is susceptible to interference from metal ions which complex with the AFB or fluoride, and anions which complex with lanthanum (in particular phosphate). It is ideally suited to the analysis of pure fluoride solutions such as pyrohydrolysates or Willard–Winter distillates but the many interference removal techniques based on precipitation, ion exchange and solvent extraction already developed for absorptiometric fluoride methods could no doubt be applied with effect.

Discussion and Summary

This method is very labour intensive and shows poor kinetics but it will produce a reliable detection limit of 40 ng l^{-1} of fluoride (0.025 ppb) with simple apparatus and reagents that can be found in every laboratory. This compares with a 'normal use' detection limit for the electrode of about 40 μg l^{-1} [34,50] although more complex procedures can lower this considerably.[20] There is no interface precipitation and reagents and products are very stable. Curve C in Fig. 3 shows that a reasonable absorbance can be achieved in a few minutes, hence the procedure could form the basis of an effective flow injection method which would obviate the two drawbacks mentioned above. Indeed, for less exacting manual use, a shaking time of 90 min can yield good results.

The main competitor is probably ion chromatography although even here the fluoride peak elutes early and is sometimes poorly resolved from other fast-moving anions. This solvent extraction procedure could act as a good independent check on ion chromatography at very low fluoride levels.

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Utility of Certain π-Acceptors for the Spectrophotometric Determination of Some Penicillins

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Two simple and sensitive spectrophotometric methods are described for the determination of six penicillin derivatives. The methods are based on the reaction of these drugs as *n*-electron donors with either 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ) or 7,7,8,8-tetracyanoquinodimethane (TCNQ) as π-acceptors, to give a highly coloured radical anion. The coloured products are quantified spectrophotometrically at 460 and 842 nm for DDQ and TCNQ, respectively. The optimization of the different experimental conditions is described. The interference from streptomycin sulphate and common degradation products was also studied. The proposed methods were applied successfully to the determination of the different penicillins investigated, either in pure or dosage forms, with good accuracy and precision. The results were compared with those given by the official United States Pharmacopeial XXI method.

Keywords: *Spectrophotometry; charge-transfer complex; π-acceptor; penicillin; drug formulation*

π-Acceptors such as 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ), 7,7,8,8-tetracyanoquinodimethane (TCNQ), tetracyanoethylene (TCNE), 2,4,7-trinitrofluoren-9-one (TNF), 2,3,5,6-tetrachloro-*p*-benzoquinone (*p*-chloranil) and 2,3,5,6-tetrabromo-*p*-benzoquinone (*p*-bromanil) are known to yield charge-transfer complexes and radical ions with a variety of electron donors such as amines,[1–3] vitamin A,[4] alkaloids,[5] procaine,[6] some drugs containing the imidazoline ring[7] antihistamines,[8] pentazocine[9] and some sulpha drugs.[10]

Apart from the work of Belal *et al.*[11] on the determination of benzylpenicillin with *p*-chloranil, this donor–acceptor interaction has not been investigated for penicillins as a group of *n*-electron donors, which were recently demonstrated to participate in charge-transfer complexation with iodine.[12,13]

This paper describes the application of the π-acceptors DDQ and TCNQ to the spectrophotometric determination of benzylpenicillin, anhydrous ampicillin, sodium ampicillin, oxacillin, amoxicillin and methicillin in pure samples and dosage forms. The interference from streptomycin sulphate and the common penicillin degradation products was also studied.

Experimental

Apparatus

A Uvidec-320 spectrophotometer (Jasco, Tokyo, Japan) and an EM-360 A proton nuclear magnetic resonance (^{1}H NMR) spectrometer (Varian, Palo Alto, CA, USA) were used.

Materials

Benzylpenicillin, anhydrous ampicillin, sodium ampicillin, oxacillin, amoxicillin, methicillin, benzylpenicillenic acid, penicillic acid, 6-aminopenicillanic acid and penicillamine were obtained from Sigma (Deisenhofen, Germany). Benzylpenicilloic acid was prepared by using a standard method.[14]

Formulations

The following commercial formulations were subjected to the analytical procedures. (1) Benzylpenicillin vials (Cid and Misr, Cairo, Egypt) containing 100.000 U of benzylpenicillin per vial (1650 U is equivalent to 1 mg of sodium benzylpenicillin). (2) Ampicillin capsules (Nile, Cairo, Egypt) containing 250 mg of anhydrous ampicillin per capsule. (3) Sodium ampicillin vials (Cid) containing 500 mg of sodium ampicillin per vial. (4) Prostaphlin capsules (Bristol Italiana, Rome, Italy, under authority of Bristol Myers, USA) containing 250 mg of sodium oxacillin per capsule. (5) Amoxicid capsules (Cid) containing 250 mg of amoxicillin trihydrate per capsule.

Reagents

*2,3-Dichloro-5,6-dicyano-*p*-benzoquinone solution*, 2 mg ml^{-1} *in methanol*. The solution was prepared fresh daily.

7,7,8,8-Tetracyanoquinodimethane solution, 1 mg ml^{-1} *in acetonitrile*. The solution is stable for at least 1 week at 4 °C.

Tetracyanoethylene, 2,4,7-trinitrofluoren-9-one, p-chloranil and p-bromanil solutions, 2 mg ml^{-1} *in acetonitrile*.

Standard Solutions

Weigh accurately 50 mg of each penicillin into a 100 ml calibrated flask. Dissolve the drug in methanol to give a final volume of 100 ml for the DDQ method. For the TCNQ method, dissolve the drug in 2 ml of methanol and dilute to 100 ml with acetonitrile.

Working Solutions

Vials and capsules

Weigh accurately 50 mg of the contents of the vial or from a composite of the mixed contents of 20 capsules. Then follow the procedure as for the standard solutions.

General Procedure

DDQ method

Place a 1 ml aliquot of a solution of the standard or pharmaceutical preparations in methanol containing 0.05–1.0 mg ml^{-1} of the drug in a 10 ml calibrated flask. Add 1 ml of DDQ solution and allow to stand at 20–25 °C for about 15 min. Dilute to 10 ml with methanol and measure the absorbance at 460 nm against a reagent blank.

TCNQ method

Place a 1 ml aliquot of a solution of the standard or pharmaceutical preparations in acetonitrile containing 0.1–0.2 mg ml^{-1} of the drug in a 10 ml calibrated flask. Add 1 ml of TCNQ solution and allow to stand at 20–25 °C for 30 min. Dilute to 10 ml with acetonitrile and measure the absorbance at 842 nm against a reagent blank.

Preparation of Charge-transfer Complexes With DDQ and TCNQ for ^{1}H NMR Measurements

Add a 0.02 mol dm^{-3} solution of DDQ in methanol or TCNQ in acetonitrile to a 0.02 mol dm^{-3} solution of benzylpenicillin in methanol. After allowing the solution to stand at room temperature for 30 min, remove the solvent by distillation under reduced pressure, dry the product over calcium chloride and use for ^{1}H NMR measurements.

Results and Discussion

The reaction of DDQ with different penicillin derivatives results in the formation of an intense orange–red product which exhibits an absorption maximum at 460 nm (see Fig. 1). This spectrum is similar to that of the radical anion obtained by reduction with iodide.[15]

In acetonitrile, a solution of the penicillins and TCNQ was found to yield an intense colour causing characteristic long wavelength absorption bands, frequently with numerous vibrational maxima in the electronic spectrum (Fig. 1). The predominant chromogen with TCNQ is the blue radical anion TCNQ$^{\cdot-}$, which was probably formed by the dissociation of an original donor–acceptor (DA) complex with the penicillins.

$$\ddot{D} + A \rightleftharpoons \underset{\text{DA complex}}{(\ddot{D}\text{–}A)} \xrightarrow[\text{solvent}]{\text{polar}} \underset{\text{radical ions}}{D^{\cdot+} + A^{\cdot-}}$$

The dissociation of the (DA) complex is promoted by the high ionizing power of the solvent, acetonitrile.[16] Further support for this assignment was provided by comparison of the absorption bands with those of the TCNQ$^{\cdot-}$ radical anion produced by the iodide reduction method.[17]

In addition to the TCNQ and DDQ radical anions, the resulting bands for the complexes of benzylpenicillin with TCNE, TNF, *p*-chloranil and *p*-bromanil, obtained by reaction in acetonitrile at 60 °C, are similar to the maxima of the radical anions of these acceptors obtained by the reduction method.[15]

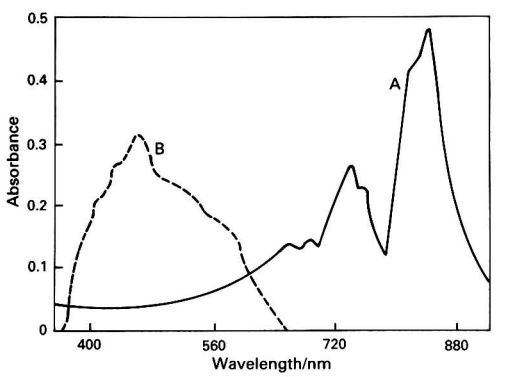


Fig. 1 Absorption spectra of A, benzylpenicillin–TCNQ; and B, benzylpenicillin–DDQ reaction products

The relative sensitivities of the six acceptors can be determined by comparing the molar absorptivities (ε) of the chromogens (Table 1) using benzylpenicillin as a reference. The compounds TCNQ and DDQ exhibited the most intense bands and were therefore selected for all further work. The most important spectral characteristics of the reaction of TCNQ and DDQ with the penicillins investigated are presented in Table 2.

As an assay solvent, methanol afforded maximum sensitivity with DDQ. On the other hand, acetonitrile was found to be the best for TCNQ because it has a high relative permittivity which ensures the maximum yield of TCNQ$^{\cdot-}$. In addition, it is a good solvent for the reagent. Of the other solvents examined, 1,2-dichloroethane and dichloromethane are possible substitutes. Benzene and chloroform were unsuitable because the reagents were not very soluble in these solvents. In 1,4-dioxane and tetrahydrofuran, other orange coloured products, and not TCNQ$^{\cdot-}$, were formed on addition of the reagent. It is likely that the formation of this product also involves interaction with solvent molecules.[18]

When various concentrations of DDQ or TCNQ were added to a fixed concentration of benzylpenicillin, 1 ml of 0.2 and 0.1% solutions of DDQ or TCNQ, respectively, was found to be sufficient for the production of maximum and reproducible colour intensity. Higher concentrations of reagent did not affect the colour intensity.

The optimum reaction time was determined by following the colour development at ambient temperature (20–25 °C). Complete colour development was attained after 15 and 30 min for DDQ and TCNQ, respectively, with all the penicillins investigated. The colour remained stable for at least a further 30 min.

The sensitivity of the TCNQ method, expressed as log ε values, varied with different penicillins in a systematic fashion which was found to be dependent on the pK_a values of the different penicillins (Table 3).

The regression equation, for the correlation between log ε and pK_a, derived by the least-squares method, was: log ε = 2.635 + 0.671pK_a (r = 0.9343).

Sodium ampicillin gives high ε values owing to the presence of a free amino group in the side chain. The amphoteric anhydrous ampicillin and amoxicillin possess properties

Table 1 Molar absorptivities of the chromogens with benzylpenicillin in acetonitrile

Reagent	ε_{max}/dm^3 mol^{-1} cm^{-1}
TCNQ	30 400 (842)*
DDQ†	2 960 (460)
TCNE	485 (393)
TNF	593 (420)
p-Chloranil	623 (540)
p-Bromanil	320 (540)

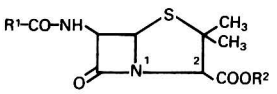
* Values in parentheses are λ_{max} values (nm).
† In methanol.

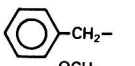
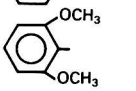
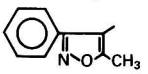
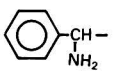
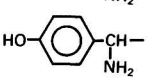
Table 2 Quantitative parameters for the reaction of different penicillins with DDQ and TCNQ

	DDQ method					TCNQ method				
Compound	Linear range/ μg ml^{-1}	ε/10^3 dm^3 mol^{-1} cm^{-1}	Intercept*	Slope*	Correlation coefficient*	Linear range/ μg ml^{-1}	ε/10^4 dm^3 mol^{-1} cm^{-1}	Intercept*	Slope*	Correlation coefficient*
Benzylpenicillin	8–80	2.96	−0.0139	0.0090	0.9996	1–7	3.04	0.0151	0.0840	0.9990
Methicillin	20–100	3.43	0.0300	0.0070	0.9995	5–20	3.09	0.0167	0.0700	0.9995
Oxacillin	20–100	3.05	0.0160	0.0068	0.9991	2–8	3.86	0.0380	0.0922	0.9998
Anhydrous amplicillin	10–80	3.67	0.0123	0.0099	0.9991	5–20	1.92	0.1950	0.0356	0.9990
Sodium ampicillin	5–60	4.97	0.0180	0.0120	0.9994	1–7	5.26	0.5189	0.0899	0.9994
Amoxicillin	10–60	4.60	0.0200	0.0100	0.9990	4–16	1.86	0.0360	0.0410	0.9990

* n = 5 in all instances.

Table 3 Correlation between log ε and pK_a values for penicillins with the TCNQ method



Penicillin	R^1	R^2	pK_a(COO$^-$)*	Log ε
Benzylpenicillin		Na	2.8	4.47
Methicillin		H	2.8	4.49
Oxacillin		H	2.8	4.59
Anhydrous ampicillin		H	2.5	4.28
Amoxicillin		H	2.4	4.27

* Reference 19.

similar to those of aliphatic amino acids. They exist essentially as zwitterions, and in this form, the basicity of the amino group is not observed. This could explain why anhydrous ampicillin and amoxicillin have ε values lower than that of sodium ampicillin and comparable to those of the other penicillins having no amino group.

Interference studies were carried out in order to investigate the effect of streptomycin sulphate and degradation products that might be present in penicillin dosage forms. Addition of a methanolic solution of DDQ to the different degradation products resulted in the formation of colourless solutions. No interference was observed from benzylpenicilloic acid, benzylpenicillenic acid, penicillic acid, 6-aminopenicillanic acid, penicillamine and streptomycin sulphate on using DDQ as the acceptor. On the other hand, benzylpenicilloic and benzylpenicillenic acids were found to interfere with the assay procedure when TCNQ was used as the acceptor. The other degradation products and also streptomycin sulphate showed no interference (Table 4).

The possible interference observed with TCNQ could be explained on the basis of the difference between the ionization potential, of the different degradation products and the intact penicillin molecules, and the electron affinity of the acceptor.[2,20]

In addition to ultraviolet–visible spectrophotometry, NMR spectroscopy could also be used to monitor the formation of the charge-transfer complexes. Generally, the protons of the donor are shifted to a lower field (paramagnetic shift). The ^{1}H

Table 4 Determination of benzylpenicillin in the presence of streptomycin sulphate and common degradation products

Substance	Amount added/ mg*	Found ± standard deviation† (%) DDQ method	TCNQ method
Streptomycin sulphate	50	99.27 ± 1.14	98.92 ± 1.43
Benzylpenicilloic acid	2	99.86 ± 0.76	High results
Benzylpenicillenic acid	2	98.65 ± 1.39	High results
Penicillic acid	2	100.00 ± 0.78	99.72 ± 0.68
6-Aminopenicillanic acid	2	99.10 ± 0.96	98.97 ± 0.85
Penicillamine	2	101.80 ± 1.07	100.56 ± 1.15

* Amount added per 50 mg of benzylpenicillin.
† Average of five determinations.

Table 5 Determination of penicillins in dosage forms. Theoretical value for t: 2.78 (p = 0.05). Theoretical value for F: 6.39 (p = 0.05)

Sample	Recovery ± standard deviation* (%) DDQ method	TCNQ method	USP-XXI method†
Benzylpenicillin vials (Cid)	99.12 ± 1.59 t = 0.045 F = 0.392	101.30 ± 0.85 t = 1.770 F = 0.112	99.18 ± 2.54
Benzylpenicillin vials (Misr)	99.43 ± 1.16 t = 1.040 F = 0.696	99.82 ± 0.93 t = 0.602 F = 0.448	100.27 ± 1.39
Ampicillin capsules	99.43 ± 1.26 t = 0.386 F = 1.102	98.92 ± 1.09 t = 1.119 F = 0.825	99.73 ± 1.20
Ampicillin sodium vials	100.08 ± 0.79 t = 0.318 F = 0.535	100.73 ± 0.92 t = 0.726 F = 0.726	100.27 ± 1.08
Prostaphlin capsules	100.21 ± 0.98 t = 1.642 F = 0.321	99.09 ± 1.33 t = 0.349 F = 0.591	98.75 ± 1.73
Amoxicid capsules	100.65 ± 1.72 t = 1.149 F = 0.531	99.98 ± 1.30 t = 0.615 F = 0.304	99.15 ± 2.36

* Mean of five determinations.
† Reference 21.

NMR spectra of benzylpenicillin–DDQ and benzylpenicillin–TCNQ were recorded in dimethyl sulphoxide-d_6 together with the spectrum of free benzylpenicillin; the 2-H proton of the benzylpenicillin in the complexes is shifted to a lower field ($\Delta\delta$ = 0.2 ppm). The chemical shifts of the other protons were unchanged. Hence, and as reported previously,[12] it could be deduced that the charge-transfer proceeds from the lactam nitrogen of benzylpenicillin.

The proposed methods and the official United States Pharmacopeial (USP) XXI titrimetric method[21] were applied to the determination of the different penicillin derivatives in capsules and vials. The DDQ and TCNQ methods gave mean values (± standard deviation) of the labelled amount ranging from 99.12 ± 1.59 to 100.65 ± 1.72% and from 98.92 ± 1.09 to 101.30 ± 0.85%, respectively (Table 5).

In conclusion, the proposed methods are simpler, less time consuming and more sensitive than the official method. Moreover, the DDQ procedure, in spite of its lower sensitivity, could be used for the routine determination of penicillins in the presence of streptomycin sulphate and the major degradation products of penicillin.

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Spectrophotometric Method for the Determination of Paraquat in Water, Grain and Plant Materials

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A sensitive spectrophotometric method for the determination of paraquat using ascorbic acid (an easily available reducing agent) is described. Paraquat is reduced with ascorbic acid in alkaline solution to give a blue radical ion with an absorbance maximum at 600 nm. Beer's law is obeyed in the range 12–96 μg of paraquat in 10 ml of the final solution (1.2–9.6 ppm). The important analytical parameters and the optimum reaction conditions were evaluated. The method was applied successfully to the determination of paraquat in water, grain and plant materials.

Keywords: *Paraquat determination; spectrophotometry; ascorbic acid; water, grain and plant materials*

Paraquat (the 1,1′-dimethyl-4,4′-bipyridylium ion), also known as methyl viologen or Gramoxone (available in the form of the dichloride or dimethyl sulphate salt), is a popular general herbicide, desiccant and defoliant. It is often used as a selective weedkiller because it is much more effective against broad-leaved vegetation than against grasses.[1,2] It is a broad-action herbicide, which is used to clear new land for planting crops, as a general weedkiller and as a means of removing vegetation from road sides. Paraquat is also a poison with no known antidote. It causes a condition known as 'paraquat lungs' (*i.e.*, honeycombing of the lungs and hardening of the breathing tract) and other diseases of the heart, kidney and liver.[3]

The wide usage, occurrence in the plant kingdom and high toxicity of paraquat together with its advantages over other herbicides have necessitated the development of a sensitive and selective method for the determination of this pesticide. The methods described in the literature include thin-layer chromatography,[4] high-performance liquid chromatography,[5] ultraviolet spectrometry[6] and polarography.[7] The available spectrophotometric methods[8,9] are based on measurement of the reduced ion obtained by the reduction of paraquat with alkaline sodium dithionite solution. These methods are less sensitive (range 4–10 ppm) than the method described here and the colour of the solution is only stable for a short period of time (5–10 min). The method proposed by Ganesan *et al.*[10] using potassium tetraiodomercurate (K_2HgI_4) gives a more stable complex (the complex is stable for up to 60 min) but uses expensive and toxic mercury compounds. At high concentrations of paraquat, the paraquat–HgI_4 complex precipitates.

In this paper, a method based on the reduction of paraquat with ascorbic acid in alkaline medium to form a blue free radical having an absorbance maximum at 600 nm is described. Beer's law is obeyed in the range 12–96 μg of paraquat in 10 ml of the final solution (1.2–9.6 ppm). The method was applied successfully to the determination of paraquat in water, grain and various plant materials. Other herbicides/pesticides such as (2,4-dichlorophenoxy)acetic acid (2,4-D), (2,4,5-trichlorophenoxy)acetic acid (2,4,5-T), kelthane, lindane, 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) and organophosphates do not interfere with the method.

Experimental

Apparatus

A Carl Zeiss Specol spectrophotometer with 1 cm matched cells was used for all spectral measurements.

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Reagents

Standard paraquat solution (Indian Explosive, India). A 1 mg ml^{-1} stock solution of paraquat was prepared in water. A working standard solution (24 μg ml^{-1}) was prepared by appropriate dilution of the stock solution.

Ascorbic acid solution, 0.5%.

Sodium hydroxide solution, 0.3 mol dm^{-3}.

Ethylenediaminetetraacetic acid (EDTA) solution, 5%.

Silica gel (BDH), 100–200 mesh for column chromatography.

Saturated ammonium chloride solution.

All other chemicals used were of analytical-reagent grade and de-ionized water was used for preparing all the solutions.

Procedure

An aliquot of a standard solution containing 12–96 μg (0.5–4 ml of the working standard solution) of paraquat was placed in a calibrated test-tube. To this solution, 1 ml of EDTA solution was added in order to mask the metal ions. Then, 1 ml of ascorbic acid solution followed by 2 ml of sodium hydroxide solution were added and the volume was made up to 10 ml by using de-ionized water. A blue free radical corresponding to reduced paraquat was obtained. The contents of the test-tube were mixed by inverting the tube two or three times. The absorbance of the blue solution at 600 nm was measured against a reagent blank.

Results and Discussion

All spectral studies were carried out at 600 nm on a final solution volume of 10 ml containing various amounts of paraquat. The blue radical was stable for about 12 h.

Effect of Variables

Time and temperature

A period of 2 min was sufficient for reduction and full colour development. The most suitable temperature range for the reaction was between 20 and 30 °C as this was the range in which maximum colour development occurred. Outside of this temperature range the absorbance value was found to decrease.

pH

Full colour development was obtained at or above a pH of 11.

Beer's Law, Molar Absorptivity, Sandell's Sensitivity and Reproducibility

Beer's law was obeyed in the range 12–96 μg of paraquat in a final volume of 10 ml (1.2–9.6 ppm). The molar absorptivity and Sandell's sensitivity were found to be 6.7×10^4 (±100) $dm^3\ mol^{-1}\ cm^{-1}$ and 0.0027 μg cm^{-2}, respectively. The reproducibility of the method was ascertained by analysing a solution containing 72 μg of paraquat per 10 ml over a period of 8 d. The standard deviation and the relative standard deviation were found to be 0.0138 A and 3.8%, respectively.

Effect of Foreign Species

The effect of various interfering co-pollutants and pesticides was studied in order to assess the validity of the method. Known amounts of metal ions and pesticides were added to the sample solution and paraquat was determined by the proposed method. Those metal ions that formed hydroxides such as Fe^{3+} and Al^{3+} interfered with the method owing to precipitation in the basic solution; this can be prevented by the addition of 1 ml of 5% EDTA solution prior to the addition of the NaOH solution. The tolerance limits (in ppm) for various foreign species in a solution containing 72 μg of paraquat per 10 ml are given in Table 1.

Table 1 Effect of foreign species. Concentration of paraquat, 72 μg per 10 ml of solution

Foreign species	Tolerance limit* (ppm)
Zn^{2+}, Cd^{2+}, Mg^{2+}, PO_4^{3-}, Sn^{2+}	500
Ca^{2+}, Al^{3+},† Fe^{3+}, Fe^{2+}, kelthane	250
Pb^{2+}, monocrotophos	1000
2,4-D, 2,4,5-T, DDT, parathion, BHC‡	2000
Cu^{2+}†	70

* Amount tolerated can vary by ±2%.
† Amount tolerated with 1 ml of EDTA solution added.
‡ Benzene hexachloride.

Application of the Method to the Determination of Paraquat in Water, Grain and Vegetables

Paraquat is ionic and cannot be extracted with organic solvents. Owing to its polar nature, it is readily absorbed by plant materials and is difficult to remove. For the determination of paraquat in water, grain and plant materials, a silica gel column was used to separate paraquat from the samples.

Preparation of the column

Silica gel (100–200 mesh) for column chromatography was used for the preparation of the column. A 25 ml glass column (9–10 mm i.d.) with a plug of glass-wool placed just above the stopcock was used. Silica gel (5 g) was weighed out, packed into the column and then washed with water. A fresh column was used for each experiment.

Determination of paraquat in water

The water sample containing paraquat was passed through the column at a flow-rate of 7–8 ml min^{-1}. The paraquat was absorbed on the silica gel and was subsequently eluted by passing 50 ml of saturated ammonium chloride through the column at a flow-rate of about 3 ml min^{-1}. The eluted

Table 2 Recovery of paraquat from water, grain and plant materials

Sample	No.	Paraquat originally found*/ μg	Paraquat added*/μg	Total paraquat found*/ μg	Difference/μg	Recovery (%)
Water†	1	8.0	12	19.50	11.50	95.83
	2	11.5	24	34.50	23.50	95.83
	3	21.0	48	67.50	96.50	96.87
Wheat‡	1	11.0	12	20.40	9.40	79.16
	2	15.0	24	36.00	21.00	87.50
	3	16.0	48	59.00	43.00	89.00
Rice‡	1	10.0	12	19.00	9.00	75.00
	2	13.0	24	34.00	21.00	87.00
	3	15.0	48	57.00	42.00	87.00
Potato‡	1	7.0	12	16.50	9.50	79.16
	2	13.0	24	33.00	20.00	83.33
	3	16.0	48	56.50	40.50	84.37
Grass‡	1	8.0	12	16.90	8.90	74.00
	2	14.0	24	35.00	21.00	87.50
	3	15.0	48	58.00	43.00	89.00

* Mean of three replicate analyses.
† Sample size, 500 ml.
‡ Sample size, 20 g.

Table 3 Comparison of the proposed method with other spectrophotometric methods

Sample No.	Method	λ_{max}/ nm	Beer's law range (ppm)	Remarks	Reference
1	Reduction with sodium dithionite	600	4–12	Reagent unstable, dye stability poor (5–10 min)	9
2	Complex formation with potassium tetraiodomercurate	400–420	3	Colour development takes 15 min, reagent toxic, complex stable for up to 60 min	10
3	Reduction with ascorbic acid	600	1.2–9.8	Method is simple and sensitive. Dye stable for about 12 h	This work

paraquat was collected in a 50 ml calibrated flask. The volume was made up to the mark with de-ionized water and paraquat was determined by the proposed method (Table 2).

Determination of paraquat in grain samples

Different samples of grain such as wheat and rice (20 g of each) were collected from fields that had been treated with paraquat. The samples were weighed, crushed and homogenized, then filtered by adding 250 ml of water. The filtrate was placed in a 500 ml separating funnel and then allowed to pass through the silica gel column at a flow-rate of 7–8 ml min^{-1}. The column was washed with 25 ml of water and the paraquat absorbed on the silica gel was eluted with 50 ml of saturated ammonium chloride at a flow-rate of about 3 ml min^{-1}. The eluted paraquat was collected in a 50 ml calibrated flask. The volume was made up to the mark with de-ionized water and paraquat was determined by the proposed method (Table 2).

Determination of paraquat in plant materials

Samples of potato and grass were collected from fields that had been treated with paraquat. The samples were macerated and homogenized in a mixer. Water (150 ml) was added in order to macerate the vegetables and the mixture was filtered through a Büchner funnel. The resulting solution was filtered using a vacuum pump and the filtrate was collected. The residue was washed with two 100 ml portions of water. The combined filtrate was placed in a 500 ml separating funnel and then allowed to pass through the silica gel column at a flow-rate of 7–8 ml min^{-1}. The column was washed with 25 ml of water and the paraquat absorbed on the silica gel was eluted with 50 ml of saturated ammonium chloride at a flow-rate of 3–4 ml min^{-1}. The eluted paraquat was collected in a 50 ml calibrated flask. The volume was made up to 50 ml with de-ionized water and paraquat was determined as described above (Table 2).

In order to establish the validity of the method, different samples of water, grain and vegetables were taken. To these samples known amounts of paraquat were added and the solutions analysed by using the proposed method. The recoveries (Table 2) ranged from 74 to 96%, which is in agreement with the reported values.[8] The proposed method was compared with the other spectrophotometric methods reported for the determination of paraquat (see Table 3) and was found to be simpler and more sensitive than the method of Calderbank and Yuen.[9]

Conclusion

The herbicide paraquat can be determined down to a level of 1.2 ppm in water and grain samples by using the proposed method. The method was applied to the determination of paraquat in water, grain and plant materials.

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Spectrofluorimetric Determination of Chromium With 8-Hydroxyquinoline-5-sulphonic Acid

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A simple spectrofluorimetric method for the determination of Cr^{VI}, based on the oxidation of 8-hydroxyquinoline-5-sulphonic acid with potassium dichromate in 6.3 mol dm^{-3} H_2SO_4, is described. The fluorescent species has excitation and emission maxima at 360 and 450 nm, respectively. The fluorescence intensity of the system is linear over the concentration range 5.6×10^{-6}–1.5×10^{-5} mol dm^{-3} Cr^{VI}. The method was applied to the determination of Cr^{VI} in industrial wastewater samples.

Keywords: *Chromium determination; 8-hydroxyquinoline-5-sulphonic acid; oxidation reaction; spectrofluorimetry*

The chemistry of Cr is very important in the field of environmental science. Recently, Jie and Yu[1] reported a direct method for the spectrofluorimetic determination of Cr^{VI}. 8-Hydroxyquinoline has been used by a number of workers for the determination of metal ions,[2–4] and the potential of some 8-hydroxyquinoline derivatives has been investigated.[5–8] Pal and Ryan have developed sensitive and selective spectrofluorimetric methods for the determination of Ag[9] and Mn.[10] Recent studies into the use of the Co–H_2O_2–8-hydroxyquinoline-5-sulphonic acid system[11] for the determination of Co showed that Cr interferes at very low levels. Hence it was envisaged that this chemical system could be used as the basis for a procedure for the determination of trace amounts of Cr^{VI}.

Experimental

Apparatus

All fluorescence measurements were made with an RF-540 spectrofluorimeter (Shimadzu, Kyoto, Japan).

Reagents

All reagents were of analytical-reagent grade, and distilled, demineralized water was used throughout.

Chromium(VI) stock solution, 1×10^{-2} mol dm^{-3}. Prepared by dissolving 2.9418 g of potassium dichromate (Yuelong Chemical Plant, Shanghai, China) in 1000 ml of water by warming the solution at 45 °C.

Stock solution of 8-hydroxyquinoline-5-sulphonic acid, 1×10^{-3} mol dm^{-3}. Prepared by dissolving 0.2252 g of the reagent (Beijing Chemical Plant, China) in 1000 ml of water. Working solutions were prepared by dilution with distilled water.

Sulphuric acid, 18 mol dm^{-3}.

Procedure

A 1 ml volume of potassium dichromate solution (1×10^{-4} mol dm^{-3}), 3.0 ml of 8-hydroxyquinoline-5-sulphonic acid solution (2×10^{-4} mol dm^{-3}) and 3.5 ml of H_2SO_4 (18 mol dm^{-3}) were added sequentially to a 10 ml test-tube. The contents of the tube were mixed; after the solution had been cooled, it was diluted to the mark with water. The tube was then placed in a boiling water-bath for 15 min, after which it was cooled under tap water and the fluorescence intensity of the solution was measured in a 1 cm quartz cell at an excitation wavelength of 360 nm and an emission wavelength of 450 nm.

Results and Discussion

Fluorescence Spectra

The excitation and emission spectra of the system investigated are shown in Fig. 1. The fluorescent species has excitation and emission maxima at 360 and 450 nm, respectively.

Effect of H_2SO_4 Concentration

Experiments indicated that maximum and constant fluorescence intensity was obtained for H_2SO_4 concentrations in the

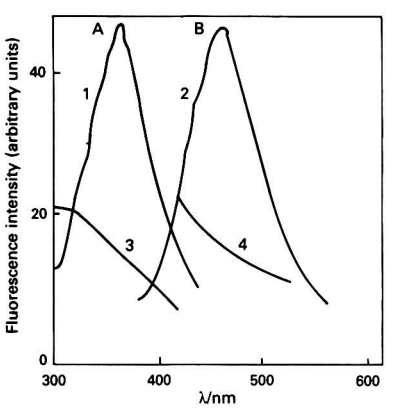

Fig. 1 Fluorescence spectra: A, excitation, 360 nm; and B, emission, 450 nm. 1 and 2, Cr^{VI}–8-hydroxyquinoline-5-sulphonic acid–H_2SO_4; and 3 and 4, 8-hydroxyquinoline-5-sulphonic acid–H_2SO_4. Conditions: 1.0×10^{-5} mol dm^{-3} Cr^{VI}; 6.0×10^{-5} mol dm^{-3} 8-hydroxyquinoline-5-sulphonic acid; and 6.3 mol dm^{-3} H_2SO_4

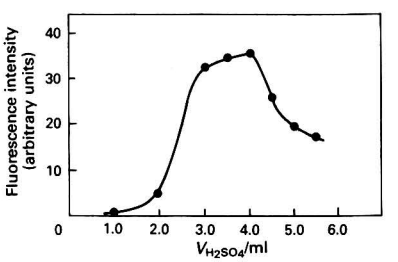

Fig. 2 Effect of H_2SO_4 concentration. Conditions: 1.0×10^{-5} mol dm^{-3} Cr^{VI}; and 6.0×10^{-5} mol dm^{-3} 8-hydroxyquinoline-5-sulphonic acid

Table 1 Tolerance of the method towards interfering ions. (Amount of Cr^{VI} taken, 5 μg)

Ion	Ratio of ion to Cr^{VI} (m/m)	Ion	Ratio of ion to Cr^{VI} (m/m)	Ion	Ratio of ion to Cr^{VI} (m/m)
Ni^{2+}	2000	Zn^{2+}	40	Cl^-	750
Be^{2+}	1600	Mn^{2+}	30	AsO_4^{3-}	600
Nd^{3+}	1200	Mg^{2+}	20	PO_4^{3-}	240
NH_4^+	1000	Cu^{2+}	20	F^-	200
K^+	1000	Co^{2+}	20	NO_3^-	100
Na^+	1000	Al^{3+}	10	NO_2^-	5
Cd^{2+}	600	Ce^{4+}	0.5	BrO_3^-	2
La^{3+}	300	V^V	0.5		
Y^{3+}	300	Sc^{3+}	40		
Ca^{2+}	100	Hg^{2+}	0.5		
Ag^+	200	Pb^{2+}	0.5		

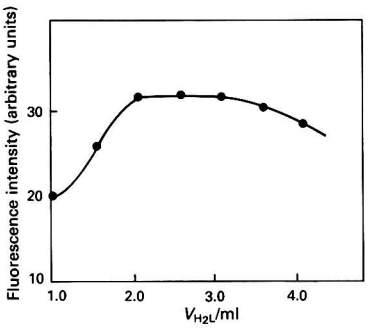

Fig. 3 Effect of 8-hydroxyquinoline-5-sulphonic acid concentration. Conditions: 1.0×10^{-5} mol dm^{-3} Cr^{VI}; and 6.3 mol dm^{-3} H_2SO_4

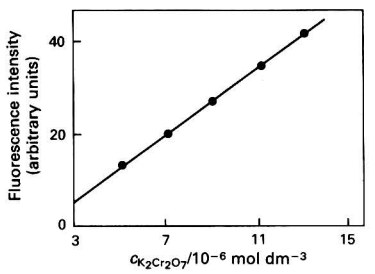

Fig. 4 Calibration graph. Conditions: 6.0×10^{-5} mol dm^{-3} 8-hydroxyquinoline-5-sulphonic acid; and 6.3 mol dm^{-3} H_2SO_4

range 5.4–7.2 mol dm^{-3} (Fig. 2). The intensity decreased for values outside this range. An H_2SO_4 concentration of 6.3 mol dm^{-3} was chosen.

Effect of 8-Hydroxyquinoline-5-sulphonic acid Concentration

The effect of the 8-hydroxyquinoline-5-sulphonic acid concentration on the fluorescence intensity of the system was studied. When the concentration of 8-hydroxyquinoline-5-sulphonic acid was between 4.0×10^{-5} and 8.0×10^{-5} mol dm^{-3}, the fluorescence intensity of the system reached a maximum and remained constant (Fig. 3). An 8-hydroxyquinoline-5-sulphonic acid concentration of 6.0×10^{-5} mol dm^{-3} was selected. In addition, it was shown that the fluorescence intensity of the system reached a maximum after heating at 100 °C for 15 min and then remained stable for 1 h.

Calibration Graph

The calibration graph for the determination of Cr^{VI} was constructed under the optimum conditions (described above).

Table 2 Analysis of industrial wastewater samples

Sample	Amount of Cr^{VI} found (ppm) Proposed method	Spectrophotometric method*
A	53.83	53.58
B	11.94	12.15
C	12.36	12.40
D	8.67	9.72

* Using 1,5-diphenylcarbazide as the colour reagent (reference 12).

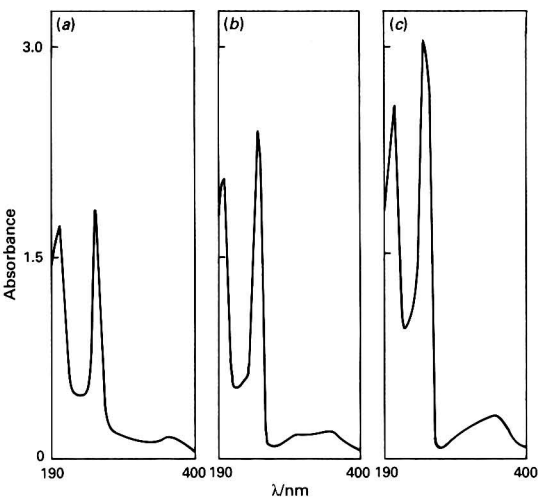

Fig. 5 Ultraviolet spectra. (*a*) Cr^{VI}–8-hydroxyquinoline-5-sulphonic acid–H_2SO_4; (*b*) 8-hydroxyquinoline-5-sulphonic acid–H_2SO_4; and (*c*) 8-hydroxyquinoline-5-sulphonic acid. Conditions: 1.0×10^{-5} mol dm^{-3} Cr^{VI}; 6.0×10^{-5} mol dm^{-3} 8-hydroxyquinoline-5-sulphonic acid; and 6.3 mol dm^{-3} H_2SO_4

Good linearity was obtained over the concentration range 5.0×10^{-6}–1.5×10^{-5} mol dm^{-3} Cr^{VI} (Fig. 4). A lower concentration of Cr^{VI} can be determined; however, at the maximum sensitivity of the instrument, the noise becomes significant and the reproducibility is poor.

Effect of Foreign Ions

The effect of foreign ions on the determination of Cr^{VI} is shown in Table 1. An error of 5% in the intensity values was considered tolerable. The largest interference effects were given by Ce^{4+}, Sc^{3+}, Pb^{2+}, V^V, Hg^{2+}, NO_2^- and BrO_3^-. The presence of Ce^{4+}, V^V, NO_2^- and BrO_3^- increased the

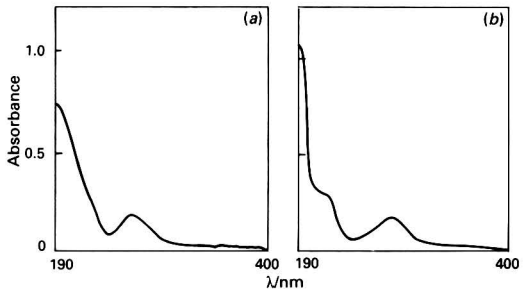

Fig. 6 Ultraviolet spectra. (*a*) Cr^{VI} (excess)–8-hydroxyquinoline-5-sulphonic acid–H_2SO_4. Conditions: 4.0×10^{-5} mol dm^{-3} Cr^{VI}; 2.0×10^{-5} mol dm^{-3} 8-hydroxyquinoline-5-sulphonic acid; and 6.3 mol dm^{-3} H_2SO_4. (*b*) *p*-Benzoquinone–H_2SO_4. Conditions: 1.0×10^{-5} mol dm^{-3} *p*-benzoquinone; and 6.3 mol dm^{-3} H_2SO_4

fluorescence intensity owing to their oxidizing nature. In 6.3 mol dm^{-3} H_2SO_4, Hg^{2+} and Pb^{2+} precipitated as their sulphates.

Analysis of Samples

The method was applied to the determination of Cr^{VI} in industrial wastewater samples. The results obtained are shown in Table 2. The recoveries of Cr^{VI} were found to be 99.2–100.4%.

Nature of the Fluorescence

Chromium(VI) is a strong oxidizing agent and can oxidize 8-hydroxyquinoline-5-sulphonic acid to give a fluorescent species. In order to investigate the nature of the fluorescent species, the ultraviolet and infrared spectra of the various systems were studied. Fig. 5 shows that the maximum absorbance values of all the systems are at the same wavelength. These results were obtained when a large excess of 8-hydroxyquinoline-5-sulphonic acid was present. The absorption band of this reagent masked that of the reaction product. In order to reduce the effect of 8-hydroxyquinoline-5-sulphonic acid, a large excess of potassium dichromate was added to the 8-hydroxyquinoline-5-sulphonic acid–Cr^{VI}–H_2SO_4 system. Fig. 6 shows that the ultraviolet spectrum of this system is similar to that of the *p*-benzoquinone–H_2SO_4 system. The infrared spectra show that the absorption bands of these systems are also very similar. These results indicate that the product might be a quinone-type compound, although the exact nature of the fluorescent species is still not known.

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Stability of Phenyl(thiocarbamoyl) Amino Acids and Optimization of Their Separation by High-performance Liquid Chromatography

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The optimization of the conditions which influence the determination of amino acids by reversed-phase high-performance liquid chromatography is described, using phenyl isothiocyanate as a pre-column derivatizing agent. The influence of the pH of the mobile phase and the eluent composition on the gradient chromatography, the effect of temperature, the characteristics of the column packing and the stability of the phenylthiocarbamoyl (PTC) amino acids with time were studied. Optimum separation of 17 amino acids was achieved using a C_{18} column, 25 cm long, with a particle size of 5 m and a 30 nm pore size; working at 40 °C, pH 6.5 and eluting with a mixture of solvents: ammonium acetate buffer, 50 mmol dm^{-3}; and ammonium acetate buffer, 100 mmol dm^{-1} (46% acetonitrile, 44% water and 10% methanol). The precision of the retention times of the PTC amino acids expressed as the percentage relative standard deviation are less than 0.5% for most of the amino acids. The PTC amino acids are relatively unstable at ambient temperature, with the stability varying widely depending on the particular amino acid. The PTC amino acids are stable at 0 °C.

Keywords: *High-performance liquid chromatography; reversed-phase; phenyl isothiocyanate; phenyl(thiocarbamoyl) amino acids*

Currently the most widely used method for the determination of amino acids is high-performance liquid chromatography (HPLC), because of its high resolution and sensitivity, and the relatively short analysis time. However, owing to the necessity to derivatize the amino acids in order to improve the sensitivity, there are many different derivatization procedures available. The most popular include the use of reagents such as phthalaldehyde,[1–3] dansyl [5-(dimethylamino)naphthalene-1-sulphonyl] derivatives,[4–6] phenyl(thiohydantoin) derivatives,[7–9] 9-fluorenylmethyl chloroformate[10] and phenyl(thiocarbamoyl) (PTC) derivatives.[11] The fact that no one reagent has displaced the others leads to one of two conclusions: either that none of them have significant advantages over the others, or that it is necessary to study the procedures in depth in order to optimize them.

Phthalaldehyde gives high sensitivity,[12,13] but it has the disadvantage that it does not react with proline or hydroxyproline and has unstable fluorescent reaction products. Dansyl chloride reacts with proline, but this frequently employed end-group reagent is less reactive than other reagents and, although its use in conjunction with amino acid determination has been described in several papers,[14,15] the method has not gained wide-spread acceptance. Derivatization with 4-(dimethylamino)azobenzene-4′-sulphonyl chloride has been described by Chang and co-workers,[16,17] but like dansylation, arylation with these sulphonyl halides presents difficulties in the form of a quantitative modification, and application of this approach has been greatly limited.

In view of the advantages and disadvantages of the above derivatization procedures, phenyl isothiocyanate (PITC) was selected as the derivatization reagent for the determination of amino acids in grape musts. In order to gain further knowledge of the derivatization and chromatographic determination with C_{18} in the reversed phase, an initial study of the derivatization conditions that do not appear in the literature, and of the parameters for optimizing the chromatographic resolution was performed.

The stability of the amino acids, and the optimization of the pH and the separation gradient at both ambient temperature and 40 °C, were studied.

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Experimental

Apparatus

A Kontron System 400 liquid chromatograph, fitted with two alternating twin-piston pumps (Model 420), a high-pressure mixing chamber (Model M-491), an automatic sample injector (Model 460), a thermostatically controlled column oven (Model 480), and a Kontron ultraviolet–visible (UV/VIS) detector (Model 430) with variable wavelength control was used.

All parameters were controlled by an Acer Plus-700 computer, to which the entire chromatographic system was connected *via* a multiport.

The column used was a Vydac C_{18} (250 mm long × 4.6 mm i.d.), filled with silica spheres with a pore size of 30 nm and particle size of 5 μm. The pre-column used had exactly the same characteristics and was 3 cm long. Also, a C_{18} column 200 mm long × 4.6 mm i.d., pore size 12 nm, and a C_{18} column 250 mm long × 4.6 mm i.d., pore size 50 nm were used for comparative purposes.

Reagents

All solvents and reagents used were of HPLC-reagent quality. Acetonitrile and methanol were supplied by Romil Chemicals. Standard 2.5 mmol dm^{-3} amino acid solutions of aspartic acid (Asp), glutamic acid (Glu), serine (Ser), glycine (Gly), threonine (Thr), alanine (Ala), histidine (His), proline (Pro), arginine (Arg), valine (Val), tyrosine (Tyr), methionine (Met), isoleucine (Ile), leucine (Leu), NH_4^+, phenylalanine (Phe) and lysine (Lys) were supplied by Pierce H, as was the PITC. Trifluoroacetic acid, pyridine and ammonium acetate were supplied by Merck, and the triethylamine by Scharlau. The coupling buffer used for derivatization was made from acetonitrile, pyridine, triethylamine and water, (10 + 5 + 2 + 3). The water used to prepare all solutions was purified using a Milli-Q (Millipore) system. Prior to use, all liquids were filtered through a membrane filter of 0.45 μm average pore size (Millipore).

The mobile phase was prepared daily, and the total flow-rate was 1 ml min^{-1}.

Solvent A. Ammonium acetate buffer, 50 mmol dm^{-3}, pH 6.5. Dissolve 3.854 g of ammonium acetate in approximately

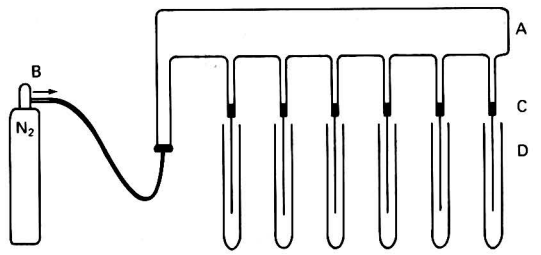

Fig. 1 Diagram of the device used to derivatize amino acids. A, 26 cm glass tube, with 6 outlets of 0.5 cm i.d. and 3 cm long; B, nitrogen inlet; C, Pasteur pipettes; D, test-tubes 12 cm long and 1 cm i.d., containing the amino acids

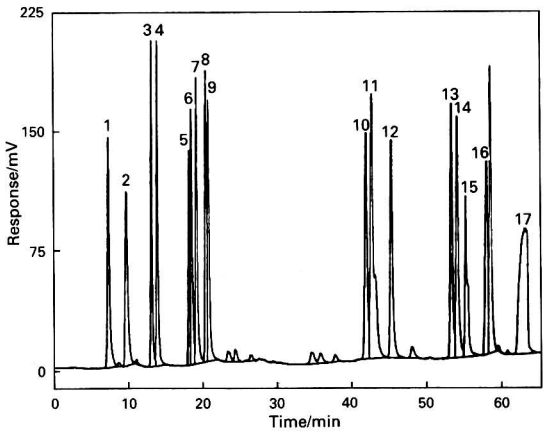

Fig. 2 Chromatograms of PTC amino acids of a standard sample of amino acids at an elution pH of 6.5. The amino acids identified are: 1, Asp; 2, Glu; 3, Ser; 4, Gly; 5, His; 6, Arg; 7, Thr; 8, Ala; 9, Pro; 10, Tyr; 11, Val; 12, Met; 13, Ile; 14, Leu; 15, NH_4^+; 16, Phe; 17, Lys

800 ml of water. Adjust the pH to 6.5 with acetic acid and dilute to 1 l with water.

Solvent B. Ammonium acetate buffer, 100 mmol dm^{-3}, pH 6.5, 46% acetonitrile, 44% water and 10% methanol. Dissolve 7.708 g of ammonium acetate in 300 ml of water and add 460 ml of acetonitrile and 100 ml of methanol. Adjust the pH to 6.5 with acetic acid and add 140 ml of water.

Procedure

Preparation of amino acid standards and derivatization with PITC

Into a 10 ml test-tube, place 20 µl of the standard mixture of amino acids. Dry under a nitrogen-gas stream, and re-suspend the dry residue in 100 µl of a coupling buffer. Add 5 µl of PITC and after leaving for 20 min at an ambient temperature, to complete the reaction, again evaporate off the solution with a stream of N_2. Dissolve the resultant PTC amino acids in 500 µl of solvent A. Centrifuge the solution for 10 min at 12000 rev min^{-1} and inject up to a maximum of 80 µl into the HPLC system at 40 °C, with detection at 254 nm.

Fig. 1 shows the laboratory-built apparatus for derivatizing the amino acids.

Table 1 Description of analytical programmes used in four reversed-phase HPLC systems for the separation of PTC amino acids

		Eluent (%)	
System	Time/min	A	B
I	1	95	5
	31	85	15
	61	50	50
	71	35	65
	76	0	100
II	1	95	5
	26	85	15
	56	50	50
	66	35	65
III	1	100	0
	20	85	15
	31	85	15
	61	50	50
	71	35	65
	76	0	100
IV	1	100	0
	45	88.5	11.5
	85	78.4	21.6
	107.7	50	50
	115	40	60
	120	0	100

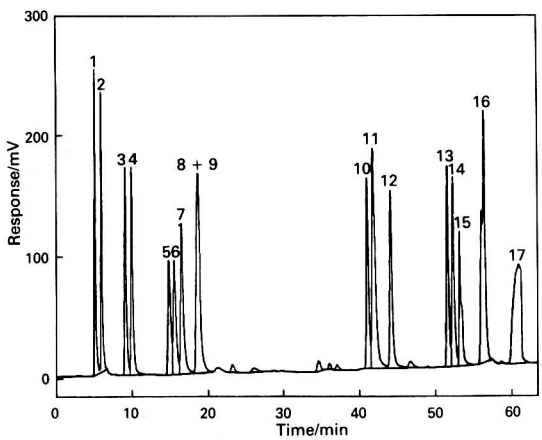

Fig. 3 Separation of PTC amino acids with the gradients shown in Table 1 (System I), at 25 °C. Peak identification as in Fig. 2

Results and Discussion

Optimization of Chromatographic Conditions

Study of the pH of the mobile phase

In order to determine the effect of pH on retention, all amino acids were subsequently determined with eluents A and B buffered at pH 5.0, 5.5, 5.7, 6.0, 6.5 and 6.8. Higher pH values were not tested as they damage the column. The more acidic pH values were found to produce large base line drifts, which tended to stabilize as the pH was increased. At pH 5, the Asp and Glu peaks are not resolved, neither are those of Arg and Thr. Above pH 5.5, these peaks are resolved, but Thr overlaps with Ala up to pH 6.0. At pH 6.0, the peaks of these compounds are perfectly resolved, but Val overlaps with Met; above pH 6.5, all the amino acids in the standard are perfectly resolved. Therefore, a value of 6.5 was adopted for the study in order to prolong the life of the column. Fig. 2 shows the chromatogram obtained at this pH.

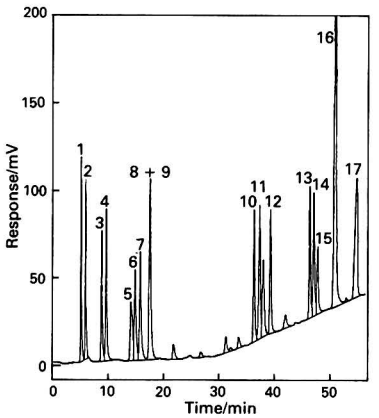

Fig. 4 Separation of PTC amino acids with the gradients shown in Table 1 (System II) at 25 °C. Peak identification as in Fig. 2

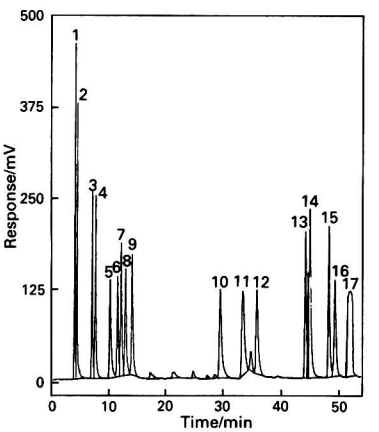

Fig. 5 Separation of PTC amino acids at 40 °C with the gradients shown in Table 1 (System I). Peak identification as in Fig. 2

The resolution of the peaks with the lowest differences in retention time (the group His, Arg, Thr, Ala and Pro and the group Tyr, Val and Met) improves as the pH increases.

Effect of gradient

As shown in Fig. 2, two groups of amino acids appear in the chromatograms, with very similar retention times. In order to increase the resolution of these two groups, the effect of varying the proportion of eluents A and B was examined.

Table 1 shows the programmes used, with systems I and II. The resolution of the amino acids is seen to be incomplete, as the gradient does not separate the peaks corresponding to Ala and Pro. System III separates all the peaks, but they are less defined than in the above systems. System IV separates all the peaks perfectly, but has the disadvantage of excessively long analysis times.

Therefore, before adopting a procedure, a study was performed of the influence of temperature on systems I and II, in order to assess whether the overlapping peaks could be resolved.

Effect of temperature

A study of the effect of the temperature on the separations of amino acids was carried out using the gradients shown in Table 1. Temperatures of up to 60 °C were tested using systems I and II. Temperatures of below 40 and above 45–50 °C were found

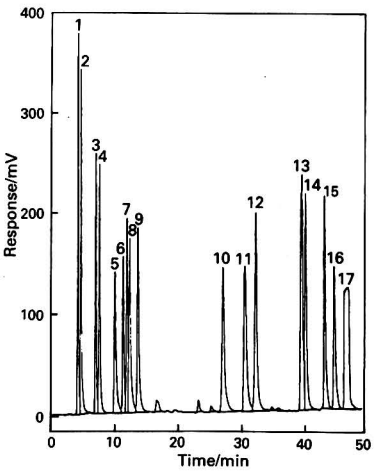

Fig. 6 Separation of PTC amino acids at 40 °C with the gradients shown in Table 1 (System II). Peak identification as in Fig. 2

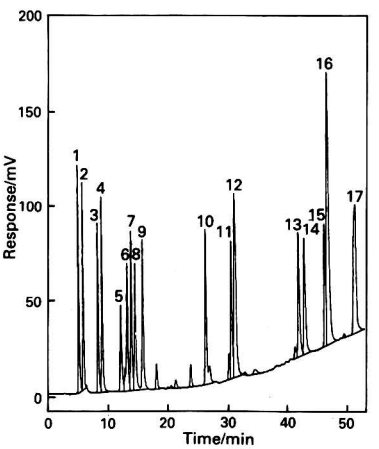

Fig. 7 Separation of PTC amino acids using a temperature programme and with the gradients shown in Table 1 (System II). Peak identification as in Fig. 2

not to modify the chromatograms appreciably. Therefore, as high temperatures deteriorate the column, a temperature of 40 °C was chosen for further work. Figs. 3 and 4 show the chromatograms obtained at ambient temperature; however, it was observed that with those chromatograms obtained at 40 °C (Figs. 5 and 6) all the peaks were perfectly resolved. System I was chosen as the working system, as it gave the best final results.

Chromatography at programmed temperatures has not been reported for HPLC, as good separation can be achieved by varying the gradient. However, as temperature plays a significant role in the separation of PTC amino acids, as has been shown, chromatograms were obtained using system II, varying the temperature of the eluent and column at a rate of 2 °C min^{-1} for the first 10 min, and then at 1 °C min^{-1} up to a temperature of 68 °C. Under these temperature-programmed conditions (Fig. 7), the resolution of Lys improved notably, but there was a slight base line drift after 40 min.

In view of the promising results obtained using temperature-programmed HPLC, further studies are currently under way and will be reported at a later date.

Table 2 Percentage decomposition of the PTC amino acids with time at ambient temperature

	Time/min							
Amino acid	0	140	280	420	560	700	840	980
Asp	100	96.57	93.26	90.37	86.49	83.26	80.65	77.87
Glu	100	98.40	96.18	95.34	91.56	89.22	88.89	85.71
Ser	100	99.33	97.58	97.21	96.21	95.63	94.10	94.09
Gly	100	97.83	99.70	99.80	99.77	100.1	99.90	101.2
Thr	100	98.37	98.04	98.00	95.70	93.19	91.87	91.26
Ala	100	94.35	92.49	92.44	90.30	90.66	89.67	88.73
His	100	96.83	95.38	96.75	93.91	92.93	92.04	90.26
Pro	100	98.87	99.11	99.50	99.02	98.91	98.80	98.46
Arg	100	98.25	97.45	95.16	93.56	91.47	90.55	88.83
Val	100	87.39	86.02	84.56	81.26	78.78	76.45	75.71
Tyr	100	102.8	100.3	98.11	96.47	94.07	90.28	88.25
Met	100	99.03	98.67	99.45	100.2	99.16	100.8	101.7
Ile	100	96.73	95.25	91.72	89.14	87.99	85.10	83.42
Leu	100	97.96	96.60	94.58	93.08	92.22	89.80	89.36
Phe	100	97.27	94.27	91.72	91.05	88.75	87.03	84.14
Lys	100	98.40	96.93	95.21	93.10	91.10	90.59	89.56
NH_4^+	100	91.17	87.18	83.44	81.04	77.42	73.79	71.74

Table 3 Retention times for PTC amino acids based on the results of eight analyses

Amino acid	Retention time*/min	RSD†(%)
Asp	6.2	1.82
Glu	7.9	2.02
Ser	14.9	0.41
Gly	16.9	0.39
His	30.6	0.41
Arg	37.4	0.39
Thr	28.3	0.36
Ala	29.7	0.30
Pro	34.7	0.29
Tyr	71.7	0.41
Val	70.3	0.23
Met	77.3	0.28
Ile	96.9	0.08
Leu	97.8	0.09
Phe	104.7	0.27
Lys	109.5	0.11
NH_4^+	101.9	0.11

* Mean values given.
† RSD, Relative standard deviation.

Influence of the chromatographic packing pore size

The influence of the pore size of the packing particles on the resolution of the chromatogram was studied. It was found that the use of 12 nm columns did not resolve the mixture of Phe, Lys and NH_4^+. However, the amino acids Asp, Glu, Ser, Gly, His, Arg, Thr, Ala and Pro were completely resolved after about 30 min. Using 30 nm columns, the retention time fell, and Lys, Phe and NH_4^+ were resolved. The use of a packing with a pore size of 50 nm also considerably reduced retention times, but no substantial advantages were found with respect to the Vydac-C_{18} column, and, therefore, the Vydac-C_{18} column was used in all subsequent work.

Study of stability

One of the limitations for the determination of amino acids with HPLC is the instability of the derivatized products. According to the literature, PTC amino acids are stable at 5 °C for at least one month,[11] but there is no reference to their stability at ambient temperature. To acquire this information, which is essential for the analysis of long series of samples with automatic injection, a stability study was performed. The same sample was injected eight times, under exactly the same chromatographic conditions and at ambient temperature, for a total of 24 h. The results obtained are shown in Table 2 and give the percentage decomposition over 16 h.

While some amino acids such as Val and Asp decayed rapidly, Ile, Phe, Glu, Arg and Ala did so more slowly. Tyrosine increased rapidly and then fell off linearly, whereas His suffered a rapid drop, then an increase and finally decayed. Leucine, Thr and Ser decayed slowly and stopped decaying after 14 h. Proline was relatively stable at ambient temperature, as there was hardly any variation. Glycine and Met suffered an initial drop in area and later increased. The conclusion was that if many amino acids are analysed together, the automatic injector cannot be programmed for long periods of time.

Precision of the retention time values

The precision of the retention times for the amino acids studied was determined by injecting the same sample and eluent eight times under optimum conditions. The results are shown in Table 3. The precision for the absolute retention time is high and, therefore, enables qualitative determinations.

Conclusions

The derivatization of amino acids with PITC leads to products which can be easily resolved using reversed-phase HPLC with a C_{18} column.

The high precision obtained for the retention times facilitates the identification of amino acids. The PTC amino acids are not stable for long at ambient temperature, so they must be analysed immediately after derivatization, or stored at temperatures below −5 °C.

Maintaining the temperature at 40 °C during the development of the chromatogram helps to resolve the peaks, and shortens the time required for analysis.

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Quality Assurance of Petrol by High-performance Size-exclusion Chromatography*

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A simple and rapid size-exclusion chromatographic method for the determination of either kerosine or diesel adulteration of petrol has been developed, based on the differences found in the average relative molecular masses of hydrocarbons present in petrol, kerosine and diesel. It is effective for detecting either kerosine or diesel adulteration of petrol down to a level of 1%. The method is ideal for serving as an 'in-field method' of analysis for monitoring the quality of petrol by using a portable gel-permeation chromatograph at delivery stations.

Keywords: *Size-exclusion chromatography; petrol; adulteration*

Petrol, also known as gasoline, is an important product of petroleum and is used as a fuel for transportation. It is often adulterated with cheaper petroleum fractions, such as kerosine and high-speed diesel oil, for monetary gains. It is known[1] that the consumption of adulterated petrol results not only in a decrease in performance, but also in serious consequences for petrol engines. These effects on engines are often recognized late, only after causing considerable damage to the vehicles. Hence, early detection and determination of these adulterants in petrol is very important for ensuring its quality and performance.

Studies on petrol adulteration were first reported by Babitz and Rocker,[2] who used semi-micro chromatography. Several specifications,[3,4] based on physico-chemical properties, such as boiling range, octane rating, oxidation stability and residue on evaporation, have been laid down for ensuring petrol quality. For example, the American Society for Testing and Materials (ASTM) method[3] is based on the distillation characteristics of petrol and is generally used for checking its quality. However, the boiling range of petrol is rather wide, as a result of which significant changes in its chemical composition are possible. Hence, it is difficult to know precisely the level of adulteration in petrol by using distillation characteristics alone. It would be much more difficult when small amounts of products with overlapping characteristics are mixed with petrol. Several methods[5–9] based on ultraviolet (UV) spectrophotometric, gas–liquid chromatographic and paper chromatographic techniques have been reported for the quality assurance of petrol. Recently, Bahari *et al.*[10] reported a phase-titration procedure for the determination of the adulteration of petrol with kerosine. However, all these methods are either time consuming, cumbersome or less informative for drawing definite conclusions. They are also not well suited to serve as 'in-field' methods of analysis.

Relative molecular mass distribution is a key property in understanding the compositional nature of hydrocarbons and also useful in studying their adulteration. High-performance size-exclusion chromatography (HPSEC) is a valuable technique in providing information about sample complexity, and accurate relative molecular masses and their distribution pattern. In an SEC column the analytes are eluted in accordance with their molecular sizes, *i.e.*, their hydrodynamic volumes in the mobile phase. Although this technique was originally developed for characterizing macromolecules, it has also been applied successfully to the study of low relative molecular mass mixtures by using more efficient column packings and making innovations in instrumentation.[11–13] Additionally, HPSEC has been successfully used for studying adulteration of oils and fats.[14–16] The present investigation describes the application of this technique to the detection and determination of the adulteration of petrol with kerosine and high-speed diesel oil.

* IICT Communication No. 2529.

Experimental

Materials

Tetrahydrofuran (THF), HPLC grade, was obtained from Spectrochem (India) and used as the mobile phase. Benzene and polyethylene glycols (150, 200, 400 and 800) were obtained from SD Fine Chemicals (India) and used as reference standards. Premium-grade petrol, kerosine and diesel were obtained from the Indian Oil Corporation and used as supplied.

Apparatus

High-performance SEC was carried out on an LC-6A high-performance liquid chromatograph (Shimadzu, Kyoto, Japan), equipped with a loop injector (capacity 12 μl) having a high-pressure six-way valve. A refractive index (RI) detector, Model RID-6A, was connected to the column for monitoring the eluate. The work was carried out on a high-speed gel-permeation chromatography (GPC) column (HSG-40H, Shimadzu), 300 × 7.9 mm i.d.; the number of theoretical plates was between and 2×10^4 and 3×10^4 m^{-1}. The column was packed with spherical beads (10 μm) of styrene–divinylbenzene copolymer. The chromatograms and the integrated data were recorded by a Chromatopac C-R3A processing system.

Procedure

Petrol–kerosine and petrol–diesel mixtures were prepared in the range 0–50% v/v kerosine and diesel (5% v/v increments), respectively. Each sample was dissolved in THF and 12 μl of the solution were injected onto the column. The chromatograms were obtained under the following conditions: sample concentration, 10 μl cm^{-3}, volume of sample injected, 12 μl; flow-rate of the mobile phase, 0.3 cm^3 min^{-1}; and detector range, 16×10^{-6} RI units.

Results and Discussion

The size-exclusion chromatograms for petrol, kerosine and diesel are shown in Fig. 1. These chromatograms, which represent the distribution of relative molecular masses for various hydrocarbons present in these products, appear to be symmetrical Gaussian peaks at their respective retention times. The retention times and average relative molecular masses of different types, *viz.*, the mass-average ($\bar{M}_m$),

number-average ($\bar{M}_n$), viscosity-average ($\bar{M}_v$) and Z-average ($\bar{M}_z$) relative molecular masses for typical samples of petrol, kerosine and diesel, are given in Table 1. The retention data were previously calibrated with benzene and polyethylene glycols (150, 200, 400 and 800) of known relative molecular masses as reference standards. The data for the calibration graph (Fig. 2) were stored on a floppy disk, FDD-1A (Shimadzu), coupled to a Chromatopac C-R3A processor for determination of the relative molecular masses of the analytes. The data regarding various relative molecular masses, *viz.*, $\bar{M}_m$, $\bar{M}_z$, $\bar{M}_n$ and $\bar{M}_v$, given in Table 1, were obtained from the retention data by using the GPC program diskette No. P/N 223-01309-91 supplied by Shimadzu. The reproducibility of the results was tested by injection of triplicate samples of standards and analytes. The relative standard deviation for relative molecular masses was found to be less than 2.5%.

The retention times for different batches of petrol, kerosine and diesel samples collected from different locations were determined and analysed by statistical methods. According to the analysis of variance there was no significant difference with respect to retention times between different batches of samples under investigation (Table 2).

It is apparent from the results given in Table 1 that the relative molecular masses of petrol, kerosine and diesel differ considerably, and this provides the basis of a precise analytical method for assessing adulteration. It is also clear that the difference between $\bar{M}_z$ and $\bar{M}_n$ values is about 120 units for petrol. For kerosine and diesel, it is about 80 and 40 units, respectively. These differences are due to the different concentrations of linear and branched-chain hydrocarbons present in these products.

Generally, two types of processes[17,18] are operative in SEC. These are separation and dispersion. The separation process controls the differential migration of solute molecules and includes any secondary retention mechanisms, additional to size exclusion, in a stationary phase. It is, therefore, responsible for the retention volume (V_R), whereas the dispersion process determines the band broadening, influenced by molecular diffusion through a column packing, and is responsible for the band width (w). Therefore, the resolution of molecules in a complex mixture depends not only on the differences in solute sizes, but also on the pore size distribution, gel capacity, eluent flow-rate, column length and porosity characteristics of the packed material. By selecting an appropriate micro-particulate column it is possible to separate petrol, kerosine and diesel satisfactorily. Typical HPSEC traces of binary mixtures of petrol–kerosine and petrol–diesel are shown in Figs. 3 and 4, respectively. Two well resolved peaks having asymmetrical relative molecular mass distributions for their respective hydrocarbons are seen in all the synthetic mixtures. It can also be seen from Figs. 3 and 4 that the peak corresponding to petrol is well separated from those of kerosine and diesel. It is further observed from Fig. 3(*a*), (*b*) and (*c*) that the kerosine component of petrol–kerosine mixtures is completely resolved. However, in Fig. 3(*d*) and (*e*) it is only partially resolved. This is perhaps due to the effect of concentration on molecular diffusion. As the concentration of kerosine in the mixtures increases progressively, it is noted that the corresponding peak is broadened [Fig. 3(*d*) and (*e*)] owing to molecular diffusion, thereby affecting the resolution. The same is valid for other mixtures containing petrol and diesel. Table 3 gives the retention data, average relative molecular masses, polydispersity indices ($\bar{M}_m : \bar{M}_n$, $\bar{M}_z : \bar{M}_n$ and $\bar{M}_v : \bar{M}_n$) and intrinsic viscosities of mixtures of petrol–kerosine and petrol–diesel. The ratios $\bar{M}_m : \bar{M}_n$, $\bar{M}_z : \bar{M}_n$ and $\bar{M}_v : \bar{M}_n$ for petrol are 1.7216, 2.7932 and 1.5603, respectively. These values are found to decrease gradually when the petrol is adulterated with small amounts of kerosine and diesel. Fig. 5 shows the linear relationship between these ratios and the amount of adulterant in petrol. As the concentration of the adulterant increases the polydispersity indices decrease corre-

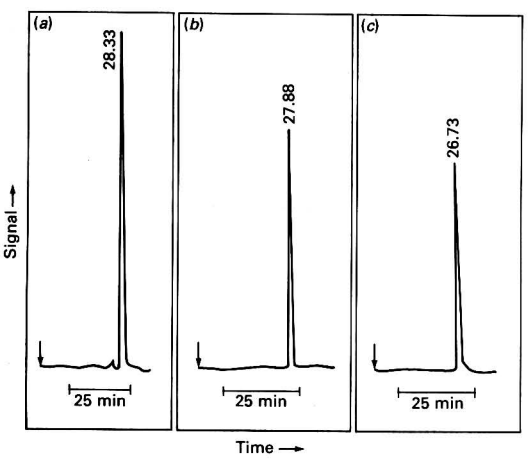

Fig. 1 Size exclusion chromatograms of: (*a*) petrol; (*b*) kerosine; and (*c*) diesel

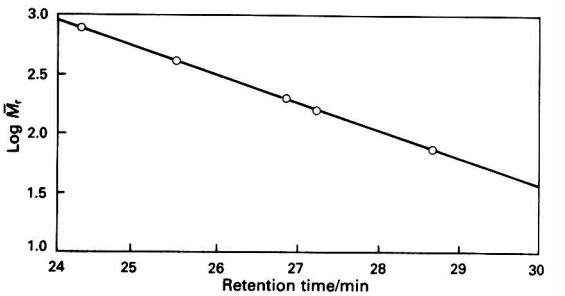

Fig. 2 Calibration graph using polyethylene glycols as standards

Table 2 Statistics of HPSEC retention data for different samples of petrol, kerosine and diesel

Characteristic	Petrol	Kerosine	Diesel
Number studied	49	36	36
Boiling range/°C	39–204	170–325	190–380
Retention time/min*	28.33 ± 0.66	27.87 ± 0.51	26.73 ± 0.54
Standard error (%)	0.13	0.10	0.11
F-test	1.53	1.20	0.45
Significance, p (%)	0.01	0.01	0.01

* Values are means ± standard deviation.

Table 1 Size-exclusion chromatographic data for petrol, kerosine and diesel

Sample	Retention time/min	Relative molecular mass				Polydispersity indices			Intrinsic viscosity
		$\bar{M}_n$	$\bar{M}_m$	$\bar{M}_z$	$\bar{M}_v$	$\bar{M}_m : \bar{M}_n$	$\bar{M}_z : \bar{M}_n$	$\bar{M}_v : \bar{M}_n$	
Petrol	28.33	69	118	194	107	1.7216	2.7932	1.5603	0.0043
Kerosine	27.87	105	140	183	135	1.3330	1.7364	1.2810	0.0051
Diesel	26.73	148	210	288	200	1.4204	1.2403	1.3511	0.0067

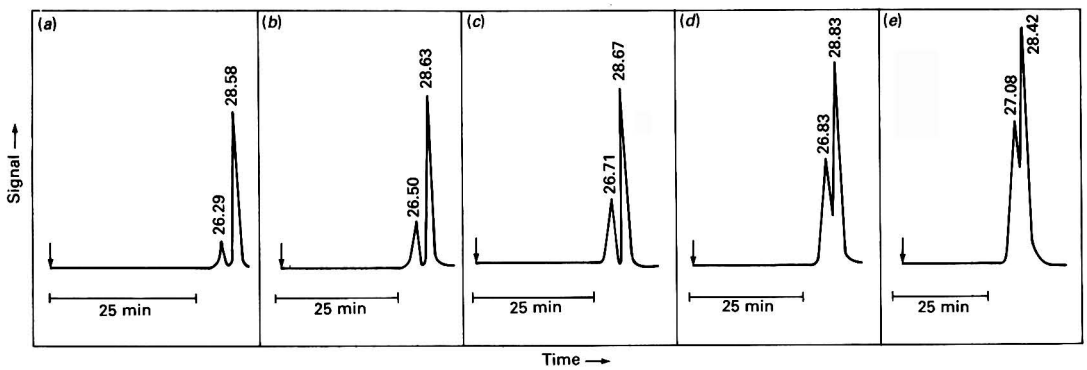

Fig. 3 Chromatograms and relative molecular mass distributions of various hydrocarbons present in binary mixtures of petrol (P) and kerosine (K). (*a*) P–K (9 + 1); (*b*) P–K (8 + 2); (*c*) P–K (7 + 3); (*d*) P–K (6 + 4); and (*e*) P–K (5 + 5)

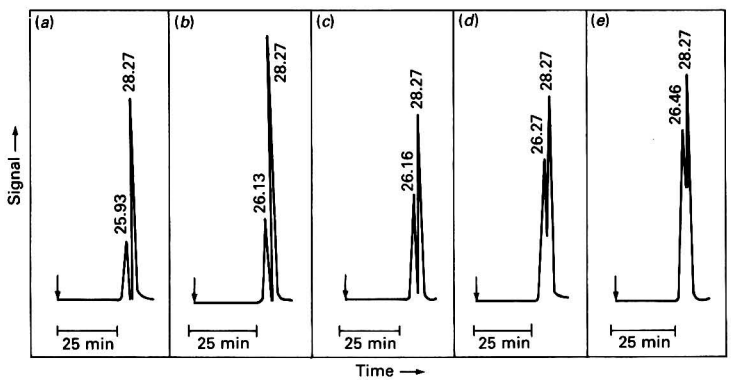

Fig. 4 Chromatograms and relative molecular mass distributions of various hydrocarbons present in binary mixtures of petrol (P) and diesel (D). (*a*) P–D (9 + 1); (*b*) P–D (8 + 2); (*c*) P–D (7 + 3); (*d*) P–D (6 + 4); and (*e*) P–D (5 + 5)

Table 3 Average relative molecular masses and polydispersity indices of binary mixtures of petrol (P) with kerosine (K) and diesel (D)

Binary mixture	Composition (v/v)	Retention time/min		Relative molecular masses				Polydispersity indices			Intrinsic viscosity
	P–K/D	P	K/D	$\bar{M}_n$	$\bar{M}_m$	$\bar{M}_z$	$\bar{M}_v$	$\bar{M}_m:\bar{M}_n$	$\bar{M}_m:\bar{M}_n$	$\bar{M}_m:\bar{M}_n$	
P–K	9.0 + 1.0	28.53	26.29	82.53	133.36	218.11	123.21	1.6158	2.6426	1.4929	0.00479
	8.0 + 2.0	28.62	26.50	84.07	133.05	212.54	123.43	1.5824	2.5279	1.4681	0.00479
	7.0 + 3.0	28.33	26.70	86.98	129.31	195.72	121.15	1.4866	2.2501	1.3927	0.00473
	6.0 + 4.0	28.83	26.83	86.84	122.39	173.06	115.94	1.4459	2.0221	1.3714	0.00459
	5.0 + 5.0	28.41	27.09	101.44	146.68	205.14	139.12	1.4093	1.9927	1.3350	0.00522
P–D	9.0 + 1.0	28.27	25.93	99.72	169.51	274.67	156.17	1.6999	2.7543	1.5660	0.00566
	8.0 + 2.0	28.27	26.13	108.57	180.57	276.77	167.69	1.6648	2.5517	1.5460	0.00595
	7.0 + 3.0	28.27	26.16	117.31	190.51	285.20	177.64	1.6239	2.4311	1.5142	0.00620
	6.0 + 4.0	28.27	26.26	125.19	194.71	282.73	182.80	1.5552	2.2583	1.4601	0.00633
	5.0 + 5.0	28.27	26.46	123.57	188.54	270.71	177.50	1.5258	2.1907	1.4364	0.00620

Table 4 Comparison of the proposed method with the UV method for determination of adulteration of petrol

Adulterant added	Amount added (%)	Amount found* (%)		Relative standard deviation (%)	
		SEC	UV	SEC	UV
Kerosine	5.0	5.19 ± 0.08	4.83 ± 0.12	1.63	2.54
	10.0	9.87 ± 0.10	9.59 ± 0.30	1.05	3.17
	15.0	15.32 ± 0.37	14.71 ± 0.43	2.48	2.95
Diesel	5.0	5.25 ± 0.05	4.77 ± 0.07	0.86	1.43
	10.0	10.09 ± 0.20	10.35 ± 0.21	2.16	1.98
	15.0	14.76 ± 0.18	14.58 ± 0.54	1.25	3.67

* Average of six determinations ± standard deviation.

spondingly. These lines, as shown in Fig. 5, are used for determining the amount of adulteration of unknown samples of petrol containing kerosine and diesel.

The precision of the chromatographic results, as shown by the low standard deviations, was found to be excellent from the results given in Table 4 for adulterated samples of petrol, and the results were found to be in good agreement with those obtained by the UV method. It is, therefore, concluded that the HPSEC method is precise and rapid, not only for the qualitative detection, but also for the quantitative determination of the adulteration of petrol with either kerosine or diesel down to a level of 1%. It appears to be suitable as an 'in-field method' for monitoring the quality of petrol by using a portable GPC system at delivery stations.

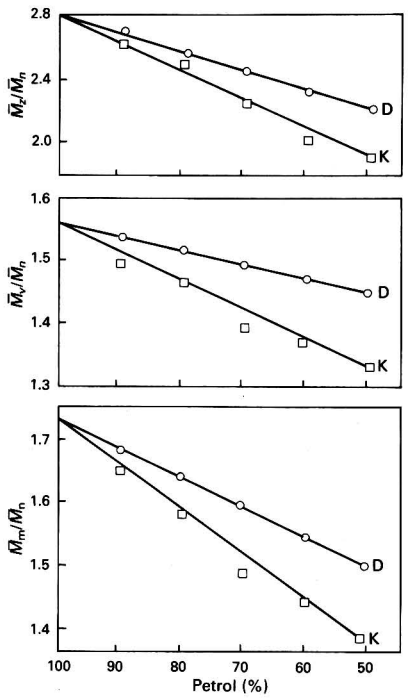

Fig. 5 Calibration graphs for calculating the amount of adulteration of petrol by: K, kerosine; and D, diesel

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Ethylenediamine–Hydrochloric Acid–Zinc(II) Eluent for the Suppressed Ion Chromatographic Separation of Strontium(II) From a Large Amount of Calcium(II): Application of the Method to the Simultaneous Determination of Magnesium(II), Calcium(II) and Strontium(II) in High Salinity Sub-surface Waters

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A new eluent has been developed for the suppressed ion chromatographic separation of strontium from large amounts of calcium. Together with a Dionex CS-2 separator column, the eluent, a mixture of 2.5 mmol dm^{-3} ethylenediamine, 5 mmol dm^{-3} hydrochloric acid and 1 mmol dm^{-3} Zn^{2+}, was successfully used for the simultaneous determination of strontium, magnesium and calcium in high salinity sub-surface waters. The results of the determination of magnesium, calcium and strontium in sub-surface Arab-D brine and its mixtures with aquifer Wasia and sea-water compare well ($p > 0.1$) with those obtained by a standard atomic absorption spectrometric method. A base line separation of magnesium, calcium and strontium from each other and from a large excess of sodium in a sub-surface brine can be achieved in approximately 8.0 min. This is a considerably shorter time than that required to achieve a reasonably good separation factor between calcium and strontium by other liquid chromatographic methods, although such methods have not been used for the determination of alkaline earth metal cations in sub-surface waters.

Keywords: *Ethylenediamine–hydrochloric acid–zinc(II) eluent; suppressed ion chromatography; alkaline earths; high salinity sub-surface waters*

Separation of alkaline earth metal ions by suppressed ion chromatography (IC) has been described in the literature.[1–3] In isocratic suppressed IC,[1] magnesium, calcium, strontium and barium can be separated from alkali metals and from each other on a Dionex CS-1 column with a mixture of 0.002 mol dm^{-3} *m*-phenylenediamine dihydrochloride and 0.002 mol dm^{-3} HCl as the eluent. Zinc(II)–nitric acid as an eluent[3] can also be used for the above separation. However, by using any of the eluents mentioned above and a Dionex CS-1 column, a base line separation of strontium from calcium cannot be achieved in a high salinity brine such as Arab-D brine or mixtures of high salinity brines with sea-water or aquifer waters, where strontium to calcium molar ratios can be as high as 1 : 100. Because IC methods for the determination of cations in sub-surface waters have not been developed, the aim of this investigation is to develop the suppressed IC conditions for the simultaneous determination of magnesium, calcium and strontium ions in high salinity sub-surface waters. The determination of these cations, in the waters mentioned above, is important in the petroleum industry.

The separation of strontium from calcium in sub-surface waters is not possible using the existing suppressed IC conditions described above. A new eluent giving a large separation factor between calcium and strontium ions was therefore sought for this purpose. In single column IC, Sevenich and Fritz[4] obtained a large separation factor between calcium and strontium on a low cation-exchange capacity column with a mixture of 0.0015 mol dm^{-3} ethylenediamine and 0.002 mol dm^{-3} tartrate as eluent. Jenke[5] has reported that ethylenediamine can be used as an eluent for suppressed IC, and by using 1.0 mmol dm^{-3} ethylenediamine solution (pH = 6) as eluent, magnesium and calcium were separated from a large excess of alkali metals on a Wescan Model 269-024 high-speed cation-exchange column. These investigations prompted the use of ethylenediamine in the present study, as the eluent for the separation and determination of alkaline earth cations on Dionex cation-exchange columns by suppressed IC.

Experimental

Reagents

Doubly distilled, de-ionized water (Nanopure II) was used to prepare the solutions. Certified atomic standards for sodium, magnesium, calcium, strontium and barium at 1000 ± 10 mg dm^{-3} were obtained from Fisher Scientific (Pittsburgh, PA, USA). Ethylenediamine (Merck-Schuchardt, Schuchardt, Germany), analytical-reagent grade hydrochloric acid (BDH, Poole, Dorset, UK) and zinc acetate dihydrate (Riedel-de Häen, Hannover, Germany) were used without further purification.

Instrumentation

Ion chromatographic analyses were carried out with a Dionex 2120i ion chromatograph at a constant temperature of 22 ± 1 °C, under the conditions summarized in Table 1. Different combinations of ethylenediamine (en), hydrochloric acid and zinc acetate were tried as eluents in an effort to achieve a base line resolution of strontium in the presence of a large amount of calcium using Dionex CS-1 and CS-2 separator columns. A Varian SpectrAA-40 atomic absorption spectrometer was also used.

Determination of Sodium, Magnesium, Calcium and Strontium by Atomic Absorption Spectrometry

Sodium was determined at 330.3 nm by using an air–acetylene flame. Potassium nitrate was added to all solutions including the blank, in order to give a final concentration of 2000 mg dm^{-3} of potassium. The instrument was calibrated with sodium standards at 100, 250 and 400 mg dm^{-3} to quantify sodium in 100-fold diluted water samples. For the determination of magnesium, calcium and strontium, a mixed standard solution containing magnesium at 2, 5 and 10 mg dm^{-3}, calcium at 10, 20 and 30 mg dm^{-3}, and strontium at 0.5, 1 and 1.5 mg dm^{-3} was used. Chemical interferences in the

Table 1 Ion chromatography conditions

Analytical column	Dionex CS-1 and CS-2
Guard column	Dionex CG-1 and CG-2
Suppressor column	Dionex Cation Micro Membrane Suppressor
Regenerent	40 mmol dm^{-3} $Ba(OH)_2$ or 100 mmol dm^{-3} tetramethylammonium hydroxide
Eluent flow-rate	1.7–2.3 ml min^{-1}
Injection volume	50 µl
Detector sensitivity	3–1000 µS full scale
Detector	Conductivity, Dionex
Peak integrator	Dionex Model 4217

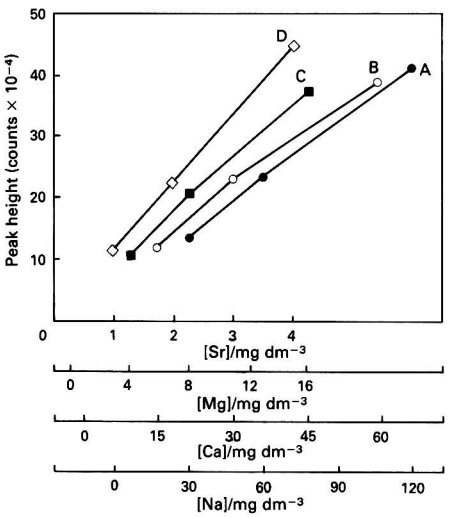

Fig. 1 Representative calibration graphs for: A, sodium B, calcium; C, magnesium; and D, strontium cations

air–acetylene flame were eliminated by the introduction of lanthanum (10000 µg ml^{-1}) in all solutions, *i.e.*, standards, samples and blank. Calcium was determined at 422.7 nm. In order to obtain a straight line calibration at fairly high calcium concentrations, the position of the burner was changed slightly from the optimum signal position. Magnesium and strontium were determined at 202.6 and 460.7 nm, respectively. The water samples were diluted 200–250-fold.

Simultaneous Determination of Sodium, Magnesium, Calcium and Strontium by IC

All the analyses were carried out on a Dionex CS-2 column with 0.0025 mol dm^{-3} ethylenediamine–0.005 mol dm^{-3} HCl–0.001 mol dm^{-3} Zn^{2+} as the eluent. For the determination of sodium, magnesium, calcium and strontium ions in sub-surface Arab-D brine by IC, the samples were diluted 250–500 times with distilled water. The ion chromatograph was calibrated with a mixed standard solution containing sodium at 30, 60 and 120 mg dm^{-3}, magnesium at 4, 8 and 16 mg dm^{-3}, calcium at 15, 30 and 60 mg dm^{-3} and strontium at 1, 2 and 4 mg dm^{-3}. The concentration *versus* peak height graphs showed non-linear relationships for all the cations in the concentration ranges mentioned above, except for strontium, which showed a linear graph (Fig. 1). Therefore, a non-linear regression routine was used to determine the concentration of the cations in water brines. The calibration was checked after every five samples by injecting a standard

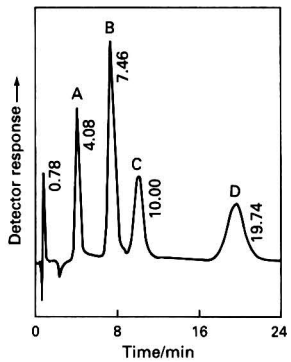

Fig. 2 Ion chromatograph showing the separation of A, Mg^{2+} (2.5 mg dm^{-3}); B, Ca^{2+} (10 mg dm^{-3}); C, Sr^{2+} (10 mg dm^{-3}); and D, Ba^{2+} (20 mg dm^{-3}) on Dionex CS-1 + CG-1 columns. Eluent = 0.001 mol dm^{-3} ethylenediamine + 0.002 mol dm^{-3} HCl; detector sensitivity = 3 µS full scale; and eluent flow-rate = 2.3 ml min^{-1}

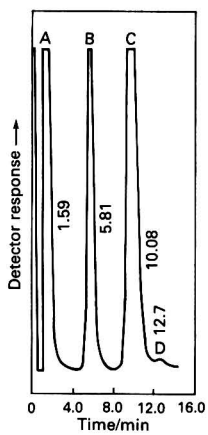

Fig. 3 Ion chromatograph showing the separation of Sr^{2+} from Na^+, Mg^{2+} and Ca^{2+} in a mixture of 60% sea-water, 30% Arab-D and 10% Wasia containing: A, 21830 mg dm^{-3} Na^+; B, 1850 mg dm^{-3} Mg^{2+}; C, 5360 mg dm^{-3} Ca^{2+}; and D, 260 mg dm^{-3} Sr^{2+}. Column = Dionex 2CG-1 + CS-1; eluent as in Fig. 2; detector sensitivity = 30 µS full scale; eluent flow-rate = 2.3 ml min^{-1}; and sample dilution = 100-fold

solution. The ion chromatograph was re-calibrated if the variation in the concentration of any of the determinands in the standard was greater than 2%.

Results

Separation of Sodium, Magnesium, Calcium and Strontium on a Dionex CS-1 Column

Fig. 2 shows the separation of Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+} ions on a Dionex CS-1 cation separator and CG-1 guard column with 0.001 mol dm^{-3} ethylenediamine + 0.002 mol dm^{-3} HCl (pH = 6) as the eluent. As shown, a base line resolution for all the alkaline earth cations is achieved and the peaks are fairly sharp and symmetrical. However, despite a good resolution between Ca^{2+} and Sr^{2+} of 1.5 (better than the 1.4 obtained with 0.002 mol dm^{-3} *m*-phenylenediamine dihydrochloride and 0.002 mol dm^{-3} HCl[1]), a base line separation of Ca^{2+} and Sr^{2+} in a mixture of Arab-D and sea-water could not be achieved with the ethylenediamine eluent with the desired sensitivity. A change in the eluent flow-rate and a small increase in the column bed did not improve the situation, as shown in Fig. 3, where a base line resolution of the strontium peak was achieved only at very low sensitivity.

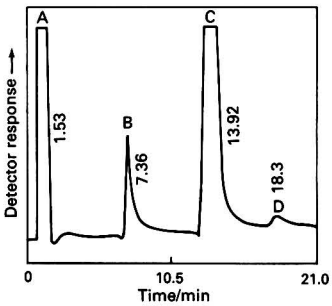

Fig. 4 Ion chromatograph showing the separation of Na^+, Mg^{2+}, Ca^{2+} and Sr^{2+} in a synthetic brine, containing: A, 50000 mg dm^{-3} Na^+; B, 1000 mg dm^{-3} Mg^{2+}; 16000 mg dm^{-3} Ca^{2+}; and D, 1000 mg dm^{-3} Sr^{2+}. Column = Dionex CS-2 + CG-2; eluent as in Fig. 2; sample dilution = 100-fold; detector sensitivity = 10 μS full scale; and eluent flow-rate = 2.0 ml min^{-1}

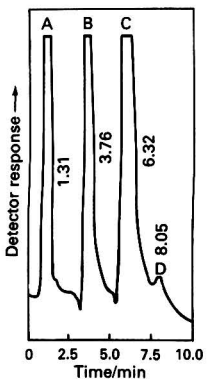

Fig. 5 Ion chromatograph showing the separation of Sr^{2+} from a mixture of 80% sea-water and 20% Arab-D, containing: A, 20500 mg dm^{-3} Na^+; B, 2000 mg dm^{-3} Mg^{2+}; C, 3650 mg dm^{-3} Ca^{2+}; and D, 160 mg dm^{-3} Sr^{2+}. Sample dilution = 100-fold; column = as in Fig. 4; eluent = 0.0025 mol dm^{-3} ethylenediamine + 0.005 mol dm^{-3} HCl; detector sensitivity = 3 μS full scale; and eluent flow-rate = 2.0 ml min^{-1}

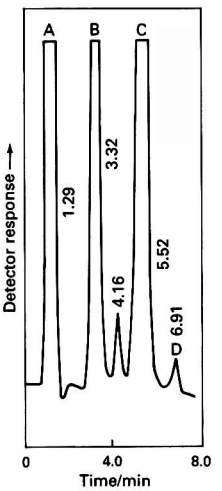

Fig. 6 Ion chromatograph showing the separation of Sr^{2+}. Sample and chromatographic conditions as in Fig. 5 except for the eluent (eluent = 0.0025 mol dm^{-3} ethylenediamine + 0.005 mol dm^{-3} HCl + 0.001 mol dm^{-3} zinc acetate dihydrate). The peak with a retention time of 4.16 min is produced by an unidentified compound

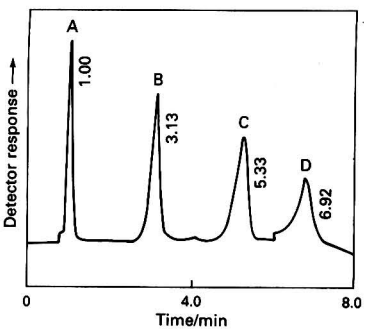

Fig. 7 Ion chromatograph showing the separation of Na^+, Mg^{2+}, Ca^{2+} and Sr^{2+}. Detector sensitivity (full scale) = 1000 μS for Na^+, 100 μS for Mg^{2+} and Ca^{2+}, and 3 μS for Sr^{2+}; sample and IC conditions as in Fig. 6

Separation of Sodium, Magnesium, Calcium and Strontium on a Dionex CS-2 Column

From the above it is clear that a base line separation of strontium from calcium in pure Arab-D sub-surface brine or its mixture with sea-water, at the desired sensitivity, cannot be achieved on a CS-1 column. Therefore, a large cation-exchange capacity column, Dionex CS-2, was tried for this purpose. Fig. 4 shows the separation of sodium, magnesium, calcium and strontium in a synthetic Arab-D water [containing Na^+ (50000), Mg^{2+} (1000), Ca^{2+} (16000) and Sr^{2+} (1000 mg dm^{-3})] on a Dionex CS-2 separator and CG-2 guard column with a 0.001 mol dm^{-3} ethylenediamine +0.002 mol dm^{-3} HCl eluent. Although a large separation factor between calcium and strontium can be seen in Fig. 4, the peaks are asymmetrical and the total analysis time of about 20 min is too long for a routine analysis.

A strong eluent containing 2.5 mmol dm^{-3} ethylenediamine + 5.0 mmol dm^{-3} HCl shortened the analysis time to about 10 min. However, even with this eluent, a base line resolution of strontium at high detector sensitivity (required for the determination of low strontium concentrations) could not be achieved (Fig. 5) owing to tailing of the calcium peak. This problem was overcome by adding Zn^{2+} ions to the eluent as a peak modifier. As can be seen in Fig. 6, the addition of 1 mmol dm^{-3} zinc acetate dihydrate to 2.5 mmol dm^{-3} ethylenediamine + 5.0 mmol dm^{-3} HCl proved to be successful in sharpening the calcium peak and the base line resolution of strontium. Although it is difficult to explain the exact role of Zn^{2+} ions in sharpening the calcium and strontium peaks, it seems that the presence of Zn^{2+} ions in the eluent modifies the exchange sites for calcium ion exchange on the column, as is discussed later.

Application of Ethylenediamine–HCl–Zn^{2+} Eluent to the Determination of Sodium, Magnesium, Calcium and Strontium in Sub-surface Arab-D Waters

As the main purpose of developing the eluent was to determine strontium and other alkaline earth cations in sub-surface brines, the IC conditions described above were used for the determination of magnesium, calcium and strontium in high salinity Arab-D water. The simultaneous determination of these ions together with sodium in sub-surface Arab-D brine was possible by changing the detector sensitivity. Fig. 7 shows the separation of these ions in a mixture of Arab-D brine with sea-water containing 20500 mg dm^{-3} Na^+, 160 mg dm^{-3} Sr^{2+}, 3600 mg dm^{-3} Ca^{2+} and 2000 mg dm^{-3} Mg^{2+}. Although all the monovalent cations will

Table 2 Concentration of Na^+, Mg^{2+}, Ca^{2+} and Sr^{2+} ions in Arab-D and its mixtures with Wasia and sea-water

Sample No.	Sodium/mg dm^{-3}		Bias*	Magnesium/mg dm^{-3}		Bias*	Calcium/mg dm^{-3}		Bias*	Strontium/mg dm^{-3}		Bias*
	AAS(X)	IC(Y)	(%)	AAS(X)	IC(Y)	(%)	AAS(X)	IC(Y)	(%)	AAS(X)	IC(Y)	(%)
1a	54220	52841	2.54	2179	2160	0.87	19040	17807	6.48	975	955	2.05
2	32880	34642	−5.36	1750	1763	−0.74	12398	12790	−3.16	655	684	−4.42
3	28380	28731	−1.24	1840	1797	2.33	9468	9678	−2.22	490	507	−3.47
4	26720	27217	−1.86	1912	1835	4.03	8208	8424	−2.63	418	439	−5.02
5	27090	27093	−0.01	1600	1708	−6.75	13480	13786	−2.27	545	551	−1.10
6	24910	25984	−4.31	1750	1737	0.74	8260	8025	2.84	418	428	−2.39
7	23440	24150	−3.03	1758	1708	2.84	6898	6792	1.54	346	358	−3.47
8	22680	22169	2.25	2048	1968	3.91	5336	5175	3.02	266	255	4.14
9	21840	21782	0.27	2040	1970	3.43	5334	5363	−0.54	266	251	5.64
10	18360	18664	−1.66	850	840	1.18	7916	7740	2.22	410	422	2.93
	Mean:		−1.24			1.18			0.53			0.51
	Standard deviation:		2.59			3.19			3.18			3.87
	Student's t value:		1.02			1.19			0.53			0.62

Student's t value at 90% confidence interval = 1.38

* Bias = $[(X - Y)/X] \times 100$.

co-elute with sodium, the determination of sodium was possible because the total concentration of K^+, NH_4^+ and Li^+ in Arab-D brine, Wasia (aquifer) and sea-water was less than 5% of the total concentration of monovalent cations.

The accuracy of the IC method for the determination of Na^+, Mg^{2+}, Ca^{2+} and Sr^{2+} in mixtures of Arab-D, Wasia and sea-water by the present method was assessed by comparing the IC results with those obtained by atomic absorption spectrometry (AAS). These results are reported in Table 2. The application of the null hypothesis (paired t-test)[6] confirms that the bias between the two techniques does not differ significantly from zero. Therefore, the IC conditions reported in the present work can be used for the accurate determination of sodium, magnesium, calcium and strontium ions in pure Arab-D brine or its mixture with other waters.

On an intra-run basis (n = 5), the maximum relative standard deviations were 1.7% for sodium, 1.3% for magnesium, 1.4% for calcium and 3.2% for strontium, showing good precision for this method.

Discussion

At neutral pH, the eluent (1 mol of ethylenediamine containing 2 mol of hydrochloric acid, pH = 6) is mainly in the form of $H_3NHC_2CH_2NH_3Cl_2$ (enH_2Cl_2) containing ethylenediammonium cations $(enH_2)^{2+}$, as the en-H^+ association constants K_1 and K_2 are reported to be 9.89 and 7.1 dm^3 mol^{-1}, respectively.[7] The high affinity of the $(enH_2)^{2+}$ for the column resin causes the elution and separation of alkaline earth metal cations. In the suppressor column (Table 1), chloride ions are exchanged for hydroxyl ions of the regenerant [tetramethylammonium hydroxide or $Ba(OH)_2$], which after reacting with $(enH_2)^{2+}$, form water and ethylenediamine and thus decrease the base line conductivity.

Examination of the magnesium and calcium peaks in Figs. 2–5 shows that the chromatographic behaviour of these ions in the presence of the ethylenediamine eluent on the Dionex CS-2 column is different from that on the Dionex CS-1 column. Based on peak shapes, the IC behaviour of these cations can be ascribed mainly to the adsorption process. In the ion chromatograph obtained on the CS-1 column (Fig. 2), the peaks are symmetrical and, therefore, the adsorption of magnesium and calcium ions followed the normal linear-type adsorption isotherm. However, for the CS-2 column (Figs. 4 and 5) the cation peaks showed a sharp front with tailing at the rear, which is typical of a Langmuir adsorption isotherm (*i.e.*, strong initial adsorption followed by a plateau due to the limited number of sites available for adsorption). Normally, an ion having very high affinity for the column material will show such behaviour. However, the ethylenediammonium cation, because of its hydrophobic nature, has a much higher affinity for the column resin than have magnesium and calcium ions. Therefore, an eluent containing ethylenediammonium cations should rapidly displace the loaded cations such as magnesium and calcium from the column, resulting in sharp and symmetrical peaks as obtained with the CS-1 column. The other possible reason for asymmetry in the magnesium and calcium peaks might be that the availability of adsorption sites on the CS-2 column, in the presence of $(enH_2)^{2+}$ cations, is not uniform. Based on the explanation given below, this seems to be the most likely reason.

The low capacity cation-exchange resins used for the CS-1 and CS-2 columns are obtained by superficial sulphonation of styrene–divinylbenzene co-polymer beads, as originally described by Small *et al.*[8] The resin beads are treated with concentrated sulphuric acid and a thin layer of sulphonic acid groups is formed on the surface (see reference 1, p. 72). The final capacity of the resin is related to the thickness of the layer. As the capacity of the CS-2 column is higher than that of the CS-1 column, the thickness of the sulphonated layer on the resin beads of the CS-2 column should be greater than that for the CS-1 column. The greater thickness of the layer of sulphonic acid groups might have led to relatively deep pores in the resin beads of the CS-2 column. In the presence of the ethylenediamine eluent, all the available exchange sites on the column are occupied by $(enH_2)^{2+}$ cations. However, because of their relatively large size, $(enH_2)^{2+}$ cations may have difficulty in reaching the exchange sites located in the deep and narrow pores on the resin particles of the CS-2 column and, as a result, the interactions between the exchange sites located in the pores and $(enH_2)^{2+}$ cations will be weaker compared with the similar interactions at the surface of the beads. Owing to their hydrophobic character, $(enH_2)^{2+}$ cations will be very strongly bound on the sites available at the surface of the resin beads, thus providing strong competition for the solute cations to adsorb onto these sites. This suggests that all the exchange sites on the separator column may not be uniformly available for the cations in the solutes to be separated in the presence of the enH_2Cl_2 eluent. Thus, during the adsorption/elution step, calcium or other cations, because of their small ionic size, will initially adsorb onto the easily available exchange sites located inside the pores, and because of weak competition from $(enH_2)^{2+}$ cations, will be held more tightly at these sites than the exchange sites available at the surface of the resin beads. This will cause the main portion of

Table 3 Separation of Mg^{2+}, Ca^{2+} and Sr^{2+} under different liquid chromatographic (LC) conditions

Column	Eluent	Type of LC detector	Analysis time/min	R*	Reference
150 × 2.6 mm Hitachi†	0.7 mol dm^{-3} sulfosalicylic acid	HPLC (spectrophotometry)	11	1.1	9
50 × 46 mm Waters	10 mmol dm^{-3} phenylethylamine	Single column IC (conductivity)	0	0.9	10
350 × 2 mm Benson§	1.5 mmol dm^{-3} + $(en)_2$‡ + 2 mmol dm^{-3} tartrate	Single column IC (conductivity)	16	2.7	4
6 × 250 mm Dionex	0.004 mmol dm^{-3} HNO_3 + 0.0025 mmol dm^{-3} Zn^{2+}	Suppressed IC (conductivity)	11	0.9	3
CS-1 + CG-1 Dionex	0.002 mmol dm^{-3} MPD¶ 0.002 mmol dm^{-3} HCl	Suppressed IC (conductivity)	7	1.4	1
CS-1 + GC-1 Dionex	0.001 mmol dm^{-3} en‡ + 0.002 mmol dm^{-3} HCl	Suppressed IC (conductivity)	12	1.5	This work
CS-2 + CG-2 Dionex	0.0025 mol dm^{-3} en‡ + 0.005 mol dm^{-3} HCl + 0.001 mol dm^{-3} Zn^{2+}	Suppressed IC (conductivity)	8	2.2	This work

* Resolution between calcium and strontium ions.
† Column was filled with cation-exchange resin 2613.
‡ Ethylenediamine.
§ Column was filled with 20 μm low cation-exchange capacity resin.
¶ *m*-phenylenediamine dihydrochloride.

the solute band to elute ahead and more rapidly than the leading front edge (the leading front edge is tightly bound in the pores) owing to the availability of a limited number of sites for adsorption, and will result in a sharp front on the eluting peak. The tailing of the peak is caused by the slow elution of Ca^{2+} or Mg^{2+} cations adsorbed in the pores. For a symmetrical peak, the part of the solute band adsorbing first should also elute first, in order to maintain the availability of sites for adsorption of the proceeding ions in the solute band. It is possible that when Zn^{2+} ions are added to the enH_2Cl_2 eluent, the exchange sites inside the pores are occupied by Zn^{2+} cations, thereby making them less available to the solute cations, which are to be separated. The presence of Zn^{2+} ions might also have modified the affinity of $(enH_2)^{2+}$ cations for the surface sites by providing more competition. Because of this, the availability of the adsorption sites, for the cations to be separated on the Dionex CS-2 column in the enH_2Cl_2–Zn^{2+} eluent, will be fairly uniform. Consequently, the eluting peak will be more symmetrical, following a linear-type adsorption isotherm. The almost symmetrical magnesium and calcium peaks, obtained with this eluent, are shown in Figs. 6 and 7. Although this explanation seems reasonable, more detailed research is required for further confirmation. Nevertheless, the applicability of the enH_2Cl_2–Zn^{2+} eluent to the separation of a small amount of Sr^{2+} from a large excess of Ca^{2+} ions is not affected.

Several papers[3,4,9,10] have been published on the liquid chromatographic determination of alkaline earth cations (Table 3). However, as can be seen in Table 3, except in one instance, the resolution between Ca^{2+} and Sr^{2+} ions is less than 1.5. Although for a complete separation of two peaks of equal size a resolution of 1.5 is sufficient, because of the much smaller size of the strontium peak compared with the calcium peak, its separation from calcium in high salinity sub-surface brines such as Arab-D brines requires a higher resolution than 1.5 between these ions. The IC conditions reported by Sevenich and Fritz[4] have revealed a resolution of approximately 2.7 between Ca^{2+} and Sr^{2+} ions and may be large enough to separate these ions in sub-surface Arab-D brine (although the IC conditions reported by these workers have not been applied to real applications and it is very unlikely that the sensitivity of a single column IC method will be good enough to determine the small concentrations of strontium normally found in sub-surface waters). However, using the IC conditions reported in this paper, a base line separation of these ions can be achieved in about half the time required to separate these ions by single column IC as demonstrated by Sevenich and Fritz.[4] The IC method reported in this work for the simultaneous determination of Na^+, Mg^{2+}, Ca^{2+} and Sr^{2+} in sub-surface waters is more convenient than standard AAS or inductively coupled plasma atomic emission spectrometry methods, because, in these spectrometric methods, interference, due to the variable concentrations of easily ionized elements, requires matrix matching of the samples, blanks and standards and thus makes accurate determination difficult. In addition, the IC method has an added advantage in that it can be used to determine the anions, chloride and sulphate, which is often required in the petroleum industry to check the compatibility of the waters.[11–14]

In conclusion, Sr^{2+} can be separated from very large amounts of Ca^{2+} on a Dionex CS-2 cation separator with the eluent 0.0025 mol dm^{-3} ethylenediamine–0.005 mol dm^{-3} HCl–0.001 mol dm^{-3} Zn^{2+}. The IC conditions developed here are particularly suitable for the separation and rapid determination of Na^+, Mg^{2+}, Ca^{2+} and Sr^{2+} in high salinity sub-surface waters, such as pure Arab-D brine or its mixture with other waters. Unlike *m*-phenylenediamine dihydrochloride, the ethylenediamine eluent is very stable and can be stored for long periods of time (at least 6 months). In addition, because of its neutral pH, the eluent can also be used with regular high-performance liquid chromatographs.

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Determination of Nitrovin in Medicated Animal Feeds by High-performance Liquid Chromatography

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Nitrovin is extracted from a medicated feed by means of a solvent mixture consisting of dichloromethane, methanol and ammonia solution and then the nitrovin is determined in the extract by high-performance liquid chromatography using a cyano-column.

Keywords: *Nitrovin; medicated animal feed; high-performance liquid chromatography*

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

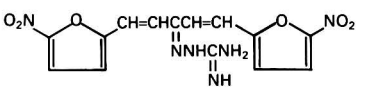
Report

The constitution of the Sub-Committee responsible for the preparation of this report was: Dr. N. T. Crosby (Chairman), Mr. P. Sanderson (Chairman until June 1987), Mr. A. Anderson, Mr. A. J. Argent (from October 1987), Mrs. C. Billingshurst (until September 1986), Mr. K. M. Bromley (from November 1988), Mr. G. C. Buddle (until October 1987), Dr. D. Campbell (from November 1988), Mr. M. Cannon (until June 1989), Mr. A. Carter (until December 1986), Mr. B. O. B. Conway (from September 1986), Mr. R. J. Davies, Mr. B. Dredge, Mr. H. W. Evans (from September 1988), Dr. J. Ganley (from December 1986), Dr. M. J. Gliddon, Miss L. M. Hughes (from February 1989), Miss A. M. Moore (until September 1986), Dr. A. Roberts (from April 1988), Dr. M. L. Robinson, Dr. S. J. Snee (March 1987 to April 1988), Dr. G. M. Taylor, Dr. M. E. Tenneson (until April 1988) and Dr. A. A. Wagland (until December 1986) with Mr. J. J. Wilson as Secretary.

The Sub-Committee gratefully acknowledges assistance from Ing. J. Koster of Orphahell B.V. and the staff of a number of continental laboratories which took part in trials of the proposed method.

Introduction

Nitrovin, 2-{3-(5-nitro-2-furanyl)-1-[2-(5-nitro-2-furanyl)-ethenyl]-2-propenylidene}hydrazinecarboxamidine, has been used as a growth promoter in animal feedingstuffs for chickens, turkeys, pigs and calves at levels varying between 10 and 25 mg of nitrovin per kilogram of feed.



After the experimental work described in this report had been completed the Sub-Committee was informed that nitrovin had been removed from Annex II and hence feeds containing nitrovin could no longer be sold within the European Community. However, it was decided to publish this report as it could be of value in areas where the use of nitrovin is still permitted.

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A method for the determination of nitrovin in medicated animal feeds was published by the Analytical Methods Committee[1] and subsequently by the Ministry of Agriculture, Fisheries and Food in regulations made under the Medicines Act 1968;[2] it will be referred to as the Statutory method in this report. According to this method, the feed was first extracted with hexane to remove fats and oils and then with dimethylformamide to dissolve the nitrovin. An aliquot of the dimethylformamide solution was placed on an aluminium oxide chromatography column and the nitrovin band eluted by means of a solution of ammonia in dimethylformamide. The nitrovin content of the eluate was determined by measurement of the absorbance of this solution at 495 nm.

Experience with this test showed that low recoveries were obtained from aged samples of medicated feeds and doubts were cast on the stability of the drug. Storage tests on the pure drug at both ambient and elevated temperatures showed no signs of drug decomposition and the Sub-Committee was asked to examine a high-performance liquid chromatography (HPLC) method proposed by the manufacturers of the drug (Method 1).

Results obtained during this investigation have confirmed that low recoveries can be obtained from feed samples by the Statutory method.

Experimental

Method 1

The drug was extracted from the finely ground feed by shaking 20 g of the sample for 1 h with 100 ml of the extraction solvent in a 250 ml conical flask. The extraction solvent was prepared by mixing 564 ml of dimethylformamide, 300 ml of water, 30 ml of methanol and 9 ml of ammonia solution (sp. gr. 0.88). After shaking, the flask and contents were allowed to stand for 15 min and then a volume of the solution was filtered through a GF/C filter. Chromatography was carried out by injecting 50 μl aliquots of the extract onto a Hypersil 5 μm ODS column (250 × 4.6 mm i.d.) at 35 °C. The mobile phase was a mixture of dimethylformamide–acetonitrile–0.225 mol dm^{-3} sulphuric acid (23 + 20 + 57, v/v) and the flow-rate was 1.5 ml min^{-1}. An ultraviolet (UV) absorbance detector was operated at 365 nm. The nitrovin content of the extract was determined from the area of the peak compared with that of a peak given by a standard solution of nitrovin in the extraction solvent.

Two 20 g samples of each of three types of animal feeds were medicated with a weighed amount of nitrovin. These medicated samples were analysed by the above method and the results obtained are given in Table 1. These results were promising, with recoveries of the order of 90% and with good agreement between duplicate analyses. Some co-extractants appeared at the start of the chromatograms but the nitrovin peaks were clear of interferents. It was considered that the

column would have a limited life when operated with such a strongly acidic mobile phase and hence an alternative method was examined.

Method 2

A literature survey revealed an HPLC method[3] which claimed good recoveries over the range 5–44 mg kg^{-1}. The drug was extracted from the feed by shaking with a solvent mixture consisting of dichloromethane–methanol–ammonia solution (950 + 50 + 1, v/v). The filtered extract was examined by chromatography on a cyano-column (cyanopropylmethylsilyl bonded to silica) using, as mobile phase, a mixture of methanol–water–acetic acid–butylamine (2500 + 50 + 10 + 1, v/v) followed by detection at 254 nm.

A preliminary trial was carried out in one laboratory on a sample of a pig grower feed medicated with nitrovin at levels of 10, 23 and 48 mg kg^{-1}. Recoveries of 84–88% were obtained; the nitrovin peaks were well resolved from any interferences. The recoveries were lower than those claimed by Chen *et al.*,[3] but sufficiently promising for a collaborative trial to be arranged in which Methods 1 and 2 could be compared.

Table 1 Nitrovin content of medicated feed by Method 1

Feed	Amount of nitrovin in 20 g of feed/μg Added	Found	Amount added/ mg kg^{-1}	Recovery (%)
Broiler finisher	653	580	32.7	89
	794	732	39.7	92
Pig grower	624	538	31.2	86
	709	613	35.5	87
Turkey grower	1075	973	53.7	91
	868	782	43.4	89

First Collaborative Trial

Samples of a broiler feed, a turkey feed and a pig feed were medicated at the 10 mg kg^{-1} level and portions of these medicated feeds were pelleted. Samples of the blank feed and of the medicated feed in both the pelleted and unpelleted form were sent to participating laboratories. Duplicate determinations were carried out on consecutive days. Laboratories were invited to comment on the methods and in some instances additional trials were carried out leading to suggestions for improvements in the methods. All samples were ground so that they passed through a 1 mm sieve before extraction.

A 10 g sample of ground feed was weighed into a 150 ml conical flask, 50 ml of the extraction solvent [dichloromethane (950 ml)–methanol (50 ml)–ammonia (1 ml)] were added and the flask was stoppered and shaken for 1 h. The extract was filtered through a GF/A filter. A Spherisorb 5 μm cyano-column (250 × 4.6 mm i.d.) (or equivalent) was fitted with a 20 μl loop injector and the mobile phase was a mixture of methanol–water–acetic acid–butylamine (2500 + 50 + 10 + 1, v/v). A standard solution of nitrovin in the extraction solvent was prepared and 20 μl aliquots were injected onto the column in order to provide reference peaks. All solutions containing nitrovin (in both methods) were prepared and stored in amber-coloured glassware.

The results obtained are given in Tables 2–4. Laboratory C reported that Method 1 gave filtration problems owing to the syrupy nature of the extract. The chromatography of Method 2 was superior; the retention time of nitrovin in Method 1 fell during the day in spite of the temperature of the column being thermostatically controlled, while in Method 2 it remained constant without the need for thermostatic control. Labora-

Table 2 Comparison of two HPLC methods for the determination of nitrovin in broiler feed

	Nitrovin content/mg kg^{-1}											
	Method 1						Method 2					
	Meal			Pellets			Meal			Pellets		
Laboratory	Determination 1	Determination 2	Mean	Determination 1	Determination 2	Mean	Determination 1	Determination 2	Mean	Determination 1	Determination 2	Mean
A	8.8	8.9	8.85	8.5	8.7	8.6	9.1	9.1	9.1	8.4	8.3	8.35
B	10.7	8.4	9.55	9.6	8.4	9.0	9.1	9.0	9.05	8.3	7.3	7.8
C	7.7	8.5	8.1	9.9	9.5	9.7	11.9	12.6	12.25	10.3	10.9	10.6
D	—	—	—	—	—	—	10.7	10.7	10.7	8.6	8.7	8.65
E*	9.6	9.2	9.4	8.1	8.7	8.4	7.4	7.9	7.65	7.5	7.6	7.55
F*	8.5	5.7	7.1	8.2	5.9	7.05	10.0	10.3	10.15	9.4	9.5	9.45
G	7.3	7.3	7.3	6.9	6.2	6.55	10.2	10.1	10.15	6.9	6.2	6.55
		Mean	8.38			8.22			9.86			8.42
	Standard deviation		1.050			1.194			1.455			1.323
1			9.3			8.3			6.1			6.4
2	7.4	7.8	7.6	7.4	7.1	7.25	8.8	9.3	9.05	8.2	7.9	8.05
3	5.5	5.4	5.45	5.3	6.2	5.75	9.2	9.2	9.2	8.7	8.4	8.55
4	7.0	7.6	7.3	6.7	6.5	6.6	7.1	7.9	7.5	6.8	6.8	6.8
5	7.8	6.7	7.25	8.1	6.9	7.5	7.4	8.9	8.15	7.3	7.7	7.5
6	9.6	9.6	9.6	8.3	8.0	8.15	7.7	7.7	7.7	7.9	6.8	7.35
7	—	—	—	—	—	—	7.3	7.7	7.5	8.0	7.6	7.8
8	8.5	7.3	7.9	8.1	6.9	7.5	10.9	10.6	10.75	9.7	9.7	9.7
9*	7.7	7.8	7.75	6.7	7.5	7.1	7.5	7.5	7.5	6.8	6.7	6.75
10	8.1	7.7	7.9	8.5	7.3	7.9	8.0	8.2	8.1	8.0	8.0	8.0
11	7.4	6.1	6.75	7.2	6.4	6.8	7.7	7.8	7.75	6.6	6.7	6.65
		Mean	7.68			7.29			8.12			7.60
	Standard deviation		1.183			0.771			1.204			0.974

* Laboratory E reported less than 1.0 mg kg^{-1} in the blank feed, laboratory F reported 0.1 and 0.4 mg kg^{-1} and laboratory 9 less than 0.3 mg kg^{-1}, the other laboratories reported 0 by Method 1. Laboratory E reported less than 1 mg kg^{-1}, laboratory F 0.1 and 0.1 mg kg^{-1} and laboratory 9 less than 0.2 mg kg^{-1} by Method 2.

tory D was unable to return results for Method 1 because of the difficulty of filtering the extract.

The Dutch manufacturers of nitrovin also received a supply of these samples for distribution amongst 11 continental laboratories. Their results are also given in Tables 2–4, where the letters A–G denote the UK laboratories and 1–11 the continental laboratories. Method 2 tended to give higher results than Method 1 and the results obtained in the UK laboratories were higher than those obtained on the continent. This difference in the results might be related to a longer time

Table 3 Comparison of two HPLC methods for the determination of nitrovin in turkey feed

	Nitrovin content/mg kg^{-1}											
	Method 1						Method 2					
	Meal			Pellets			Meal			Pellets		
Laboratory	Determination 1	Determination 2	Mean	Determination 1	Determination 2	Mean	Determination 1	Determination 2	Mean	Determination 1	Determination 2	Mean
A	8.8	9.0	8.9	8.4	8.4	8.4	9.2	9.1	9.15	8.3	8.6	8.45
B	12.1	10.2	11.15	7.8	7.6	7.7	9.1	7.8	8.45	8.2	7.2	7.7
C	8.9	8.9	8.9	8.3	8.3	8.3	11.0	10.7	10.85	10.4	10.1	10.25
D	—	—	—	—	—	—	9.3	8.9	9.1	8.6	8.6	8.6
E	10.4	9.2	9.8	8.5	9.3	8.9	9.1	8.5	8.8	8.0	8.5	8.25
F	8.0	8.9	8.45	8.2	8.1	8.15	10.0	9.8	9.9	9.6	9.3	9.45
G	6.8	6.3	6.55	6.2	6.3	6.25	10.0	9.8	9.9	8.5	9.7	9.1
		Mean	8.96			7.95			9.45			8.83
		Standard deviation	1.522			0.919			0.816			0.845
1			9.0			7.3			7.1			5.9
2	7.7	8.0	7.85	6.7	6.3	6.5	8.7	8.8	8.75	8.1	7.6	7.85
3	5.4	6.0	5.7	5.6	6.1	5.85	8.8	8.9	8.85	7.9	8.5	8.2
4	8.4	8.0	8.2	6.8	6.5	6.65	6.9	7.5	7.2	6.3	6.3	6.3
5	6.7	9.6	8.15	9.3	7.2	8.25	8.3	9.3	8.8	7.4	7.6	7.5
6	9.0	9.6	9.3	9.0	7.7	8.35	7.2	7.2	7.2	6.6	6.2	6.4
7	—	—	—	—	—	—	8.1	7.5	7.8	7.3	6.7	7.0
8	8.7	7.7	8.2	8.1	7.1	7.6	10.5	10.6	10.55	9.8	9.5	9.65
9	7.6	8.3	7.95	6.4	7.3	6.85	8.0	7.9	7.95	7.1	7.1	7.1
10	7.8	7.5	7.65	7.3	7.7	7.5	7.8	8.7	8.25	6.7	7.8	7.25
11	8.7	6.5	7.6	7.8	6.1	7.95	7.9	8.3	8.1	6.9	7.1	7.0
		Mean	7.96			7.28			8.233			7.21
		Standard deviation	0.965			0.811			1.002			1.03

Table 4 Comparison of two HPLC methods for the determination of nitrovin in pig feed

	Nitrovin content/mg kg^{-1}											
	Method 1						Method 2					
	Meal			Pellets			Meal			Pellets		
Laboratory	Determination 1	Determination 2	Mean	Determination 1	Determination 2	Mean	Determination 1	Determination 2	Mean	Determination 1	Determination 2	Mean
A	8.8	8.7	8.75	8.2	8.4	8.3	9.0	8.8	8.9	8.3	8.3	8.3
B	8.1	8.1	8.1	7.7	7.9	7.8	7.7	7.2	7.45	7.6	7.9	7.75
C	9.7	10.4	10.05	8.6	8.0	8.3	8.7	8.6	8.65	6.9	8.0	7.45
D	—	—	—	—	—	—	8.7	8.9	8.8	8.6	8.5	8.55
E	7.1	7.7	7.4	8.5	8.5	8.5	9.0	9.0	9.0	8.7	8.8	8.75
F	7.9	8.6	8.25	7.4	5.0	6.2	10.0	9.0	9.5	8.7	9.3	9.0
G	7.0	6.9	6.95	6.4	6.3	6.35	10.1	9.4	9.75	8.2	8.7	8.45
		Mean	8.25			7.58			8.86			8.32
		Standard deviation	1.089			1.034			0.737			0.548
1			6.7			7.0			6.7			5.5
2	7.8	7.7	7.75	7.5	7.1	7.3	8.2	8.3	8.25	7.7	7.4	7.55
3	6.1	6.3	6.2	6.1	6.1	6.1	11.0	10.9	10.95	8.6	8.2	8.4
4	7.1	6.8	6.95	5.8	6.8	6.3	6.6	6.8	6.7	5.8	6.4	6.1
5	6.2	7.7	6.95	8.2	7.3	7.55	7.6	8.3	7.95	7.1	7.5	7.3
6	9.3	8.3	8.8	8.3	9.0	8.65	7.9	7.2	7.55	6.8	6.6	6.7
7	—	—	—	—	—	—	7.6	7.4	7.5	7.4	7.3	7.35
8	8.8	7.7	8.25	8.1	7.5	7.8	10.6	10.4	10.5	9.7	9.7	9.7
9	7.4	7.6	7.5	7.2	7.6	7.4	8.0	8.1	8.05	7.3	7.4	7.35
10	9.6	9.1	9.35	7.2	7.3	7.25	7.4	8.7	8.05	6.66	7.5	7.05
11	8.6	8.4	8.5	8.0	5.7	6.85	8.2	8.3	8.25	7.3	7.2	7.25
		Mean	7.70			7.22			8.22			7.30
		Standard deviation	1.016			0.732			1.355			1.102

interval betweeen preparation and analysis of the samples on the continent. A few laboratories obtained low results which were rejected as outliers. The recoveries from the pellets were lower than from meal. Some laboratories using high-resolution columns reported that the nitrovin peak was split into two peaks. This appeared to be a pH effect, as it could be avoided by adjustment of the pH of the solution. Further work by one laboratory showed a drop of about 10% in the results after storage of the samples for 2 weeks. With Method 1, the difference between the results obtained from the meals and the pellets was less than that obtained with Method 2. Some results with the Statutory method obtained by one laboratory were considerably lower than those given by either Method 1 or 2.

The results reported by the 11 continental laboratories were examined statistically and it was reported that, overall, similar results were obtained with the two methods. Method 2 tended to produce higher results than Method 1 but the differences were not statistically significant. The repeatability of Method 2 was better than Method 1. It was noted that three laboratories produced consistently higher results with Method 2 while three others produced higher results using Method 1. Some work by one Dutch laboratory investigated peak splitting or the production of broad peaks. They concluded that the area of a peak due to nitrovin was strongly influenced by the nature of the solvent and by its pH. It was suggested that a better shaped peak for the standard was obtained when the stock standard solution was diluted with an extract from a blank feed in place of the extraction solvent. However, in practice, laboratories that were required to examine samples of feeds in order to determine the nitrovin content would rarely be able to obtain samples of the corresponding feed before medication.

Laboratory C repeated their work on the chromatography of the reference solution without experiencing peak splitting. They also changed the amount of ammonia in the extraction solution and found that it changed the shape but not the area of the nitrovin peak.

Second Collaborative Trial

An exercise was arranged in which a sample of feed medicated at the 10 mg kg^{-1} level, in meal and pelleted form, was circulated and laboratories were asked to determine the nitrovin content by Method 2 and by the Statutory method. The results obtained in this exercise are given in Table 5. The results reported by all the laboratories show that the recovery by the Statutory method was considerably less than that by Method 2.

The work in laboratory C on the Statutory method indicated

Table 5 Comparison of Method 2 and the Statutory method for the determination of nitrovin in feeds

	Nitrovin content/mg kg^{-1}											
	Method 2						Statutory method					
	Meal			Pellets			Meal			Pellets		
Laboratory	Determination 1	Determination 2	Mean	Determination 1	Determination 2	Mean	Determination 1	Determination 2	Mean	Determination 1	Determination 2	Mean
A	8.9	8.9	8.9	8.3	8.2	8.25	6.9	6.3	6.6	5.9	6.2	6.05
B	8.1	8.6	8.35	8.0	8.4	8.2	3.3	3.7	3.5	2.6	3.3	2.95
C	9.0	9.0	9.0	8.5	8.4	8.45	4.3	1.9	3.1	3.7	3.9	3.8
D			9.4			9.1	—	—	—	—	—	—
E			8.5			8.0			4.8			5.4
F	10.7	10.3	10.5	9.1	9.3	9.2	3.7	3.4	3.55	2.5	2.9	2.7
G	8.9	8.0	8.45	6.8	7.4	7.1	4.6	4.6	4.6	2.6	2.6	2.6
		Mean	9.01			8.33			4.36			3.92
	Standard deviation		0.752			0.708			1.285			1.477

Table 6 Nitrovin content of feed samples by Method 2, results from continental laboratories

	Nitrovin content/mg kg^{-1}											
	Mash						Pellets					
		Analyst 1			Analyst 2			Analyst 1			Analyst 2	
Laboratory	Date			Date			Date			Date		
1*	28/9	13.2	12.3	2/10	13.1	12.7	28/9	12.5	13.0	2/10	12.6	13.1
2	30/9	9.3	9.4	30/9	9.2	9.4	30/9	10.1	11.0	30/9	8.9	9.3
3	28/9	7.4	7.1	28/9	7.2	7.0	1/10	7.4	6.9	1/10	6.7	6.6
4	28/9	7.2	7.2	29/9	7.7	7.3	28/9	6.0	6.6	29/9	6.8	6.7
5	22/9	7.9	7.9	23/9	8.1	8.1	22/9	7.4	7.6	23/9	7.2	7.2
6	1/10	7.2	7.0	6/10	8.6	8.3	1/10	7.6	7.5	6/10	8.4	7.2
	(2/10	8.0	8.2)†				(2/10	7.3	8.2)†			
7	22/9	8.3	7.8				22/9	7.4	7.4			
8	23/9	8.0	8.0	23/9	7.4	7.4	23/9	8.6	7.6	23/9	7.0	7.1
9	22/9	9.5	8.2‡				22/9	9.3	9.3			
10	22/9	6.4	6.4	23/9	6.9	6.8	22/9	6.0	6.1	23/9	6.4	6.4
11	22/9	10.8	10.5	24/9	10.2	10.3	22/9	10.5	10.9	24/9	10.7	10.9
12	21/9	7.3	7.2	30/9	7.9	7.5	21/9	6.7	6.0	30/9	7.1	6.6
13	7/10	9.1	9.1	13/10	9.4	9.5	7/10	8.7	8.8	13/10	8.9	8.6
14	29/9	8.0	8.3				29/9	9.7	9.2			

* Straggler according to Dixon's test.
† Results not included in statistical evaluation.
‡ Outlier according to Cochran's test.

Table 7 Statistical examination of results in Table 6. Values given are the mean, standard deviation (SD) and relative standard deviation (RSD) for the repeatability (denoted by subscript r), reproducibility (denoted by subscript R), analyst (denoted by subscript an) and between laboratories (denoted by subscript lab)

	Mash		Pellets	
	All laboratories included	Without laboratories 7, 9 and 14	All laboratories included	Without laboratories 7, 9 and 14
Mean	8.50	8.54	8.33	8.27
RSD_r (%) (SD_r/mg kg^{-1})	2.4 (0.21)	2.3 (0.20)	4.5 (0.37)	4.3 (0.36)
RSD_{an} (%) (SD_r/mg kg^{-1})	4.0 (0.34)	4.0 (0.34)	4.3 (0.36)	4.3 (0.36)
RSD_{lab} (%) (SD_{lab}/mg kg^{-1})	18.5 (1.58)	21.0 (1.79)	22.1 (1.84)	24.8 (2.05)
RSD_R (%) (SD_R*/mg kg^1)	19.2 (1.63)	21.5 (1.84)	22.9 (1.91)	25.5 (2.11)
RSD_R (%) (SD_x†/mg kg^{-1})	18.9 (1.60)	21.2 (1.81)	22.5 (1.87)	25.0 (2.07)

* $SD_R = \sqrt{SD_{lab}^2 + SD_{an}^2 + SD_r^2}$

† $SD_x = \sqrt{SD_{lab}^2 + SD_{an/2}^2 + SD_{r/4}^2} = S_{lab\text{-}average}$

that nitrovin could be lost in the hexane wash that was used to remove fat and oil from the sample. Dimethylformamide was a less effective extraction solvent than the alkaline mixtures used in Methods 1 and 2. Some laboratories attempted to modify the Statutory method by adding ammonia to the dimethylformamide extraction solvent, which gave increased recoveries, but the results were still below those given by Method 2.

Portions of medicated feed samples examined in this exercise were also distributed to 14 continental laboratories where they were examined according to Method 2. Determinations were carried out in duplicate by two analysts, and in many instances on different days. The results obtained are reported in Table 6 and a statistical examination of these results is given in Table 7.

In Method 2, the concentration of nitrovin in the working standard was higher than in the extract prepared from a feed containing 10 mg kg^{-1} of nitrovin and it was proposed to amend the method in order to reduce the concentration of nitrovin in this solution. This change would also change the pH of the solution and reduce the tendency to peak splitting reported by some laboratories. The amended method is given in the Appendix.

Discussion

The performance of the Statutory method in this trial has been inferior to that obtained when the method was developed in 1974[1] and the reports of low recoveries have been supported in this work. One possible explanation of this difference is that, in the earlier work, the recovery trials were carried out on samples of feed that had been medicated by the addition of a pre-mix shortly before starting the determination. Other suggestions point to variations in the activity of the aluminium oxide used for the chromatographic separation of the nitrovin. One member reported that when carrying out the Statutory method the red nitrovin band did not move when the yellow band due to the feed material was being washed off the alumina. Method 1 represented a definite improvement over the Statutory method, but it was the opinion of the Sub-Committee that Method 2 was to be preferred as the accuracy appeared to be better, filtration of the extract was easier, column life would be longer and the chromatography was superior.

It will be noted that the recovery of nitrovin from pelleted feeds was lower than that from meal; this effect has been found with all medicinal additives examined by the Committee and is probably due to some form of binding between the drug and a component of the feed. In some instances it has been suggested that the drug is broken down by the heat generated during pelleting but the same effect has been found with additives that have been shown to be stable when heated. In addition, the chromatograms from meal and pellets are similar with no signs of degradation products.

Recommendation

The Analytical Methods Committee recommends that the Statutory method should be replaced by the method given in the Appendix for the determination of nitrovin in medicated animal feedingstuffs.

APPENDIX

Determination of Nitrovin in Medicated Animal Feeds

Purpose and Scope

The method is for the determination of nitrovin in feeds containing 10–25 mg kg^{-1} of nitrovin.

Principle

Nitrovin is extracted from the ground feed by shaking with a mixture of dichloromethane–methanol–ammonia solution. The extract is filtered and the nitrovin determined in the extract by HPLC, using a cyano-column and a UV detector at 365 nm.

Reagents

Dichloromethane. Analytical-reagent grade.

Methanol. HPLC grade.

Ammonia solution (sp. gr. 0.88).

Acetic acid, glacial.

Butylamine.

Extraction solvent. Dichloromethane–methanol–ammonia solution (950 + 50 + 1, v/v).

Nitrovin, standard substance.

Nitrovin, stock standard solution. Weigh accurately 10 mg of nitrovin into a 250 ml amber-coloured calibrated flask, dissolve and dilute to the mark with extraction solvent. This solution will contain 40 μg ml^{-1} of nitrovin.

Nitrovin, working standard solution. Transfer by pipette 2.00 ml of stock standard solution into a 25 ml calibrated flask and dilute to the mark with extraction solvent. This solution will contain 3.2 μg ml^{-1} of nitrovin.

HPLC mobile phase. Methanol–water–acetic acid–butylamine (2500 + 50 + 10 + 1, v/v). De-gas before use.

Apparatus

HPLC system. Pump, UV detector operating at 365 nm and valve injector fitted with a 20 μl loop.

Column. Spherisorb 5 μm cyano-column, 250 × 4.6 mm i.d. (or equivalent).

Glass fibre filter, GF/A.

Extraction

Weigh 10 g of sample, ground so that it passes through a 1 mm sieve, into a 150 ml conical flask and add 50 ml of extraction solvent. Stopper the flask and shake on a mechanical shaker for 1 h. Filter the extract through a GF/A filter and collect the filtrate in an amber-coloured vessel. Transfer, by pipette, 2.00 ml of mobile phase into a 25 ml amber-coloured calibrated flask and make up to the mark with the filtered sample extract.

Chromatography

Set the flow-rate of the pump to 2.0 ml min^{-1} and stabilize the system for at least 30 min. Adjust the wavelength of the detector to 365 nm and inject 20 μl of the working standard solution and record the area (or height) of the nitrovin peak. Repeat this injection several times until consistent areas (or heights) are obtained. Make several injections of 20 μl aliquots of the sample extract and record the area (or height) of the nitrovin peak.

Calculation

Compare the sample area (or height) with the standard area (or height) and determine the concentration of nitrovin in the sample extract in μg ml^{-1} and use this figure to calculate the amount of drug in the feed.

$$\text{Concentration of nitrovin/mg kg}^{-1} = \frac{S_a \times W_{sc} \times V \times 25}{M \times W_{sa} \times 23}$$

where S_a = peak area or height of the sample extract; W_{sc} = concentration of the working standard; M = mass of sample (g); W_{sa} = peak area or height of the working standard; and V = volume of the extract.

NOTE: All solutions containing nitrovin should be protected from actinic light by the use of amber-coloured glassware.

References

1 Analytical Methods Committee, *Analyst*, 1974, **99**, 70.
2 The Medicines (Animal Feeding Stuffs) (Enforcement) Regulations 1985. (S.I. 273) HM Stationery Office, London, 1985, p. 82.
3 Chen, J., Wang, F., and Yang, S., *Fenxi Huaxue*, 1985, **13**, 935; *Anal. Abstr.*, 1986, **48**, 7G15.

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Determination of Vitamin E in Animal Feedingstuffs by High-performance Liquid Chromatography

Analytical Methods Committee*
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A method is given for the determination of vitamin E in animal feeds and pet foods. The sample is saponified with ethanolic potassium hydroxide solution and the vitamin E extracted into light petroleum (boiling range 40–60 °C). This solution is evaporated to dryness under reduced pressure and the residue dissolved in hexane. The concentration of vitamin E in this solution is determined by high-performance liquid chromatography using a silica column and an isooctane–propan-2-ol mixture as the mobile phase. An alternative method using hexane–1,4-dioxane as the mobile phase is also given.

Keywords: *Vitamin E determination; animal feedingstuffs; high-performance liquid chromatography; collaborative study*

The Analytical Methods Committee has received and has approved for publication the following report from its Animal Feedingstuffs Sub-Committee.

Report

The constitution of the Sub-Committee responsible for the preparation of this report was Mr. G. H. Smith (Chairman), Miss I. B. Agater (from July 1985), Mr. G. R. Andrews (until June 1984), Mr. C. W. Ashby (from January 1989), Mr. S. Bailey (until January 1986), Mr. I. D. Craig (from January 1985), Dr. N. T. Crosby, Mr. D. Crudgington, Mr. S. Guffogg (from June 1984), Mr. R. M. Hughes (from January 1987), Mrs. A. Jones (from August 1988), Mr. J. J. Kay (until November 1986), Mr. J. S. McCall (until June 1984), Mr. G. S. Meadows (until November 1986), Mr. G. H. J. Merson (until July 1985), Mr. K. D. Mitchell (from June 1984 until August 1988), Mr. A. A. Moulder, Mr. D. A. Shirling-Rooke (from June 1987), Mr. G. Topp (until December 1988), and Dr. A. A. Wagland with Mr. J. J. Wilson as Secretary. In addition, members of the staffs of the following laboratories took part in some of the experimental work: A. H. Allen and Partners; Aspland and James; Clayton, Bostock, Hill and Rigby; The County Analyst for Lancashire; Moir and Palgrave; Ruddock and Sherratt Ltd.; and the Wickham Laboratories.

Introduction

Following the development of a method for the determination of vitamin A in animal feedingstuffs by high-performance liquid chromatography (HPLC),[1] the Animal Feedingstuffs Sub-Committee examined the methods for the determination of vitamin E. It was decided to adopt the extraction procedure that had proved to be satisfactory for extracting vitamin A from the feeds and to concentrate on an investigation of the chromatography of the extract. Laboratories that wished to determine both vitamins A and E in a feed sample would be able to carry out both determinations on portions of the one extract from the feed. Of the various isomers present in vitamin E, α-tocopherol is the only one that is nutritionally important and there is a legal requirement for the amount of this isomer present in the feed to be declared.

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Experimental Development

First Preliminary Trial

From an examination of the methods, both published and unpublished, known to the committee members, two were selected for a preliminary trial. Method 1 used normal phase HPLC[2] and Method 2 reversed-phase HPLC.[3] Three samples were circulated for a trial; these consisted of a blank feed and a feed fortified with 25 and 50 U kg^{-1} of α-tocopherol.

Extraction procedure

Samples (50 g) of finely ground feed were placed in a 1 l conical flask, 2 ml of sodium ascorbate solution (100 g l^{-1}), 50 ml of potassium hydroxide solution (500 g l^{-1}) and 200 ml of ethanol were added and the flask was swirled in order to mix the contents. A reflux condenser was fitted to the flask which was then placed in a boiling water-bath, allowed to reflux for 30 min and then cooled under a stream of cold water. The contents of the flask were transferred into the cylinder shown in Fig. 1; the flask was rinsed with two 25 ml portions of ethanol followed by two 125 ml portions of light petroleum (boiling range 40–60 °C) and one 250 ml portion of water. Each of these washings was added to the contents of the cylinder which was stoppered, shaken for 1 min and then

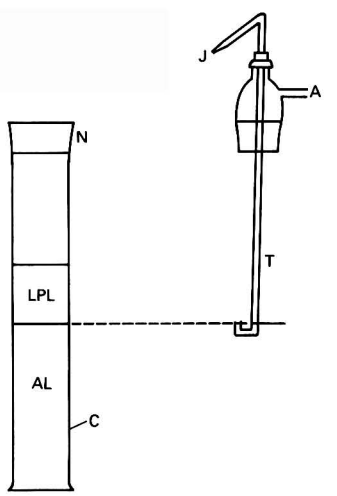

Fig. 1 Extraction apparatus. J, Jet; A, side arm; T, adjustable tube; N, ground-glass neck and stopper; LPL, light-petroleum layer; AL, aqueous layer + saponified feed; and C, 1 l cylinder

allowed to stand for the phases to separate. The cylinder was cooled in water before the stopper was removed and the light petroleum layer siphoned off into a 1 l separating funnel. A further portion (125 ml) of light petroleum was added to the contents of the cylinder which was stoppered, shaken well for 1 min, allowed to stand and the light petroleum layer siphoned off into the separating funnel. This operation was repeated with a further portion (125 ml) of light petroleum. The combined petroleum extracts were washed with four 100 ml portions of water and than transferred into a 1 l flask of a rotary evaporator. The interior of the separating funnel was washed with two 25 ml portions of light petroleum and the washings were added to the flask. The light petroleum was then removed by evaporation under vacuum at a temperature not exceeding 40 °C and the vacuum released by the admission of an inert gas. The residue in the flask was dissolved in a small volume of hexane and transferred into a 25 ml calibrated flask. The interior of the evaporation flask was washed two or three times with small portions of hexane; these washings were added to the contents of the calibrated flask and made up to 25 ml with hexane. (These operations are described in more detail in reference 1.) Portions of this solution were filtered through a 0.5 μm Millipore filter before being chromatographed.

During all these operations, care was taken to protect the material from the effects of light and atmospheric oxygen.

Chromatography

Normal phase HPLC (Method 1). A hexane–1,4-dioxane mixture (97 + 3, v/v) was used as the mobile phase with a 125 × 5 mm i.d. column packed with 5 μm silica particles (*e.g.*, LiChrosorb Si 60). A fluorescence detector was used at wavelengths of 293 nm (excitation) and 326 nm (emission). Portions (20 μl) of the filtered extract were injected onto the column. Calibration was carried out by analysing portions (20 μl) of solutions containing 8, 16, 24 and 32 μg ml^{-1} of α-tocopherol in hexane and a graph prepared showing peak height against concentration of α-tocopherol.

Reversed-phase HPLC (Method 2). The mobile phase used was methanol–water (95 + 5, v/v) and the column was 250 × 5 mm i.d. packed with a stationary phase consisting of 10 μm particles of octadecyl groups bonded to silica. A fluorescence detector was used at 295 and 330 nm. Aliquots (20 μl) of the filtered extract dissolved in propan-2-ol were injected onto the column and calibration was carried out with solutions containing 8, 16, 24 and 32 μg ml^{-1} of α-tocopherol in propan-2-ol.

Clean-up procedure. The effect of a preliminary clean-up of the extract was examined by carrying out a series of determinations in parallel, with and without a clean-up of the extract. The extract from the feed was passed through a Sep-Pak silica cartridge when the tocopherol was retained on the silica and the co-extractants were removed by washing the cartridge with hexane. The tocopherol was then eluted from the cartridge with hexane–ethyl acetate (99.5 + 0.5, v/v). This solution was then evaporated to dryness in a rotary evaporator under vacuum and the residue dissolved in hexane and made up to 25 ml with hexane. This solution was then injected onto the HPLC column. When the determination was carried out by reversed-phase chromatography, the residue in the rotary evaporator was dissolved in propan-2-ol instead of hexane.

Results

The results obtained in this exercise are shown in Table 1. Some laboratories (D and F) did not have a fluorescence detector for their chromatograph and substituted an absorbance detector operating at 295 nm.

The ultraviolet (UV) detector used gave satisfactory results on these samples, but it was pointed out that these detectors might not be sufficiently selective and sensitive and could be subject to considerable errors when analysing some commercial products. Where laboratories returned results obtained by the use of absorbance detectors, these have been included in the tables as they might be of interest to other workers who do not have fluorescence detectors.

The general view of the members was that the normal-phase method (Method 1) was preferred and that the clean-up stage was not required, although one member thought it made the readings easier when reversed-phase chromatography was used. Some laboratories had problems in obtaining supplies of 95% ethanol and it was agreed that the equivalent grade of industrial methylated spirit (IMS) could be used.

Second Preliminary Trial

An exercise was arranged in which Method 1 would be tried on a range of samples containing 5, 25 and 75 U kg^{-1} added to an unfortified feed. This feed already contained a low level of

Table 1 First preliminary trial: determination of α-tocopherol in feeds by HPLC. Comparison of normal- and reversed-phase chromatography, with (+) and without (−) preliminary clean-up

Laboratory	Amount found in blank feed*/U kg^{-1}				Amount† found less amount found in blank/U kg^{-1}				Amount‡ found less amount found in blank/U kg^{-1}			
	Normal-phase		Reversed-phase		Normal-phase		Reversed-phase		Normal-phase		Reversed-phase	
	−	+	−	+	−	+	−	+	−	+	−	+
A	28.2	26.1	27.9	23.6	30.1	33.1	33.6	32.3	51.6	52.4	52.8	52.0
	28.1	25.5	26.9	23.3	32.0	32.2	31.6	37.5	52.3	47.8	51.9	50.1
C	27.2	26.8	26.2	25.5	31.0	30.5	31.5	29.8	48.1	46.9	51.2	49.4
	26.0	26.0	26.5	25.5	30.5	30.5	31.0	29.5	49.4	48.1	51.2	49.4
D§	27.2	23.8	23.6	22.0	31.2	26.2	29.4	24.0	50.6	46.3	48.0	38.2
	27.4	22.5	25.0	20.3	29.4	24.8	25.5	26.7	49.6	43.8	45.9	36.5
F§	—	—	21.3	1.9	—	—	28.3	0.1	—	—	48.8	ND¶
	—	—	22.9	ND¶	—	—	30.3	28.1	—	—	49.2	44.6
H	27.4	27.4	27.1	25.7	30.4	30.8	32.4	31.9	49.5	42.7	47.3	43.9
	27.2	26.9	28.0	23.8	30.4	30.8	32.4	31.6	48.5	43.7	47.2	43.9
L	24.8	22.8	22.3	22.1	28.7	28.2	28.5	25.5	48.4	43.2	45.5	40.4
	24.6	21.7	22.3	22.2	28.3	26.0	29.2	25.5	48.7	43.7	46.2	40.4
K	26.6	25.7	17.0	13.1	31.4	31.2	21.0	15.5	50.9	50.4	32.5	27.1
	26.4	24.9	17.0	13.2	31.3	30.7	20.3	15.9	51.4	50.4	32.6	15.9

* No correction made for moisture content of feed.
† Blank feed plus 25 U kg^{-1} of α-tocopherol.
‡ Blank feed plus 50 U kg^{-1} of α-tocopherol.
§ Laboratories D and F used absorbance detectors.
¶ Not detected.

Table 2 Second preliminary trial: determination of vitamin E in ground wheat with added vitamin E

Laboratory	Ground wheat blank				Blank + 5 U kg^{-1} of vitamin E				Blank + 25 U kg^{-1} of vitamin E				Blank + 75 U kg^{-1} of vitamin E			
	Vitamin E level/U kg^{-1}				Vitamin E level/U kg^{-1}				Vitamin E level/U kg^{-1}				Vitamin E level/U kg^{-1}			
	As received		On dry matter		As received		On dry matter		As received		On dry matter		As received		On dry matter	
A	9.9	10.4	11.4	12.0	14.3	14.0	16.4	16.2	30.8	30.7	35.6	35.4	76.5	74.5	88.5	86.2
B	9.6	9.6	11.1	11.1	13.0	13.1	15.1	15.2	24.3	27.9	28.2	32.3	64.0	68.6	74.2	79.5
C	9.2	8.8	10.7	10.2	13.9	13.2	16.1	15.3	27.2	27.5	31.5	31.9	71.9	73.1	83.3	84.7
D*	8.4	8.8	—	—	13.9	14.2	—	—	30.8	32.5	—	—	76.0	77.0	—	—
E	9.3	9.7	10.7	11.2	14.8	15.3	17.0	17.6	29.0	27.6	33.5	31.9	78.8	74.3	91.0	85.8
F*	7.6	8.9	8.7	10.2	13.4	13.5	15.4	15.5	28.0	27.5	32.1	31.5	67.9	64.9	77.8	74.3
G	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
H	10.2	10.9	11.8	12.6	14.8	13.9	17.2	16.1	33.9	32.4	39.3	37.6	73.2	76.0	84.9	88.1
J	8.0	8.4	9.3	9.7	12.6	12.9	14.6	14.9	26.4	26.6	30.5	30.8	80.0	79.5	92.6	92.0
K	8.7	8.7	—	—	13.4	13.5	—	—	30.3	29.9	—	—	76.3	79.1	—	—
L	9.1	8.9	10.5	10.3	13.4	13.8	15.5	15.9	27.8	27.7	31.9	31.9	69.4	69.1	80.0	79.7
Mean:	9.16		10.72		13.75		15.88		28.9		32.9		73.5		83.91	
SD:	0.82		1.01		0.69		0.85		2.40		2.81		4.81		5.87	
RSD (%):	8.9		9.3		5.0		5.4		8.3		8.54		6.5		7.0	

* These calculations omit the results from Laboratories D and F.

α-tocopherol and participating laboratories were asked to determine this amount. The feed material was known to contain 10–15% of moisture and as this might change during storage, despatch and handling of the samples, participants were asked to determine the moisture content of their sample at the time of the test and to calculate the α-tocopherol content on a dry matter basis.

NOTE:—This procedure was followed throughout this investigation as it was thought that it would facilitate comparison of the results, but when feed samples are examined for enforcement purposes the vitamin E content must be calculated on the sample as received. [Thanks to the cooperation of the Association of Public Analysts (APA) and the United Kingdom Agricultural Supply Trades Association (UKASTA), a number of analysts who were not members of the Sub-Committee agreed to take part in this and later work.]

Where laboratories encountered problems with this method they were encouraged to look for possible improvements which might be examined in the next exercise.

Results

The results obtained in this exercise are shown in Table 2, together with the standard deviation (SD) and relative standard deviation (RSD). The laboratories which did not have fluorescence detectors continued to use absorbance detectors. Some laboratories used isooctane–tetrahydrofuran (975 + 25, v/v) in place of hexane–1,4-dioxane as this mixture had been reported to give a better separation of the beta- and gamma-isomers, but some of these reported badly shaped peaks or calibration lines that did not pass through the origin. Some laboratories had problems in obtaining sufficiently pure reagents. Laboratory L tried the isooctane–tetrahydrofuran mixture without success and reverted to the hexane–1,4-dioxane mixture. There was general agreement that raising the flow-rate from 1.0 to 1.5 ml min^{-1} gave improved separations.

Two laboratories carried out a limited comparison of these two mobile phases without finding any significant difference.

First Collaborative Trial

This was based on Method 1, with some amendments, in the light of experience gained in the two previous exercises. The method was amended to include the step of drying the solution in light petroleum by filtration through anhydrous sodium sulphate and the flow-rate of the mobile phase was increased from 1.0 to 1.5 ml min^{-1}. The method, as previously circulated, did not include a warning that care should be taken to avoid the formation of an emulsion when washing the light petroleum solution with water. Emulsions, once formed, were stable and hard to break. The recommended procedure was to use gentle inversion for the first wash, followed by gentle shaking.

Samples of a cattle ration, a poultry ration and a pig ration, containing 25, 30 and 65 U kg^{-1} of α-tocopherol, respectively, were finely ground before distribution together with a specimen of pure α-tocopherol to the laboratories. The α-tocopherol was included so that all the laboratories used the same standard material for calibration. Recipients were asked to analyse the samples within 2 weeks of receipt. It was hoped that these precautions would reduce any uncertainty caused by possible inhomogeneity of the samples and loss of vitamin E on storage, which was known to occur with certain feeds. Participants were asked to determine the moisture content of all samples so that the α-tocopherol content could be calculated on a dry matter basis.

Results

The results for the first collaborative exercise are given in Table 3. The feeds contained 5–6% of fat and it was thought that the presence of this amount of fat might have made the saponification time inadequate. In most instances, the agreement between duplicate results obtained in the same laboratory was good, although the range of results between the different laboratories was high.

Second and Third Collaborative Exercises

Two further samples of animal feeds were circulated, followed by three samples of different types of pet foods. The pet foods were either hard, dry material or supplied in sealed cans and the results were expressed on the samples as received.

Results

The results for the second and third exercises are given in Tables 4 and 5. A similar pattern of results was found as in the exercise reported in Table 3, where the majority of the laboratories produced results close to the means with little difficulty, while others were unable to obtain satisfactory results. The most common problem was non-linear calibration graphs and poor, long-trailing peaks. Laboratory E, which was unable to obtain good peaks, by using the proposed

Table 3 First collaborative exercise: vitamin E content of animal feed samples

Laboratory	Ration 125 (cattle)				Ration 831 (poultry)				Ration 470 (pig)			
	Vitamin E level/U kg^{-1}				Vitamin E level/U kg^{-1}				Vitamin E level/U kg^{-1}			
	As received		On dry matter		As received		On dry matter		As received		On dry matter	
A	22.5	22.8	25.6	25.9	26.6	27.1	30.5	31.0	54.7	54.7	60.0	60.0
B	22.3	22.1	25.3	25.1	27.5	28.6	31.5	32.8	64.9	68.4	71.5	75.3
C	23.1	23.4	26.2	26.6	26.2	24.0	30.3	27.8	58.3	58.8	64.4	65.0
D*	23.5	24.8	26.8	28.3	30.0	31.5	34.5	36.2	60.0	72.0	66.2	79.5
E	21.9	20.8	24.9	23.7	28.3	28.8	32.4	33.0	65.3	63.0	72.1	69.5
F†	—	—	—	—	—	—	—	—	—	—	—	—
H	23.8	24.5	27.2	28.0	30.9	30.9	35.5	35.5	70.5	70.5	78.4	78.4
J	24.2	23.3	27.7	26.7	29.5	29.5	34.3	34.3	68.2	69.3	75.5	76.7
K‡	18.0	18.1	20.4		22.5	26.4	28.0		61.6	59.6	66.0	
L	24.5	24.3	27.9	27.7	34.8	34.9	39.9	40.0	72.2	69.2	79.8	76.5
M§	32.0	31.0	36.5	35.4	33.0	34.0	37.9	39.0	66.0	65.0	72.7	71.6
Mean:	23.1		26.2		29.8		34.1		64.3		71.7	
SD:	1.11		1.31		2.97		3.65		5.80		6.37	
RSD (%):	4.8		4.98		9.95		10.7		9.03		8.88	

* D omitted, UV absorbance detector.
† F omitted, gross difference of results.
‡ K omitted at request of collaborator.
§ M results for ration 125 rejected as outliers by Dixons test.

Table 4 Second collaborative exercise: α-tocopherol content of samples of animal feeds

Laboratory	Ration 722				Ration 748			
	α-Tocopherol level/U kg^{-1}				α-Tocopherol level/U kg^{-1}			
	As received		On dry matter		As received		On dry matter	
A	71.7	73.7	77.5	79.7	124.8	126.8	141.2	143.5
B	69.2	68.9	74.7	74.4	124.5	125.4	140.7	141.7
C	—	—	86.0	85.0	—	—	124.0	125.0
D*	91.3	93.5	99.2	101.6	150.7	154.0	170.9	174.6
E	65.8	68.3	70.2	72.9	124.8	138.2	138.5	153.4
F*	—	—	68.1	70.1	—	—	129.4	127.8
H	76.0	78.1	82.2	84.4	131.4	132.0	148.6	149.3
K	70.5	72.1	75.6	77.4	142.3	138.8	160.1	156.1
L	—	—	79.4	84.2	—	—	149.0	143.2
M	63.0	62.0	68.0	67.0	104.0	92.0	118.0	104.0
P	—	—	84.0	82.0	—	—	150.0	145.0
Q*	—	—	55.0	51.5	—	—	134.7	134.8
Mean:	69.9		78.0		125.4		140.6	
SD:	4.84		5.97		14.4		14.3	
RSD (%):	6.93		7.64		11.5		10.2	

* Omitted, as UV absorbance detectors used.

Table 5 Third collaborative exercise: α-tocopherol content of pet food samples

Laboratory	Sample A		Sample B		Sample C	
	α-Tocopherol/U kg^{-1}		α-Tocopherol/U kg^{-1}		α-Tocopherol/U kg^{-1}	
A	22.4	22.2	183.9	180.6	75.5	79.2
B	18.5	18.2	171.2	174.0	67.8	68.2
C	19.8	18.8	194	195	82	84
D*	23.0	21.3	189	196	87	85
E	20.1	20.3	184.4	175.6	76.9	77
F*	21.2	21.3	151.3	150.8	80.4	72.6
H	22.2	22.2	175.5	180.4	77.9	80.1
K†	20.9	20.9	165.8	166.9	74.6	79.5
L	24.1	24.2	188.6	187.3	82.4	82.3
M	17.1	17.6	160.0	175.0	83.0	85.0
Q*	16.1	15.5	129.4	144.0	45.7	46.0
Mean:	20.6		176.7		78.4	
SD:	2.20		13.3		5.23	
RSD (%):	10.1		7.5		6.9	

* D, F and Q omitted as UV absorbance detector used; also, Q had a delay between extraction and chromatography.
† Isooctane–tetrahydrofuran used as mobile phase.

method, regularly carried out determinations of vitamin E using a reversed-phase system. Their own method gave results close to the means of the results obtained by the other laboratories.

Exploration of Further Alternatives

The hexane–1,4-dioxane mobile phase had been used in the first trial and the comparison with the isooctane–tetrahydrofuran mixture had not shown any grounds for change. However, the attention of the Sub-Committee was drawn to a French standard[4] in which a mixture of isooctane and propan-2-ol was used as a mobile phase and the extract in light petroleum was treated with sulphuric acid. One laboratory explored aspects of the French work by testing a range of pet foods using hexane–1,4-dioxane (97 + 3, v/v) and isooctane–propan-2-ol (99 + 1, v/v) as the mobile phases and concluded that the isooctane–propan-2-ol mixture could be a satisfactory alternative to the first mixture. However, the member concerned did not think there would be a sufficient improvement to justify a change; it was also noted that an aged sample of cattle ration apparently contained an impurity that was not resolved from the α-tocopherol peak by the icooctane–propan-2-ol mixture. A mixture in which the propan-2-ol content was reduced (99.5 + 0.5, v/v) gave longer retention times with no improvement in resolution. The same laboratory also carried out a trial of the effect of treatment of the extract with sulphuric acid. A portion of ground wheat was extracted and the amount of α-tocopherol present determined in a portion of the extract using the hexane–1,4-dioxane mobile phase. Another portion of the extract was treated with sulphuric acid, washed with water until free of acid, dried and the α-tocopherol content determined. The recovery was 56% of the amount recovered from the untreated extract. The chromatogram was cleaner with fewer late eluting peaks but the low recovery was a serious objection. When a solution of α-tocopherol in hexane (10 μg ml^{-1}) was treated with sulphuric acid in this way, the recovery was 79%. It was also noted that if any tocotrienols were present they might be converted by the acid treatment into compounds that might not be separated from the α-tocopherol, leading to erroneously high results.

Table 6 Fourth collaborative exercise: comparison of two mobile phases for chromatography of a solution of α-tocopherol. α-Tocopherol content peak areas normalized

Concentration/ μg ml^{-1}	Laboratory					
	A1*	A2*	B	G	H	J
Hexane–1,4-dioxane (97 + 3); *flow-rate*, 1.5 ml min^{-1}—						
2	18.9	17.3	22.4	17.2	11.0	15.7
4	39.0	35.0	39.5	37.9	30.2	37.3
6	61.0	57.7	61.3	59.0	54.0	59.2
8	78.4	77.6	81.8	81.1	79.3	79.2
10	100.0	100.0	100.0	100.0	100.0	100.0
Intercept:	−1.020	−4.830	1.750	−3.600	−13.240	−4.870
Regression:	0.999	0.999	0.999	1.000	0.999	1.000

	Laboratory						
	A1	A2	B	H	E1†	E2†	J
20	21.3	17.7	21.6	17.2	0.9	10.9	27.2
40	42.6	37.7	40.0	37.3	12.5	28.7	37.7
60	65.3	59.2	58.1	57.5	36.5	48.9	58.5
80	84.1	80.1	83.6	77.2	65.4	71.5	79.0
100	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Intercept:	2.990	−3.160	0.540	−3.810	−32.310	−14.290	−3.590
Regression:	0.998	1.000	0.996	1.000	0.995	0.996	1.000

	Laboratory						
	A1	A2	B	E1	E2	H	J
Isooctane—propan-2-ol (99 + 1); *flow-rate*, 1 ml min^{-1}—							
2	18.3	22.2	22.0	13.4	11.7	19.0	19.0
4	27.8	41.3	40.0	32.5	30.8	38.7	38.9
6	58.9	63.0	53.0	55.2	54.7	59.4	58.7
8	77.4	78.6	82.2	77.7	80.4	79.2	78.8
10	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Intercept:	−7.410	3.150	−0.200	−9.760	−12.340	1.490	−1.490
Regression:	0.991	0.989	0.993	−1.00	− 0.999	1.000	1.000

	Laboratory						
	A1	A2	B	E1	E2	H	J
20	20.2	20.7	22.2	18.7	17.0	19.2	17.5
40	39.9	41.4	40.2	38.6	36.8	39.5	37.1
60	59.0	60.7	60.8	59.6	56.9	60.0	56.4
80	79.3	80.9	71.6	79.1	78.2	78.7	76.5
100	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Intercept:	−0.020	1.310	2.860	−1.730	−4.440	−0.760	−3.820
Regression:	1.000	1.000	0.993	1.000	1.000	1.000	0.990

* Laboratory A used two columns containing the same packing from different suppliers.
† Laboratory E used two columns containing equivalent packings from the same supplier.

Fourth Collaborative Exercise

Members decided to carry out a short trial of the two mobile phases, hexane–1,4-dioxane (99 + 1, v/v) and isooctane–propan-2-ol (99 + 1, v/v). A solution of α-tocopherol in hexane was prepared and portions were distributed to the participants who were asked to dilute it with hexane to give a range of solutions with a nominal α-tocopherol content of 2–100 μg ml^{-1}. Portions of these solutions were then chromatographed using each of the mobile phases.

Results

The results obtained in this exercise are given in Table 6. The areas of the peaks were normalized so that the areas in all instances should be 20, 40, 60, 80 or 100. Statistical examination of the results showed virtually no regression curves passing through the origin. The use of the isooctane—propan-2-ol mobile phase gave steeper calibration graphs, and fewer difficulties were experienced by the users than with the hexane–1,4-dioxane system. Laboratory E obtained more nearly linear calibration graphs with the isooctane–propan-2-ol mixture than with the other, but their graphs were further from the origin than those obtained by the other laboratories.

The Sub-Committee was reluctant to make a major change and to discard the hexane–1,4-dioxane system in favour of the isooctane–propan-2-ol mixture at this stage of the investigation. Both systems performed satisfactorily in a number of laboratories but a minority experienced difficulties with the hexane–1,4–dioxane system. It was noted that one laboratory (E) was the only one that obtained 1,4-dioxane and hexane from one particular supplier and when they changed their supplier and used a new column they obtained satisfactory results with both systems. It was found that the unsatisfactory results were related to the presence of some substance in the hexane and/or 1,4-dioxane which they had used, even though these solvents were supplied for chromatographic use. Further, after the passage of these materials through the column, its properties were adversely affected so that it did not give satisfactory results when used with solvents from another source or when tested by another member. The 1,4-dioxane originally used by Laboratory E had been in their laboratory for some considerable time and it was thought that their problems might have been due to the age of this material, especially as it was known that 1,4-dioxane readily formed peroxy compounds when stored in a partly filled bottle. An improvement could be effected by passing a stream of nitrogen through the 1,4-dioxane for a short period of time but it would be preferable to purchase this material in such amounts that it is not stored for any length of time in a partly filled bottle.

The Sub-Committee was advised that users could expect to obtain isooctane and propan-2-ol of more consistent quality than hexane and 1,4-dioxane.

Fifth Collaborative Exercise

A representative range of five animal feeds and three pet foods were examined using the isooctane–propan-2-ol mobile phase. Full details of the method used are given in the Appendix as Method A.

Results

The results obtained in this exercise are given in Tables 7 and 8. The results from Laboratory K were regarded as outliers and omitted from the statistical treatment.

Sixth Collaborative Exercise

One member with considerable experience in the determination of vitamins by HPLC considered that there was a possible source of error in the use of calibration graphs, as the properties of a column could change between operations. It was his practice to bracket each unknown sample by the injection of a pair of standard solutions one above and the

Table 7 Fifth collaborative exercise: α-Tocopherol content of animal feeds. All values in U kg^{-1}

	Feed A			Feed B			Feed C		
Laboratory			Mean			Mean			Mean
A	63.4	59.9	61.7	81.5	88.2	84.9	134.0	118.0	126.0
B	70.6	69.0	69.8	99.6	105.9	102.8	151.2	159.9	155.6
C	69.5	68.4	69.0	94.6	100.0	97.3	144.9	142.7	143.8
E	67.5	63.2	65.4	101.1	99.7	100.4	137.9	138.2	138.1
F*	58.9	56.3	57.6	85.6	93.8	89.7	138.0	138.4	138.2
G	67.4	72.0	69.7	99.5	101.0	100.3	139.9	142.7	141.3
H	67.7	68.1	67.9	83.6	78.8	81.2	151.2	151.3	151.3
J	57.9	55.1	56.5	89.8	87.8	88.8	125.0	123.1	124.1
K†	32.0	56.0	44.0	86.0	70.0	78.0	114.0	111.0	112.5
Mean:			65.7			93.7			114.0
SD:			4.99			8.58			11.8
RSD (%):			7.6			9.1			8.5

	Feed D			Feed E		
Laboratory			Mean			Mean
A	152.0	146.0	149.0	69.8	65.5	67.7
B	178.2	186.6	182.4	84.6	90.2	87.4
C	206.3	205.4	205.9	79.6	78.3	79.0
E	150.2	148.3	149.3	83.0	82.2	82.6
F*	172.6	171.8	172.2	79.8	77.5	78.7
G	182.1	177.5	179.8	82.3	85.1	83.7
H	171.7	170.8	171.3	82.1	78.4	80.3
J	158.5	155.5	157.0	72.3	73.1	72.7
K†	159.0	163.0	161.0	67.0	69.0	68.0
Mean:			170.7			79.1
SD:			20.7			6.77
RSD (%):			12.1			8.6

* F omitted, UV absorption detector used.
† K omitted, spread of results too great.

Table 8 Fifth collaborative exercise: α-tocopherol content of pet foods. All values in U kg^{-1}

	Pet food F			Pet food G			Pet food H		
Laboratory			Mean			Mean			Mean
A	—	—	135.5	—	—	323.5	—	—	10.7
B	201.9	193.2	197.6	300.6	306.2	303.4	16.8	15.7	16.3
C	205.0	196.0	200.5	340.6	339.0	339.5	11.0	11.4	11.2
E	173.4	175.2	174.3	345.3	339.6	342.5	11.8	11.8	11.8
F*	172.4	170.5	171.5	303.3	315.6	309.5	11.2	10.8	11.0
G	191.2	188.7	190.0	325.3	325.3	325.3	12.3	12.3	12.3
H	191.6	192.1	191.9	336.7	340.2	338.5	12.2	12.0	12.1
J	175.8	172.0	173.9	271.1	259.1	265.1	10.7	10.2	10.5
K*	175.0	140.0	157.5	167.0	220.0	193.5	5.0	8.0	6.5
Mean:			180.5			319.7			12.1
SD:			22.4			27.6			1.96
RSD (%):			12.4			8.6			16.2

* F and K omitted.

Table 9 Sixth collaborative exercise: determination of α-tocopherol in animal feeds and pet food by Method B. All values in U kg^{-1}

	Animal Feed A			Animal Feed B			Pet food		
Laboratory			Mean			Mean			Mean
A	85.3	87.6	86.5	229.3	223.8	226.6	190.6	195.1	192.9
B	79.1	78.0	78.6	233.6	232.9	233.3	184.6	185.8	185.2
C	89.4	91.6	90.5	218.6	220.1	219.4	194.4	197.9	196.2
D*	84.0	85.0	84.5	225.1	227.0	226.1	198.9	195.2	197.1
E	81.1	79.7	80.4	220.4	232.9	226.7	202.4	200.6	201.5
F	—	—	—	—	—	—	—	—	—
G	78.9	80.3	79.6	218.8	219.7	219.3	193.6	204.1	198.9
Mean:			83.1			225.1			194.9
SD:			5.15			5.88			6.31
RSD (%):			6.2			2.6			3.2

* D omitted as UV absorbance detector used.

other below the concentration of vitamin in the unknown sample. Some members thought that this procedure would be more time consuming than the use of a calibration graph as it could involve the repetition of a determination and the preparation of two more standard solutions if the unknown did not fall within the standards. On the other hand, the time required for the preparation of the calibration graph would be saved and in most instances the analyst would know the expected level of vitamin in the sample and would prepare the standard solutions accordingly. Many laboratories handling a number of samples would have automatic injection equipment so that the repeated injection of the standards would present no problems and would provide a continual check on the performance of the column. It was agreed to follow this procedure in a limited exercise. It was also decided to specify that the 1,4-dioxane used to prepare the mobile phase should be taken from a freshly opened bottle, as it seemed likely that this material would be free from peroxy compounds and should avoid the problems previously experienced by laboratory E. Two samples of animal feeds and one of a dry pet food were circulated to be examined by the method given in the Appendix as Method B.

Results

The results are given in Table 9 and show very good agreement between the laboratories.

Discussion

High-performance liquid chromatographic methods employing both isooctane–propan-2-ol and hexane–1,4-dioxane have been examined and shown to be capable of giving satisfactory results. The earlier unexplained problems with the hexane–1,4-dioxane mobile phase led to considerable extra work culminating in the exercise using isooctane–propan-2-ol. (Tables 7 and 8). The Sub-Committee confidently recommended the adoption of this method (Method A).

However, as it had been clearly shown that only some aged samples of 1,4-dioxane had led to poor results and column damage and the final exercise had confirmed that material from a freshly opened bottle gave satisfactory results, the Sub-Committee can recommend the adoption of Method B. If Method B is employed, care should be taken that the 1,4-dioxane has not been exposed to the air in a partly filled bottle. Occasional users would be well advised to purchase their supplies in smaller bottles and open a fresh one before use.

Animal feeds contain up to 14% of moisture and as mentioned above, it was decided to ask the laboratories to determine the moisture content of their samples and to report the α-tocopherol content on a dry matter basis. This would eliminate any errors caused by changes in the moisture content of the samples. However, in the UK, the Statutory requirement is for the tocopherol content to be determined on the sample as received and no allowance to be made for the moisture present.

Samples of some feeds have shown a 'shoulder' on the α-tocopherol peak when examined by Method A. This shoulder might be due to the presence of tocotrienols in the sample which can be removed by treatment of the extract with sulphuric acid, but, as indicated in this work, this treatment can cause loss of α-tocopherol and is not recommended. Method B is recommended for these samples.

The Analytical Methods Committee is particularly grateful to those laboratories that are not members of the Sub-Committee for their co-operation, especially as some of these laboratories experienced problems with the method, which they were unable to resolve.

Recommendation

The Sub-Committee recommends the use of Method A for the determination of α-tocopherol in animal feeds and pet foods. Quantification should be carried out using either the calibration process or, alternatively, using the bracketing method. Some samples might produce chromatograms with interfering peaks, under which circumstances Method B should be employed. If Method B is employed, care should be taken to ensure that the 1,4-dioxane used is free from peroxy compounds.

APPENDIX

Determination of α-Tocopherol in Animal Feeds by Normal-Phase High-performance Liquid Chromatography, Method A

Purpose and Scope

This method is for the determination of α-tocopherol in animal feedingstuffs including pet foods.

Principle

The sample is hydrolysed with ethanolic potassium hydroxide solution and the α-tocopherol extracted into light petroleum. The light petroleum is removed by evaporation and the residue dissolved in hexane. The α-tocopherol content of the sample extract is determined using normal-phase high-performance liquid chromatography.

Reagents

Ethanol, 95% v/v, or industrial methylated spirit (IMS).

Sodium ascorbate solution, 10 g per 100 ml.

Potassium hydroxide solution. Dissolve 500 g of potassium hydroxide in water and make up to 1 l.

Sodium sulphate, anhydrous.

Light petroleum, boiling range 40–60 °C; *residue on evaporation less than* 2 mg per 100 ml.

Inert gas, e.g., *nitrogen.*

Hexane, HPLC grade.

Standard substance. Pure ±-α-tocopherol.

α-Tocopherol stock standard solution, 1 mg ml^{-1}. Weigh, to the nearest 0.1 mg, 100 mg of α-tocopherol, dissolve in hexane in a 100 ml calibrated flask and make up to volume with hexane and mix.

α-Tocopherol intermediate standard solution, 100 μg ml^{-1}. Transfer, by pipette into a 50 ml calibrated flask, 5.0 ml of α-tocopherol stock standard solution and dilute to volume with hexane and mix.

Mobile phase. Mix 990 ml of isooctane and 10 ml of propan-2-ol.

Apparatus

High-performance liquid chromatograph. This featured the following: a pump, delivering a constant eluent flow-rate of 1.0 ml min^{-1}; a column 250 × 5 mm i.d., packed with silica, particle size 5 μm, *e.g.*, Partisil 5 Si; and a fluorescence detector set at excitation and emission wavelengths of 293 and 326 nm, respectively.

Boiling water-bath.

Rotary film evaporator, with water-bath at 40 °C.

Apparatus for the extraction of α-tocopherol from the saponified feed (see Fig. 1). This consisted of the following: a cylinder (of either the graduated or Dreschel bottle type) of 1 l capacity and fitted with a ground-glass neck and stopper; and a ground-glass joint, fitting the cylinder and equipped with an adjustable tube (T) passing through the centre and a side arm (A). The adjustable tube should have a U-shaped lower end and a jet (J) at the opposite end so that the upper liquid layer in the cylinder may be transferred to a separating funnel *via* the jet, on application of pressure at the side arm.

Separating funnel, 1 l capacity.

Rotary evaporator flask, 1 l capacity.

Procedure

NOTE:—α-Tocopherol is extremely sensitive to decomposition by UV light and air. All operations should be carried out away from natural and fluorescent light and as rapidly as is consistent with accurate working. Use amber-coloured glassware or glassware protected with aluminium foil throughout. Failure to observe these precautions will result in low recoveries of α-tocopherol.

Saponification

Weigh, to the nearest 0.1 g approximately 50 g of the feedingstuff, previously ground to pass through a 1 mm sieve. Transfer into a 1 l conical flask and add 200 ml of ethanol or IMS. Add 2 ml of sodium ascorbate solution, 50 ml of potassium hydroxide solution and mix by swirling. Fit a reflux condenser to the flask and immerse the latter in a boiling water-bath and allow to reflux for 30 min, swirling occasionally. Cool the flask to room temperature under a stream of cold water.

Extraction

Transfer the contents of the saponification flask into the extraction cylinder. Rinse the saponification flask with 2 × 25 ml portions of ethanol or IMS and transfer the rinsings into the cylinder. Repeat this procedure with 2 × 125 ml portions of light petroleum and one 250 ml portion of water. Stopper the cylinder and shake well for 1 min. Allow the phases to separate and cool the cylinder under a stream of cold water before removing the stopper. Insert the adjustable tube into the cylinder positioning the U-shaped lower end so that it is just above the level of the interface. By application of pressure to the side arm, using an inert gas, transfer the upper, light-petroleum layer into a 1 l separating funnel. Add 125 ml of light petroleum to the cylinder, stopper and shake well for 1 min. Allow the phases to separate and transfer the upper layer into the separating funnel by using the adjustable tube as before. Repeat the extraction with a further 125 ml of light petroleum, transferring the upper layer again into the separating funnel.

Wash the combined light-petroleum extracts with 4 × 100 ml portions of water. It is important to avoid the formation of an emulsion and the first wash should be by gentle inversion, followed by gentle shaking. Transfer the washed extract, through a medium/fast filter-paper containing approximately 60 g of anhydrous sodium sulphate, into a 1 l rotary evaporator flask. Rinse the funnel with 2 × 25 ml portions of light petroleum, and add these, through the filter, to the rotary evaporator flask. Rinse the filter with 2 × 25 ml portions of light petroleum and collect the washings in the rotary evaporator flask.

Evaporate the light petroleum extract to dryness, under vacuum and at a temperature not exceeding 40 °C. Restore atmospheric pressure by admitting the inert gas. Dissolve the residue immediately in a minimum amount of hexane and transfer the solution quantitatively into a 25 ml calibrated flask. Make the contents of the flask up to volume and mix. If necessary, dilute an aliquot of the sample extract with hexane so as to obtain an expected α-tocopherol concentration of between 5 and 10 μg ml^{-1}. Filter the sample extract through a 0.5 μm Millipore filter before subjecting the extract to HPLC.

High-performance liquid chromatography

By using isooctane–propan-2-ol as the mobile phase, set the flow-rate of the chromatograph pump to 1.0 ml min^{-1}, and

inject onto the column 20 μl of the sample extract. Measure the height of the α-tocopherol peak and determine the concentration of α-tocopherol in the extract by reference to the calibration graph.

Calibration graph

Into a series of 50 ml calibrated flasks, transfer by pipette 1, 2, 3, 4 and 5 ml of the α-tocopherol intermediate standard solution, make up to the mark with hexane and mix. These solutions contain 2, 4, 6, 8 and 10 μg ml^{-1} of α-tocopherol respectively. Inject 20 μl of each solution onto the column and measure the height or area of the α-tocopherol peak. Plot a calibration graph using the peak heights or areas of α-tocopherol, as the ordinates and the corresponding concentrations in μg ml^{-1} as abscissae.

Calculation of Results

The α-tocopherol content of the sample in U kg^{-1} is given by the equation:

$$\frac{25c \times 1.1F}{m}$$

where c = concentration of α-tocopherol (μg ml^{-1}) in the sample extract; F = dilution factor described under Extraction; and m = mass of the test portion saponified (g) (1.1 U = 1 mg of ±-α-tocopherol).

Alternative Procedure Using Bracketing Method

Prior to the actual determination, carry out five injections of a standard solution of the approximate expected α-tocopherol content of the sample. These results should be consistent and within ±5% of the expected value. When performing the actual analysis of a sample, inject a standard solution, followed by two replicate injections of the unknown sample and finally a second standard. The two standards should give peaks of similar areas/heights and the mean should be used for the calculation of the α-tocopherol content of the sample.

Determination of α-Tocopherol in Animal Feeds by Normal Phase High-performance Liquid Chromatography, Method B

Purpose and Scope

This method is for the determination of α-tocopherol in animal feedingstuffs including pet foods.

Principle

The sample is hydrolysed with ethanolic potassium hydroxide solution and the α-tocopherol extracted into light petroleum. The light petroleum is removed by evaporation and the residue dissolved in hexane. The α-tocopherol content of the sample extract is determined using normal-phase HPLC.

Reagents

Ethanol, 95% v/v or industrial methylated spirit (IMS).

Sodium ascorbate solution, 10 g per 100 ml.

Potassium hydroxide solution. Dissolve 500 g of potassium hydroxide in water and make up to 1 l.

Sodium sulphate, anhydrous.

Light petroleum, boiling range 40–60 °C; *residue on evaporation less than* 2 mg per 100 ml.

Inert gas, e.g., *nitrogen.*

Hexane, HPLC grade.

Standard substance. Pure ±-α-tocopherol.

Standard solution. Accurately weigh a suitable amount of pure α-tocopherol into a calibrated flask, dissolve and make up to the mark with hexane.

1,4-*Dioxane, HPLC grade.*

Mobile phase. Mix 970 ml of hexane and 30 ml of 1,4-dioxane.

Apparatus

High-performance liquid chromatograph. This featured the following: a pump, delivering a constant eluent flow-rate of 1.5 ml min^{-1}; a column, 250 × 4.6 mm i.d., packed with silica, particle size 5 μm, *e.g.*, LiChrosorb Si 60; and a fluorescence detector set at excitation and emission wavelengths of 293 and 326 nm, respectively.

Boiling water-bath.

Rotary film evaporator, with water-bath at 40 °C.

Apparatus for the extraction of α-tocopherol from the saponified feed (see Fig. 1). This consisted of the following: a cylinder (of either the graduated or Dreschel bottle type) of 1 l capacity and fitted with a ground-glass neck and stopper; and a ground-glass joint, fitting the cylinder and equipped with an adjustable tube (T) passing through the centre and a side arm (A). The adjustable tube should have a U-shaped lower end and a jet (J) at the opposite end so that the upper liquid layer in the cylinder can be transferred into a separating funnel *via* the jet, on application of pressure at the side arm.

Separating funnel, 1 l *capacity.*

Rotary evaporator flask, 1 l *capacity.*

Procedure

Carry out the saponification and extraction procedures as detailed in Method A.

High-performance liquid chromatography

Set the flow-rate of the hexane–1,4-dioxane mobile phase at 1.5 ml min^{-1}. Inject 20 μl of the standard solution onto the column and wait until the tocopherol peaks have eluted. Repeat the injection and elution five times. The peak heights or areas of the α-tocopherol peaks should not differ by more than 5%.

Inject 20 μl of the sample solution. Wait until elution is complete and repeat the injection. Finally, inject a further 20 μl of the standard solution onto the column. The peak height or area should be essentially identical with that of the earlier standard injections.

The peak heights or areas of the standard and sample solution should not differ by more than 10%.

Calculations

Calculate the concentration of the sample by comparing the peak height or area of the sample with the mean peak height or area of the standard, using the known concentration of α-tocopherol in the standard.

General Comments

In general, it is more convenient to adjust, in as far as is convenient, the concentration of the various sample solutions to be as close as possible to that of the standard. This enables a number of different samples to be analysed sequentially, using the same standard. It is suggested that a standard injection be made between each 2–5 sample injections, always completing the exercise with a standard injection.

The purity of the hexane and 1,4-dioxane, especially the latter is important. It is recommended that only material taken from a freshly opened bottle be used.

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BOOK REVIEWS

Detection-Oriented Derivatization Techniques in Liquid Chromatography

Edited by Henk Lingeman and Willy J. M. Underberg. *Chromatographic Science Series. Volume 48.* Pp. xv + 389. Marcel Dekker. 1990. Price $99.75 (USA and Canada); $119.50 (Export). ISBN 0 8247 8287 9.

During the past decade derivatization techniques have become increasingly important in liquid chromatography as analysts have responded to the demands for greater analytical specificity and sensitivity. This is particularly evident in the fields of biomedical and environmental analysis. Many of the innovations in derivatization chromatography have originated in the various Schools of Analytical Chemistry of Dutch Universities. Therefore, it is hardly surprising that the editors of this splendid overview have, in the main, called upon local experts. Some may argue that a broader cross-section of international scientific opinion should have been sought. In the reviewer's opinion, however, it is doubtful that by doing so a more useful text would have resulted.

The book consists of ten chapters, each written by a different team of authors, covering the essential theoretical, instrumental and practical aspects of the topic, as follows.

(*i*) Derivatization in liquid chromatography: an introduction by H. Lingeman and W. J. M. Underberg; (*ii*) Derivatization reactions and kinetics in liquid chromatography by A. N. Fruijtier, H. Lingeman, J. H. Beijnen and W. J. M. Underberg; (*iii*) Sample pre-treatment procedures by H. Lingeman and U. R. Tjaden; (*iv*) Post-chromatographic reaction detection by G. J. de Jong, U. A. Th. Brinkman and R. W. Frei; (*v*) Enantiomeric derivatization by S. Görög; (*vi*) Enzymatic derivatization by P. C. van Krimpen and M. A. J. van Opstal; (*vii*) Ultraviolet derivatization by J. A. P. Meulendijk and U. T. M. Underberg; (*viii*) Electrochemical derivatization by J. A. Lisman, W. J. A. Underberg and H. Lingeman; (*ix*) Fluorescence derivatization by J. Goto; and (*x*) Chemiluminescence derivatization reactions in liquid chromatography by K. Imai.

The first part of the book deals with the fundamentals of derivatization liquid chromatography. In the first chapter a brief review of derivatization chromatography is presented. Here, situations where derivatization is appropriate are identified and the improvements in selectivity and chromatographic behaviour delineated. Guidance is given on the choice of pre- and post-column techniques, their associated advantages and disadvantages, and the selection of appropriate derivatization reagents. Somewhat surprisingly, the computer-aided optimization of derivatization processes is hardly mentioned. The second chapter is devoted to a discussion of reaction mechanisms, kinetics, reaction conditions and characteristics of derivatization reagents. Reactions commonly used for solute modification including alkylation, acylation, arylation, silylation, esterification and cyclization are treated. Much of this material is standard organic chemistry, nevertheless, the inclusion does much to enhance the usefulness of the text to the practising analyst.

Methods for the isolation of analytes from various matrices, in particular biological fluids and tissues, are covered in Chapter 3. Included are details of solvent extraction, de-proteination, ion-pair and solid-phase procedures, and their effective link-up to pre-column derivatization is discussed. Various reactors are described and their application related to the kinetics of derivatization reactions. In addition to the advantages of this approach, mention is made of the attendant problems, in particular zone dispersion, pressure drop and phase separation. Newcomers to derivatization chromatography could find the ideas put forward in this chapter, and those in Chapter 6 on enzymatic processes, particularly rewarding.

Chapter 5 provides useful information on the application of derivatization reactions to facilitate the assay of enantiomeric substances. Both chiral and achiral reagents are considered for use with conventional and chiral liquid chromatographic columns, respectively.

The last four chapters describe derivatization procedures to enhance the detectability of analytes that are appropriate to the most widely used detector systems available for use in liquid chromatography, namely ultraviolet, electrochemical fluorescence and chemiluminescence. In each instance guidelines are given to the choice of reagent together with sound practical advice. In common with the earlier chapters in the book these chapters include useful bibliographies.

In conclusion, this is an excellent overview of a topic which is becoming increasingly important in contemporary liquid chromatography. The various authors are to be complemented on the way that they have combined the theoretical and practical aspects of the subject to produce a very readable text. Although not inexpensive, purchase by analytical chemists using chromatographic techniques is recommended.

M. B. Evans

L'Atomisation Électrothermique en Spectrométrie d'Absorption Atomique

H. Hoenig and A. M. de Kersabiec. Pp. 295. Masson. 1990. Price Fr 380.00. ISBN 2 225 81937 8.

This is a very modern text, the last references date from late 1988 and the book was published in 1990. Written by Michel Hoenig of the Institute of Chemical Research of the Belgian Ministry of Agriculture and Ann-Marie de Kersabiec of the CNRS Laboratory of Geochemistry and Metallurgy Engineering of the University Paris VI, it represents a major contribution to the science and practice of atomic absorption spectrometry. Dedicated particularly to electrothermal atomization, the authors set out to give the reader not only a theoretical and scientific basis for the technique, but also the most important practical details which transform textbook description into practicable methodology. In this respect, this is one of the most successful texts I have read in recent years, technique-oriented texts often being either entirely theoretical and leaving the reader with little knowledge of how exactly to start applying the method, or alternatively so practically oriented towards specific instrumentation and particular matrices, that the textbook rapidly ages and it is difficult to translate the approaches into other applications. These authors have avoided this trap by concentrating on the important details of both theory and practice and in so doing, given the reader the sort of foundation he needs to apply the technique generally. The concise text is divided into 11 chapters followed by subject and author indices.

What I particularly like about the book is the correct emphasis given to what in my opinion are the most important aspects of electrothermal atomization. The atomizer itself is treated in considerable detail, with both the chemistry of the surface and the all-important rates of heating being treated in some detail. Sample introduction and the treatment of the signals obtained is particularly well described in Chapter 1. The first chapter covering these aspects is one of the longest in the book, the fifth chapter on interference correction being of approximately equal length. Both spectral and chemical interferences are dealt with and particular emphasis is made of Zeeman correction. Chapter 2 deals with the apparatus for measurements of electrothermal atomic absorption and some details given of modern analytical equipment. Chapter 3 gives

a useful description of calibration procedures and some useful tutorial comments on accuracy and precision. Chapter 4 discusses interferences, both spectral and chemical, leading on to Chapter 5 with a detailed description of interference correction. Chapter 6 describes methods of matrix modification in order to optimize efficiency of atomization and Chapter 7 goes on to discuss the particular problems of refractory elements with particular reference to molybdenum, barium, chromium and vanadium. There then follows two chapters on the very important aspects of sample preparation and the analysis of solid materials. Reading these chapters reinforces the belief that these authors have a very wide and detailed knowledge of the application of electrothermal atomization to a wide range of target elements and matrices. I was particularly impressed by their insight into the problems, many of which we have experienced in this laboratory over two decades of work with the technique. The last two chapters are devoted to the application of the methods to a very wide range of trace elements and matrices, giving ultrabrief descriptions of the sample preparation. In my view this chapter is a little short to be totally useful, but on the other hand gives one some key references as a starting point. The final chapter, Chapter 11, describes the essential details of the elements, their principal analytical wavelengths, typical electrothermal programmes and typical sensitivities. This is a useful compilation of information which I am sure will be handy for the practising analyst.

Altogether I was very impressed by this text, it is well set out, easy to read and written in a modern French which is quickly accessible to anybody with a grounding in that language. The price of the hardback edition is moderate and one hopes that it will appear eventually in paperback and English text. The French version will I am sure be extremely useful to those analytical chemistry departments that are teaching 'Chemistry with French' courses and I am confident that it will find itself on the bookshelves of many practising analysts around the world.

J. F. Alder

Radioanalytical Chemistry: Volumes 1 and 2.
J. Tolgyessey and M. Kyrs. *Ellis Horwood Series in Analytical Chemistry*. Pp. 354 (Vol. 1) and 498 (Vol. 2). Ellis Horwood. 1990. Price £59.95 (Each). ISBN 0 85312 745 X (Vol. 1, Ellis Horwood) 0 85312 747 6 (Vol. 2 Ellis Horwood); 0 470 21301 9 (Vol. 1, Halsted Press) 0 470 21302 7 (Vol. 2, Halsted Press).

These two books deal with a range of aspects of Radioanalytical Chemistry, covering both theoretical considerations and practical applications. The two volumes are individually sufficiently self-contained to be used separately but together provide a comprehensive overview of the subject. The books are well produced and are of high quality with a minimum number of typographical errors. Basic aspects of radioactivity and of radiation detection are not treated in detail and a prior familiarity with these subjects would be required of the reader. The authors present an interesting historical and philosophical background to the material, and provide rigorous definitions of the concepts and conventions used in radioanalytical chemistry. This aspect of the work will be of interest to those actively involved in radioanalytical chemistry but may be too detailed for a wider readership. The main strength of this work lies in the thorough treatment of the principles and applications of radioanalytical methods. The major weakness is that the treatment of instrumentation and the application of computers in radioanalytical chemistry is obsolete.

Volume 1 contains 7 chapters, each of which is followed by a set of references and a selected bibliography. Chapter 1 provides a brief introduction to the subject along with a classification of radioanalytical methods. Chapter 2 deals with analysis by means of activity. Both natural and man-made radionuclides are considered and, while useful information is provided, a definite weakness of this section is the outdated description of the instrumentation used in radioanalytical work. Chapter 3 provides an excellent treatment of isotope dilution analysis. After a general introduction to the principles of the technique, detailed consideration is given to reverse isotope dilution analysis, derivative isotope dilution analysis and substoichiometric isotope dilution analysis. Accuracy, precision and applications of the technique are also fully discussed. Finally, the chapter ends with a section on isotope dilution analysis using stable isotopes and applications of this technique in organic, inorganic and nuclear chemistry. While not falling strictly within the bounds of radioanalytical chemistry, this section does fit well with the rest of the chapter and is a useful inclusion. Chapter 4 deals with radio-reagent methods in which the authors consider the use of radioactive species in reactions and the measurement of the change in activity of the radioactive species in the course of the reaction. A detailed description is given of the authors classification of radio-reagent methods and to the distinction between isotope dilution analysis and radioactive reagent methods. The information provided in this section will be of interest to practising radiochemists but will probably be too detailed for the general reader.

Chapter 5 is devoted to radiometric titrations and includes sections based upon precipitation, complex formation, redox reactions, neutralization and thermoradiometric methods. The final two chapters of this volume are concerned with radiobiology, with Chapter 6 dealing with radioimmunoassay and Chapter 7 with radiochemical methods for determination of biological activity of enzymes. The volume concludes with a comprehensive index.

Volume 2 contains 3 chapters dealing with instrumental methods and automation in radioanalytical chemistry and could be used independently from Volume 1 by those with a specific interest in instrumental radioanalytical chemistry. The format is the same as that of Volume 1, with each chapter being followed by a set of references and a selected bibliography.

The sequence of chapters continues from Volume 1 so Volume 2 starts with Chapter 8 on activation analysis. This is a useful chapter covering the principles of activation analysis as well as practical aspects of the subject. Irradiations using neutrons (both thermal and fast), charged particles and gamma photons are considered and both destructive (radiochemical) and non-destructive (instrumental) methods are described. The chapter concludes with a description of application of activation analysis in a diverse range of subjects including organic and inorganic chemistry, metallurgy, geochemistry, archaeology and others.

Chapter 9 describes non-activation interaction analysis and includes sections on charged particle scattering; absorption and scattering of beta particles and gamma photons; radionuclide induced X-ray fluorescence analysis; neutron thermalization, scattering and absorption plus other specialized techniques. Finally, Chapter 10 is devoted to automation in radioanalytical chemistry and ranges from general considerations to some highly specific applications.

A 172 page Appendix is provided which contains 2 sections: (*i*) a data table on the radioactive nuclides of importance in radioanalytical chemistry; and (*ii*) a table for attaining maximum precision of isotope dilution analysis. The first of the nuclear data tables provides information of the energies and half-lives of radionuclides arranged in order of increasing radiation energy for each of alpha, beta and gamma radiation. These are undoubtedly useful tables but the use of energy ranges rather that discrete energies means that these tables would have to be used in conjunction with more detailed

compilations of exact radiation energies in many instances. As with volume 1, the index for volume 2 is both comprehensive and useful.

The content of these books clearly reflects the theoretical and empirical interests of the authors with the main emphasis being firmly upon classical radioanalytical methods and they make a useful and interesting contribution to the literature. The books will be of most value to those directly involved in radioanalytical chemistry but will also be of value to others such as geochemists and archaeologists who make use of radioanalytical techniques. Some aspects of the work will be extremely useful in the context of a textbook for undergraduate teaching but the value is unfortunately restricted by the obsolete treatment of instrumentation and the application of computers.

These books would make a useful addition to libraries in institutions with an interest in radioanalytical chemistry or its applications. They are priced at a level where they may also be of individual interest to those working in this area in universities or industry.

A. B. Mackenzie

DECHEMA Corrosion Handbook. Volume 5. Corrosive Agents and Their Interaction With Materials
Edited by Dieter Behrens. Pp. ix + 323. VCH Verlagsgesellschaft. 1990. Price DM775.00; £286.00. ISBN 3 527 266569 (VCH Verlagsgesellschaft); 0 89573 626 8 (VCH Publishers).

Volume 5 is the latest contribution to the DECHEMA Corrosion Handbook series, and effectively covers aliphatic amines, alkaline earth chlorides, alkaline earth hydroxides, fluorine, hydrogen fluoride and hydrofluoric acid and hydrochloric acid. The Editor's objective is to compile state-of-the-art knowledge of materials to selected corrosive media, employing corrosion experts to review and evaluate the world-wide literature, including patents, commercial publications and internal publications of universities and research institutes. As in earlier volumes, information and recommendations on the appropriate use of construction materials is given, together with their behaviour during the handling and processing of corrosive agents.

The approach in each chapter is uniform, first an introduction and then the effects of a particular corrosive substance on metallic materials, non-metallic inorganic materials, organic materials and materials with special properties. The introductions are generally short, but not all mentioned the toxicity and hazards—given for the amines, but not to my surprise, for fluorine, hydrogen fluoride and hydrofluoric acid. The interaction with metallic materials, both elements and alloys, is reported systematically (and alphabetically according to their German names). The Non-Metallic Inorganic Materials section includes building materials, glass and quartz, enamel, porcelain, stoneware, and ceramic materials whereas Organic Materials includes natural fibres, bituminous compositions, fats, wood and all the various polymers and resins. Materials with Special Properties includes coatings and linings, seals and packings, various composite and powdered metallurgical materials. Each of these main sections ends with a useful expanded overview of the limits of use, in tabular form, and each chapter concludes with a bibliography. There is also a good subject index.

The intended purpose of this handbook is to help the reader who is considering a particular material in order to answer such questions as: is information available on the behaviour of the material in a particular medium? Which materials cannot be used? Which can be used without hesitation in the medium concerned? What are the conditions under which a less resistant material will give satisfactory service? Also, what protective measures are available (inhibitors, coatings, *etc.*)? Answers to such questions are generally quickly found, and one useful feature is that each material is given the same reference number throughout the volume. For example, nickle–chromium alloys are always found under A27, and if they are not covered in one chapter this designation is missing in the chapter contents.

Most of the chapters are written for a compilation (and translation) of this broad scope. The latest reference regarding aliphatic amines was in 1986; for alkaline earth chlorides, 1981; for alkaline earth hydroxides, 1985; for fluorine, hydrogen fluoride and hydrofluoric acid, 1981 (except for one reference in 1986); and the chapter on hydrochloric acid had several references in 1988.

The book is liberally sprinkled with useful diagrams and tables to illuminate the text. I would, however, recommend the reader turn to the references given in the text he is interested in, to see how recent they are. I noted, for example, that a clear explanation was said to be unresolved, which seemed strange, but the reference was to a 50s paper: the chemical explanation, I expect, has now appeared in a chemical journal.

I am sure that the translaters faced the same problems as those that face translators of the Bible: should it be word-for-word, or thought-for-thought, to name but two. They have done an excellent job, but occasionally a remark concerning the German economy could have been replaced by one relevant or equivalent in the English-speaking world. Presumably they also concluded that to reorganize topics according to the English alphabet for metals etc., was too time-consuming, and this must therefore be recognized when using this Handbook.

The combination of topics is more sensible than in the previous volume, and despite the price, it should be acquired by (or for) corrosion specialists and professionals concerned with large scale handling and usage of acids, alkalis and amines, to whom it will be most welcome.

T. R. Griffiths

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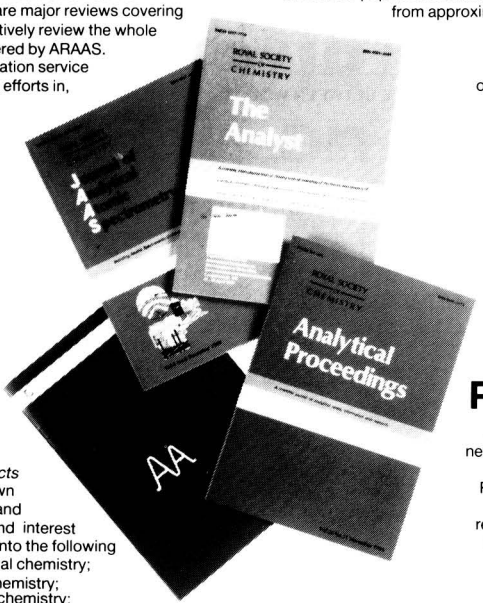

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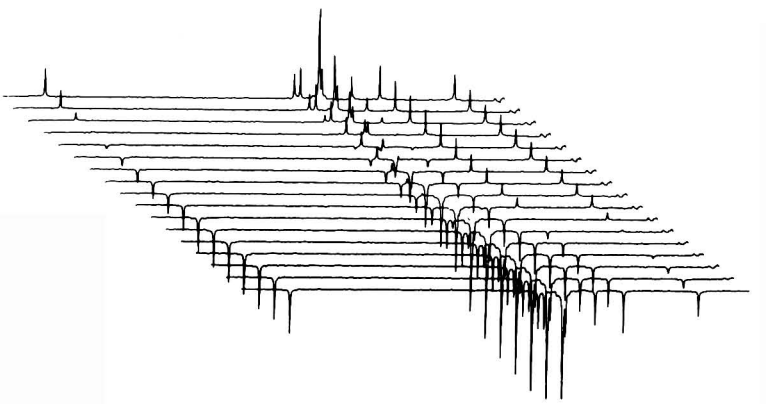

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