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A PHOTO-ELECTROANALYZER FOR DETERMINATION OF VOLATILE NITROSAMINES

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SUMMARY

An apparatus is described in which an aqueous stream of 5 mM NaOH is irradiated by a xenon arc lamp. Nitrosamines injected into the aqueous stream are photochemically decomposed to give nitrite as a product. The nitrite is concentrated by adsorption on an anion-exchange column and subsequently stripped with 0.01 M $HClO_4$. The effluent stream containing nitrite is mixed with a stream of 9 M HCl and the nitrite is detected electrochemically by a flow-through platinum electrode. The detection limit corresponds to injection of approximately 1 ng of *N*-nitrosodipropylamine.

Electrochemical methods of analysis can be highly sensitive, accurate and economical when applied to selected systems of well-defined composition. Difficulties resulting from the lack of selectivity of electroanalytical techniques, when applied to complex mixtures, have been successfully circumvented for many examples by use of chromatographic separations with continuous electroanalysis of the chromatographic effluent in situ (see, e.g. [1—3]). A complete listing of references to applications of amperometric and coulometric detection in liquid chromatography has been compiled [4]; copies are available on request. Commonly reported are detection limits of 10 ng or less for electroactive analytes at the ppb level of concentration.

A sensitive procedure is described here for determination of nitrosamines based on the photochemical decomposition of the nitrosamines to produce nitrite with cathodic detection of the nitrite. Anion-exchange columns are used to minimize interferences, and photolysis of the sample occurs within the chromatographic flow-system in a coiled quartz tube irradiated by a xenon arc lamp.

Two absorption bands are observed in the ultraviolet—visible absorption spectra for nitrosamines in aqueous solutions [5]. The more intense absorption band has a maximum at 220 nm and a molar absorptivity of approximately 7,000 l mol^{-1} cm^{-1}; this band has been identified as resulting from a $\pi \rightarrow \pi^*$ transition [5]. The less intense band at 360 nm, corresponding to a $n \rightarrow \pi^*$ transition, has a molar absorptivity of approximately 100 l mol^{-1} cm^{-1}.

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An early method for the determination of nitrosamines was based on thin-layer chromatography [6]. After separation, the chromatographic plate was exposed to u.v. light, and the nitrosamines were photolytically decomposed with production of nitrite, which was visualized by use of color-forming reagents. As an analytical technique for nitrosamines, this method has the disadvantage that the color-forming reagents can also react with fatty acids, tocopherols, pigments and other compounds to give false identification [7].

Daiber and Preussmann [8] reported a method for the determination of nitrosamines based on the photochemical decomposition of nitrosamines. Nitrosamines were irradiated with light from a Hg lamp for 15 min in aqueous solutions containing Na_2CO_3 or a phosphate buffer at pH 6.5. The nitrite produced was then visualized by production of an azo compound with Griess reagent. The nitrosamines were photolyzed at efficiencies greater than 95% for the concentration range 2×10^{-5}—2×10^{-4} M. The detection limit was 1 μg of *N*-nitrosodipropylamine. Several interferences are expected as discussed for the t.l.c. method [7].

Fan and Tannenbaum [9] modified the procedure introduced by Daiber and Preussmann, using parts from a Technicon AutoAnalyzer. To eliminate interference from nitrate [10], a longer-wavelength source can be used (ca. 350 nm from a General Electric germicidal lamp). A residence time of 30 min was required to photolyze 50% of the nitrosamines. The method gave linear response for concentrations of nitrosamine less than 1 ppm. The detection limit was 30 ng of *N*-nitrosodipropylamine. Interferences noted by Walters et al. [11] were acknowledged but their extent was not determined. Sander [10] stated that photolysis at 350 nm is more specific for compounds having the N—NO structure than photolysis at 250 nm.

Doerr and Fiddler [12] have proposed the use of photolysis in the confirmation of peaks obtained in gas chromatography with a thermal-energy analyzer [13—15]. Seven different nitrosamines were found to be completely photolyzed in methylene chloride, hexane, methanol and water by exposure to radiation at 366 nm for 1 h. Chromatographic peaks obtained prior to photolysis which disappeared after photolysis were presumed to correspond to nitrosamines.

Polarography has been used for the determination of nitrosamines [11, 16, 17], but the method suffers from interferences by pyrazines which are very common in foods [18, 19]. Differential pulse polarography has been investigated for the determination of nitrosamines [20, 21] but suffers from interferences caused by reduction of oxygen and nitrite [20]. Walters et al. [11] used the photolytic reaction to confirm the presence of nitrosamines in polarographic determinations. Derivative polarograms of nitrosamines in 0.2 M HCl were recorded before and after photolysis; disappearance of a peak indicated that the original peak could be ascribed to a nitrosamine. The production of nitrite by photolysis of several compounds in alkaline solutions was investigated. Several compounds underwent photochemical decomposition with production of nitrite in alkaline media including *N*-nitroso-*N*-methylurea, *N*-nitrodimethylamine, nitromethane, pentyl nitrite and ethyl nitrate.

Burns and Alliston [22] studied the rate of photolysis of various nitrosamines in different solutions. A 15-W u.v. lamp (254 nm), for chromatographic detectors, was used to measure the half-lives of photolytic decomposition. The half-lives for nitrosamines decreased as the pH was decreased, e.g., 3.5 h at pH 9.2 and 2.2 h at pH 4 for *N*-nitrosodimethylamine.

EXPERIMENTAL

Photo-electroanalyzer

The photo-electroanalyzer constructed is shown schematically in Fig. 1. Apart from the components related to photolysis, the methods and materials for construction of the analyzer were consistent with accounts given in the literature for ion-exchange liquid chromatography [1, 23] and will not be described here. Light for the photolysis of nitrosamines originated from a 500-W, high-pressure, xenon arc lamp (Model 959C-98; Hanovia Inc.) powered by a Model LPS-255 supply (Schoeffel Instrument Corp.). The lamp housing was Model C-60-51 (Oriel Optics Corp.). Radiation was taken from a side port on the lamp housing without focusing or wavelength discrimination.

The photolysis cell was constructed from quartz tubing (1 mm i.d. and 2 mm o.d.) which was tightly coiled with all loops of the coil being in a single plane. The diameter of the coil was 1.5 in. and the internal volume was about 0.25 ml. Attachment of the quartz photolysis cell to Teflon tubing in the photo-electroanalyzer was made in the conventional manner with plastic connectors [23]. To maximize the total flux of light on the photolysis cell, the cell was positioned so that the plane of the coil was parallel to the axis of the xenon arc, and an imaginary line drawn perpendicular to the plane of the coil passed

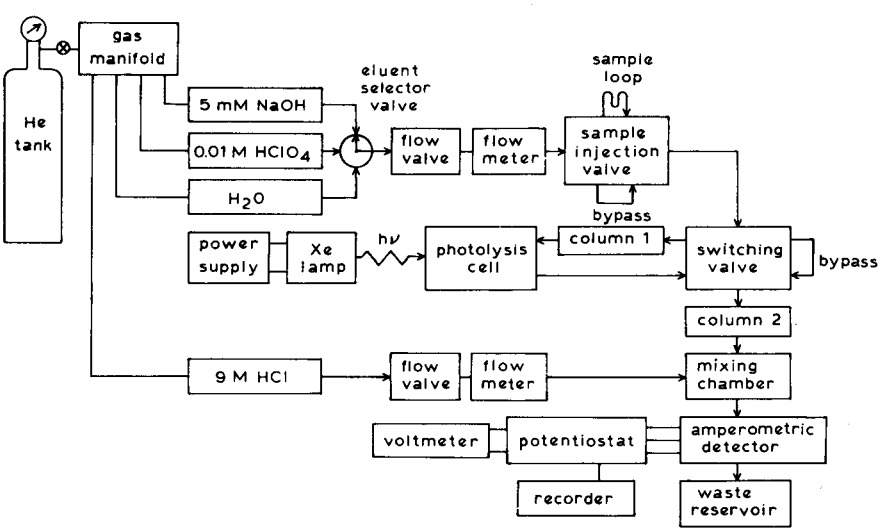


Fig. 1. Schematic diagram of photo-electroanalyzer.

through the center of the arc. The distance from the arc to the coil was ca. 15 cm.

The two Pyrex chromatographic columns (1.5 mm i.d., 10 cm long) included in the photo-electroanalyzer were packed in the conventional manner to about 5 cm with Amberlite IRA-900 anion-exchange resin (60—100 mesh; Rohm and Haas Co.). In some preliminary experiments, these columns were replaced by Teflon tubing (i.d. 0.8 mm).

Two flow-through amperometric detectors were used. For preliminary experiments at high concentrations of nitrosamines, a Pt tubular electrode [1] was used. A Pt wire electrode was used for most of the work reported here. The design of this detector is shown in Fig. 2. The housing was constructed of silica-loaded Teflon and the surface area of the Pt wire indicating (working) electrode was approximately 0.5 cm^2. The counter electrode was a Pt wire and the reference electrode was a miniature Beckman saturated calomel electrode with a fiber junction. The filling solution of saturated KCl was replaced by saturated NaCl. The potential, E_{ref}, was 0.234 V vs. NHE.

Control of electrode potential in preliminary experiments was by a three-electrode potentiostat (Model RDE-3; Pine Instrument Co.). For electrochemical detection with the Pt wire detector for very low concentrations of nitrosamines, the RDE-3 was replaced by a two-electrode potentiostat constructed from a mercury cell and a 10 kΩ, ten-turn potentiometer. The Beckman SCE was used as the auxiliary electrode for this arrangement. Electrical current was monitored by recording the iR drop across a standard resistor in series with the Pt wire detector electrode. The standard resistor was chosen so that the value of iR did not exceed 50 mV. The advantage of using the power supply constructed from the mercury cell for detection at low concentrations was a decreased level of electrical noise in the detector signal.

A strip-chart recorder (Model SR-255B, Heath-Schlumberger Co.) was used. The current—time peaks were integrated with a Keuffel-Esser dual compensating planimeter.

Chemicals and reagents

Nitrosamines were obtained from Aldrich Chemical Co., Eastman Organic Chemicals, and Matheson, Coleman and Bell. (Nitrosamines are potentially

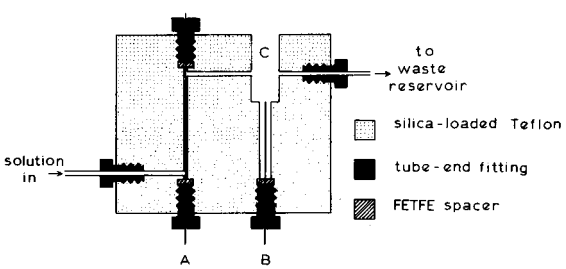


Fig. 2. Diagram of Pt-wire detector. (A) Pt wire working electrode. (B) Pt wire counter electrode. (C) SCE reference.

carcinogenic and should be handled with care.) Solutions of supporting electrolyte were prepared from reagent-grade acids (Fisher Scientific Co.). The water was triply distilled with demineralization after the first distillation; the second distillation was from alkaline permanganate solution. Stock solutions of $NaNO_2$ were prepared with reagent-grade chemical (J. T. Baker Co.) previously dried at 90°C for 4 h and stored in a desiccator over $Mg(ClO_4)_2$.

Preconditioning of platinum detectors

At the start of each working day, the detector electrode was preconditioned to assure maximum and reproducible sensitivity by the following procedure: (i) 0.01 M $HClO_4$ was passed through the detector; (ii) the electrode potential was scanned between +2.0 V and −2.0 V for 15 min at a rate of about 10.0 V min^{-1}; (iii) on completion of a negative sweep, the potential was set to 0.10 V; (iv) a 1-cm^3 aliquot of 10^{-2} M NaI was injected into the stream flowing through the detector; (v) the electrode potential was set to the value of 0.40 V appropriate for detection of nitrite [24].

Analytical procedure for aqueous solutions of nitrosamines

The eluent selector valve was switched so that 5 mM NaOH flowed through the analyzer. The sample injection valve was switched to allow the sample to be drawn into and to fill the sample loop. The switching valve was set to allow flow through column 1 and the rate of flow through columns 1 and 2 was adjusted to 0.5 cm^3 min^{-1}. The rate of flow of the 9 M HCl was then adjusted to 0.5 cm^3 min^{-1}. After a 5-min waiting period, the sample was injected by appropriate switching of the sample injection valve. After a second 5-min waiting period, the switching valve was set to bypass column 1 and the photolysis cell. The eluent selector valve was then switched to 0.01 M $HClO_4$ and nitrite was eluted from column 2 and detected. After elution of column 2 was complete, the switching valve was set so that the stream of 0.01 M $HClO_4$ flowed through column 1 and the photolysis cell. Finally, elution of column 1 by 0.01 M $HClO_4$ was continued for 5—10 min to remove completely any adsorbed anionic species.

Isolation of volatile nitrosamines

The procedure for extraction of volatile nitrosamines from food samples and preparation of aqueous samples as required for analysis was based on the procedure of Fine et al. [14]. This procedure was modified in the extraction step and in the subsequent removal of the organic extractant. In the procedure of Fine et al., methylene chloride is used to extract nitrosamines from food samples. An aqueous solution of nitrosamine is required for the photo-electroanalysis, thus the organic phase must be extracted with water. Methylene chloride is soluble in water to an extent of about 2%. This amount of methylene chloride in the aqueous samples injected resulted in a large interference corresponding to ca. 10^{-6} M *N*-nitrosodipropylamine. Petroleum ether was satisfactorily substituted for methylene chloride without interference.

The isolation procedure was as follows. The sample (100 g) was minced in a Waring Blender and placed in a 500-ml round-bottom flask. After addition

of 20 ml of 0.1 M NaOH and 100 ml of mineral oil, the contents of the flask were heated slowly under reduced pressure (1 mm Hg) at a rate such that the distillate temperature was 110°C after 45 min. The distillate was collected in a series of three cold fingers placed in liquid nitrogen. The combined distillates from the three cold fingers were extracted three times with a volume of petroleum ether equal to that of the distillate and washings. The petroleum ether was removed with a Kuderna-Danish evaporator over a steam-bath, and the nitrosamines were left in the water (2 cm^3) previously added to the evaporator. Any oily residues left after removal of the petroleum ether were extracted three times with an equal volume of acetonitrile, and the acetonitrile was removed by the same procedure for removal of petroleum ether. Finally, the water and washings were transferred to a 10-cm^3 volumetric flask, 0.1 cm^3 of 0.10 M NaOH was added to make the final solution 5 mM in NaOH, and the solution was diluted to volume with water.

RESULTS AND DISCUSSION

Detection of photolytic products

Nitrite produced by photolysis of nitrosamines is reduced at a platinum electrode in 4.5 M HCl at an electrode potential in the range 0.7—0.4 V by a cathodic process limited by convective—diffusional mass transport [24]. The reaction involves one electron per molecule of HNO_2 and NO is produced.

$$R_2N{-}NO + OH^- \xrightarrow[5\ mM\ NaOH]{h\nu} NO_2^- + R_2NH$$

$$H_2O + NO^+\ (NOCl) \xleftarrow{4.5\ M\ HCl} \qquad (1)$$

$$NO^+ + e \rightarrow NO \qquad (2)$$

The cathodic response of the Pt detector at 0.40 V was studied in the photo-electroanalyzer under conditions of continuous flow of *N*-nitroso-dipropylamine in 5 mM NaOH. The anion-exchange columns were disengaged from the analyzer for this study. The sensitivity of the detector for HNO_2 produced by continuous photolysis of the nitrosamine in 5 mM NaOH steadily decreased with time over a period of 2—3 h if NaI was not used in the preconditioning of the Pt electrode. The sensitivity of the detector did not decrease for continuous detection of nitrite over similar periods in a purely inorganic solution. Apparently, the organic product of the photochemical reaction of *N*-nitrosodipropylamine is adsorbed at the electrode surface with a resultant loss of surface activity.

Iodide ion in aqueous solution is known to be strongly adsorbed onto a platinum surface as a neutral atom and the adsorption will cause displacement of other adsorbed ions. The adsorbed iodine is not desorbed even when the platinum surface is washed for an extended period of time by water or solutions of electrolyte free of I^- or I_2. The effect of adsorbed iodine was tested

in the hope that adsorption of organic matter at the electrode surface might thus be blocked. This idea was verified and the sensitivity of the platinum detector was stabilized by exposing the electrode surface as specified in the pretreatment procedure. The response of the electrode with and without adsorbed iodine is shown in Fig. 3 for intermittent photolysis of a continuous stream of *N*-nitrosodipropylamine in 5 mM NaOH. In this study, the shutter on the xenon lamp housing was alternately opened and closed for 10-min periods. With iodine adsorbed on the detector (curve B), the sensitivity is larger and the shift of the baseline is significantly less than for the absence of adsorbed iodine (curve A). Therefore the specified pretreatment was used for all remaining studies.

Photolysis

For the work described here, the photolysis cell was placed directly in the beam of polychromatic radiation from the xenon arc lamp so that a maximum photon flux would be obtained.

The efficiency for photolysis of *N*-nitrosodipropylamine was determined as a function of the pH of the photolysis media. The area of the detection peak for injection of 0.5 μg of the nitrosamine at each pH value was compared to that for injection of an equivalent amount of nitrite at the same pH. The peak areas for the nitrosamine and for nitrite would be identical if the efficiency of the photolysis were 100%. Columns 1 and 2 were disengaged from the photo-electroanalyzer for this study. Clark and Lubs buffers were used for pH 3—9 and a Bates and Bower buffer for pH 11. The results (Table 1) show that the efficiency for photochemical conversion of *N*-nitrosodipropylamine to nitrite approaches 100% only above pH 11. Retention of nitrite by the anion-exchange resin in column 2 is not quantitative at high pH and the 5 mM NaOH was selected as the media for photolysis.

The residence time for the sample in the photolysis cell is controlled by the volume of the cell and the flow rate of the sample stream. At a flow rate of

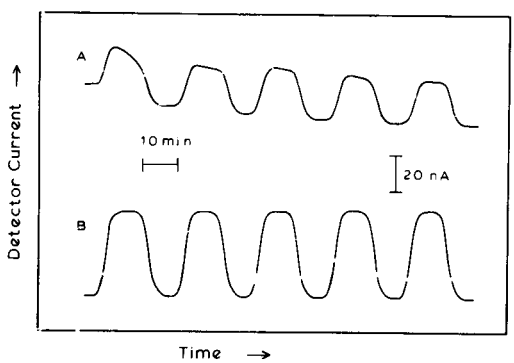


Fig. 3. Detector response as a function of surface coverage of electrode by adsorbed iodine, θ_I. (A) $\theta_I = 0.0$; (B) $\theta_I = 1.0$.

TABLE 1

Efficiency of photolysis of 0.5 μg of *N*-nitrosodipropylamine as a function of pH at a flow rate of 0.5 cm^3 min^{-1}

pH	3	5	7	9	11
Eff. (%)	7	21	38	53	92

0.5 cm^3 min^{-1}, the residence time was about 30 s. The efficiency for photolytic conversion of 0.5 μg of *N*-nitrosodipropylamine to nitrite in 5 mM NaOH was determined as a function of flow rate. The results (Table 2) show clearly that an efficiency of almost 100% can be obtained for 5 mM NaOH at very low rates of fluid flow (ca. 0.3 cm^3 min^{-1}) but at the expense of the time required for the analysis. A flow rate of 0.5 cm^3 min^{-1} was chosen as a compromise between expediency and sensitivity. The average efficiency for photolysis of *N*-nitrosodipropylamine at a flow rate of 0.5 cm^3 min^{-1} was found to be 64.9% with a relative standard deviation of 2.5% (4 injections). The corresponding efficiencies for *N*-nitrosodimethylamine and *N*-nitrosodiphenylamine under the same conditions were 71% and 89%, respectively. These efficiences are sufficiently large to indicate that the photo-electroanalyzer should be applicable as an analytical screening method for volatile nitrosamines.

Calibration and detection limit

A linear calibration curve with zero intercept was obtained for peak area as a function of concentration of *N*-nitrosodipropylamine in the range 5×10^{-8}—1×10^{-5} M. The volume of the sample loop was 0.80 cm^3 and the linear range corresponds to 5 ng—1 μg of *N*-nitrosodipropylamine. The detection limit (50% uncertainty) was estimated to be ca. 1 ng of *N*-nitrosodipropylamine. The detection limit could probably be significantly decreased by use of a larger sample volume.

Interferences

To constitute an interference in the photo-electroanalysis for nitrosamines, a species in the aqueous sample must be eluted through column 1 with 5 mM NaOH and photolyzed to produce an electroactive product which must be anionic in 5 mM NaOH, retained by column 2, and eluted from column 2 with 0.01 M $HClO_4$. Nitrite present in the aqueous sample will be quantitatively retained on column 1 and will not interfere; column 1 is switched out of the

TABLE 2

Efficiency of photolysis of 0.5 μg of *N*-nitrosodipropylamine in 5 mM NaOH as a function of flow rate

Flow rate (cm^3 min^{-1})	0.3	0.4	0.5	0.8
Efficiency (%)	97	76	65	54

flow system prior to elution of column 2 by 0.01 M $HClO_4$. Following elution from column 2, and detection of the nitrite produced by photolysis, column 1 is again engaged into the system and both columns 1 and 2 are eluted with 0.01 M $HClO_4$ until the electrical current in the detector has decayed to the residual value.

Electroactive neutral or cationic species not involved in any photolytic reaction are not retained by column 1 or 2 and do not interfere with the determination of nitrosamines. Likewise, neutral or cationic products of photolytic reactions are not retained by column 2 and do not interfere.

The compounds tested for possible interference included inorganic nitrogen compounds, anionic inorganic compounds known to be electroactive near 0.4 V (vs. E_{ref}), and organic amines and nitro compounds. Aqueous 0.01 M (or saturated) solutions of each compound were prepared. A 0.800-cm^3 sample of each solution was analyzed by the procedure described for nitrosamines. The sensitivity of the detector system was set so that the nitrite produced by photolysis of 10^{-7} M *N*-nitrosodipropylamine could be detected. A compound was judged to interfere if the peak observed was larger than the peak obtained for 10^{-7} M *N*-nitrosodipropylamine in a similar run. The results are summarized in Table 3. Other compounds tested which are not electroactive themselves and do not give electroactive photoproducts are hydroxylamine, benzaldehyde, glutamic acid, glycine and pyrazine.

The three compounds that interfere are nitromethane, nitrobenzene and methylene chloride. Response ratios (RR) were calculated from RR = nC $n$mol^{-1} (interferent)/nC $n$mol^{-1} (*N*-nitrosodipropylamine). The RR value for methylene

TABLE 3

Results of interference study

Compound	Compound electroactive	Photoproduct electroactive	Interference	Comment
KNO_3	no	yes	no	NO_3^- retained on column 1
NH_3	no	yes	no	NH_3 removed by deaeration
$K_2Cr_2O_7$	yes	n.p.[a]	no	Retained on column 1
$KMnO_4$	yes	n.p.	no	Retained on column 1
KIO_3	yes	n.p.	no	Retained on column 1
Cysteine	yes	n.p.	no	Not retained on column 2
Cystine	yes	yes	no	Neither is retained on column 2
Citric acid	yes	n.p.	no	Not retained on column 2
Nitromethane	no	yes	yes	NO_2^- produced
Nitrobenzene	no	yes	yes	NO_2^- produced
Methylene chloride	no	yes	yes	OCl^- produced (?)
Amyl nitrite	no	yes	no	Retained on column 1
Propyl nitrate	no	yes	no	Retained on column 1
Petroleum ether	no	n.p.	no	

[a]n.p. means that no photoreaction occurred.

chloride (saturated solution) was 4×10^{-6}, which is not serious but does preclude methylene chloride as the extractant; the anionic electroactive photoproduct is probably hypochlorite.

The most serious interference was observed for the two organic nitro compounds, both of which were tested at concentrations of 1.0×10^{-6} M and are photolyzed with production of nitrite. The RR values were 1.2 for nitromethane and 0.3 for nitrobenzene. Organic nitro compounds are not likely to be present in foods because of the rather harsh conditions necessary for production of the nitro compounds [25]. If they were present, they would probably represent a health hazard [26], so that their detection in a screening procedure is probably desirable.

It is surprising that photolysis of NH_3 in 5 mM NaOH resulted in production of an electroactive anionic species; an RR value of 1.0 was found. The photoproduct was concluded to be nitrite on the basis of electrochemical evidence. A possible photochemical reaction for the conversion is given by

$$NH_3 + 2H_2O \xrightarrow{h\nu} \underbrace{HNO_2 + 3H_2}_{\longrightarrow} NO_2^- + H^+$$

The photochemical conversion of nitrate to nitrite has been investigated by Daniels et al. [27]; the reactions are probably

$$NO_3^- \xrightarrow{h\nu} NO_2^- + O; \quad O + NO_3^- \xrightarrow{k_1} O_2 + NO_2^-; \quad O + NO_2^- \xrightarrow{k_2} NO_3^-.$$

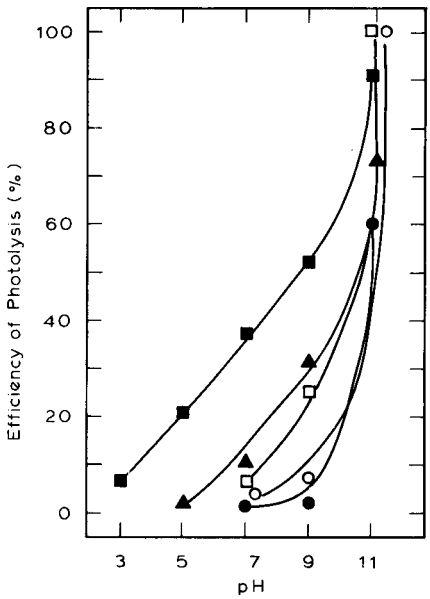
At 300 nm, $k_2/k_1 = 2 \times 10^4$.

The efficiency for photolytic production of nitrite from nitromethane, nitrobenzene, ammonia and nitrate was determined as a function of pH. The results are shown in Fig. 4, which also includes values for *N*-nitrosodipropylamine from Table 2. The interferences from the organic nitro compounds could be substantially decreased if photolysis were done at pH 5. However, the sensitivity for nitrosamines would also be much smaller at pH 5.

Elution of nitrite from column 2

Nitrite ion produced by photolysis of nitrosamines in 5 mM NaOH is quantitatively adsorbed on the strong-base anion-exchange resin in column 2. Whereas the efficiency for photolysis of *N*-nitrosodipropylamine in 5 mM NaOH is not 100%, the use of a solution containing a larger concentration of NaOH results in some elution of nitrite from column 2 because of displacement by hydroxide ions. Adsorbed NO_2^- from column 2 was eluted quickly with 0.01 M $HClO_4$. The ClO_4^- strongly competes with NO_2^- for adsorption sites [28] and the decreased pH produces HNO_2 ($pK_a = 3.4$) which is not retained. The elution peak observed for nitrite had a bandwidth of less than 5 min for a flow rate of 0.5 cm^3 min^{-1}.

A sudden change in ionic composition of a fluid stream in an amperometric flow-through detector produces changes in the residual current measured in the detector [29]. The customary procedure for ion-exchange chromatography



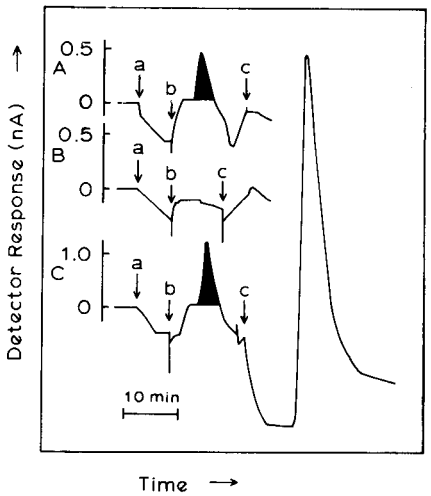


Fig. 4. Photolytic efficiency as a function of pH: (■) *N*-nitrosodipropylamine; (▲) NO_3^-; (□) CH_3NO_2; (○) nitrobenzene; (●) NH_3.

Fig. 5. Chromatograms for low levels of nitrosamines (A) Injection of 1×10^{-7} M *N*-nitrosodipropylamine (0.5 nA/in.); (B) injection of 5×10^{-3} M NaOH (0.5 nA/in.); (C) injection of extract from bacon grease (1.0 nA/in.). (a) Sample injected; (b) eluent switched from 5 mM NaOH to 0.01 M $HClO_4$ with bypass of column 1 and the photolysis cell; (c) switching valve changed to elute column 1 and the photolysis cell with 0.01 M $HClO_4$.

is the use of controlled, step-wise changes in the ionic composition of the eluting stream to achieve selective elution of adsorbed ionic species. Hence, the analyte species emerge from the chromatographic column simultaneously with the ionic front. The uncertainty involved in determination of the baseline as a result of the ionic front is ultimately responsible for setting the detection limit of the method. In the present case, the eluent stream is mixed with a stream of 9 M HCl to produce the solution conditions appropriate for detection of nitrite at the Pt detector and change in composition of the eluting stream from 5 mM NaOH to 0.01 M $HClO_4$ produces a minimal change in the ionic composition of the stream in the detector.

Analysis of food samples

The analytical procedure described was applied to the determination of volatile nitrosamines in fried bacon, fried bacon grease and tuna. The results summarized in Table 4 were corrected for the blank, which was obtained by running a total analysis without a sample. The concentrations of nitrosamines

TABLE 4

Results for determination of volatile nitrosamines in food samples (calculated as *N*-nitrosodipropylamine)

Sample	Nitrosamine found (ppb)	Sample	Nitrosamine found (ppb)
Fried bacon	1.3	Tuna	0.5
Bacon grease	3.0	Blank	<0.2

found were extremely low. Concentrations of nitrosamines in food are typically about 5 ppb [30]. In some cases, the nitrosamines are produced predominantly during cooking. Fazio et al. [31] found *N*-nitrosopyrrolidine in fried but not raw bacon at levels of 10—108 ppb. The concentration of *N*-nitrosopyrrolidine remaining in the bacon grease was 45—207 ppb. A more complete discussion of nitrosamines in foods is given elsewhere [7, 19].

The current—time curves shown in Fig. 5 were obtained in the process of analyzing fried bacon grease. Curves are also shown for analysis of a standard solution of *N*-nitrosodipropylamine and for the blank. The peak corresponding to nitrite is clearly marked.

The precision of the determination at the ppb level was calculated from results for injection of three aliquots of the bacon extract. The relative standard deviation was 15 ppb. The recovery for 6.5 ppb *N*-nitrosodipropylamine was 50%.

A source of error was the adsorption of nitrosamines by the first column after several injections (more than 10) of sample extract. There was always a small amount of oily material (0.5 cm^3) left at the top of the volumetric flask after all the sample pretreatment steps had been performed. Perhaps, a little oily material was injected into the photo-electroanalyzer and adsorbed on the first column so that the nitrosamine then partitioned between the aqueous phase and the adsorbed oily material. This problem was detected by comparing the peak height for the injection of standards with those obtained previously. The easiest solution to the problem was to replace the column packing. The use of chemibonded ion-exchange resins, where there are no aromatic parts of the resin for adsorption to occur, could solve the problem.

Occasionally, a large anodic peak was observed prior to the elution of nitrite. The peak was so large that the current did not return to the background value before the nitrite was eluted by the 5 mM NaOH. The problem was solved by allowing only the minimum amount of time for the nitrosamine to pass through the photolysis loop before switching column 1 and the photolysis loop out of the flow stream. Another solution was to use water as the photolysis medium. A small sacrifice in photolysis efficiency resulted.

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POTENTIOMETRIC STRIPPING ANALYSIS FOR LEAD IN URINE

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SUMMARY

Potentiometric stripping analysis is based on the preconcentration of analytes by means of potentiostatic reduction and amalgamation at a thin-film mercury electrode. After preconcentration, the potentiostatic circuitry is disconnected and the amalgamated metals are oxidized either by mercury(II) ions or by dissolved oxygen. Lead can be determined in acidified urine samples by potentiometric stripping analysis after the addition of Triton X-100. In deaerated samples the detection limit is 1 $\mu g\ l^{-1}$, and in non-deaerated samples 12 $\mu g\ l^{-1}$, the preconcentration time being 16 min.

The concentration of lead in urine normally ranges between 5 and 20 $\mu g\ l^{-1}$; concentrations above 50 $\mu g\ l^{-1}$ may indicate lead poisoning [1]. Lead in urine has been determined spectrophotometrically with dithizone [2, 3], and by flame atomic absorption spectrometry after solvent extraction [4—9], coprecipitation [10—12] or ion-exchange [13]. Delves' method [14] has been modified for the determination of lead in urine by standard addition procedures [15—19] or by special calibration plots [20]. Carbon rod atomizers have been used in combination with solvent extraction [21] or directly after addition of EDTA to the urine sample, in order to prevent the formation of volatile lead chloride [22]. Potentiometric analysis for lead in urine has proved successful only for very high concentrations [23].

Anodic stripping voltammetric techniques are sufficiently sensitive for the determination of lead at natural levels [24]. This technique is, however, affected by water-soluble proteins present in varying concentrations in different urine samples. Such proteins affect the transport rate of metal ions to the mercury electrode surface and, consequently, the analytical signal. For accurate results, acid digestion would appear to be necessary. This paper suggests the use of a new technique, potentiometric stripping analysis [25, 26] for the determination of lead in urine. As in anodic stripping voltammetry, lead is preconcentrated by means of potentiostatic reduction and amalgamation at a mercury electrode. After preconcentration (plating), however, the potentiostat is disconnected and the oxidation—reduction processes occurring at the thin-film mercury electrode are registered on a high input impedance x-t recorder. Mercury(II) ions,

added to the sample before the commencement of the plating, are normally used as oxidizing agent. Apart from the simple instrumentation, the main advantage of this technique is that the transport mechanisms exploited are the same during plating and during oxidation. The effect of proteins is thus largely cancelled and acid digestion is unnecessary.

EXPERIMENTAL

Chemicals

Stock solutions (1000 mg l^{-1}) of mercury(II) and of lead(II) in 0.005 M nitric acid were prepared from analytical-grade metal nitrates. The lead(II) content of the mercury(II) stock solution was less than 0.5 μg l^{-1}. Triton X-100 stock solution was prepared by diluting 5 g of Triton X-100 with 95 ml of ethanol. All mineral acids used were of Suprapur grade (Merck).

Apparatus

The Potentiometric Stripping Analyzer (Radiometer prototype, REA 120, REC 61 and TTA 60) could be programmed to plating times of 1, 2, 4, ... 64 min and plating potentials between 0 and −2 V vs. SCE. Two seconds before the end of the plating time the recorder was automatically started and terminated after 1/16 of the plating time chosen. A new plating/stripping cycle was then initiated automatically.

Polyethylene beakers of total volume 70 ml (Radiometer 956—178) were used as electrochemical cells. Constant-rate stirring was achieved by a three-edged Teflon stirrer operated mechanically by the Analyzer or by a magnetic stirrer.

The glassy carbon working electrode (Radiometer F 3500) had a total surface area of 8 mm^2 and was pressure-fitted into a Teflon rod with an outer diameter of 7 mm. A saturated calomel electrode (Radiometer K 4040) was used as reference and a platinum foil (Radiometer P 101) as counter electrode. All electrodes were screwed tightly into the lid of the electrochemical cell.

The atomic absorption measurements were made on a Perkin-Elmer 370 spectrometer equipped with an HGA 2100 graphite furnace atomizer and a deuterium arc background corrector. Analyses were done by the procedure specified by Ebert and Jungman [22].

An Original Hanau quartz lamp TQ 1200 with a radiation flux of 110 W in the wavelength region 200—400 nm was used to provide ultraviolet radiation.

Pre-treatment of the working electrode. Prior to each analysis, the glassy carbon working electrode was polished vigorously with 3-μm diamond paste for 20—30 s and rinsed carefully with acetone in order to remove polishing lubricant.

Sampling

Samples were taken from laboratory personnel, none of whom had been occupationally exposed to lead. The samples were acidified with hydrochloric acid to a total concentration of 0.2 M and analyzed within a week after sampling.

Procedures

For the preconcentration of lead in urine, by means of potentiostatic reduction and amalgamation, the surface of the glassy carbon electrode must be coated with a film of mercury thick enough to dissolve all metals reduced. The simplest way of coating the electrode is by adding mercury(II) ions to the sample. During the subsequent stripping, these ions can then be exploited as the major oxidant. In order to achieve this, the sample must, however, be de-oxygenated; otherwise, the major oxidant will be dissolved oxygen and the oxidation rate will be much higher, resulting in a decreased detection limit. Depending on the specific analytical problem, lead in urine can therefore be determined either in deaerated (for lower concentrations of lead), or in non-deaerated samples (for concentrations above approximately 10 μg Pb l^{-1}).

Procedure with deaeration. To 20 ml of acidified urine sample, 2 ml of Triton X-100 stock solution and 80 μl of mercury(II) stock solution are added. The solution is deaerated by passing a stream of nitrogen through the sample for 20 min. The sample is then transferred to the Analyzer in which nitrogen is passed over the sample surface. The Analyzer is adjusted to a plating potential of —0.95 V vs. SCE with a plating time of 1 min. A minimum of four plating/stripping cycles, each cycle comprising a 1-min plating time and a 15-s stripping time, is then performed automatically. During this procedure, the glassy carbon electrode is pre-coated with a thin film of mercury. After the completion of the last cycle, the plating time is adjusted to the value to be used during the subsequent analysis. Plating for 4, 8 or 16 min yields detection limits of approximately 4, 2 and 1 μg l^{-1}, respectively. After the recording of the potentiometric stripping curve, an aliquot of lead(II) solution corresponding to 10 μg l^{-1} of urine is added and the plating/stripping cycle is repeated. The lead concentration is then calculated from the usual equation for standard addition. It is advisable to minimize the time during which the mercury-coated glassy carbon electrode is allowed to be in contact with the mercury-containing sample in the absence of an applied reducing potential. Prolonged exposure under such conditions may result in the formation of calomel on the electrode surface.

Procedure in non-deaerated samples. The sample is pre-treated as above except that nitrogen purging is omitted. After mercury pre-coating by means of the four plating/stripping cycles described above, the Analyzer is immediately adjusted to the plating time to be used during analysis. Plating times of 8, 16 and 32 min yield detection limits of approximately 24, 12 and 6 μg l^{-1},

respectively. The lead concentration is evaluated from a standard addition aliquot corresponding to 100 μg Pb l^{-1} of urine.

RESULTS AND DISCUSSION

Deaerated samples

Deaerated samples from ten different people were analyzed by plating at —0.95 V for plating times varying between 4 and 32 min. The results obtained varied between 2.3 and 11.2 μg l^{-1}. Figure 1 shows the potentiometric stripping curve, obtained after plating for 32 min in a sample containing 2.3 μg Pb l^{-1} (curve II). Also shown in Fig. 1 is the background curve recorded after plating for 5 s (curve I). Curve III shows the potentiometric stripping after the addition of a standard aliquot corresponding to 10 μg Pb l^{-1}. The evaluation of the analytical signal from the potentiometric stripping curve is illustrated on curve III of Fig. 1.

Accuracy and precision were investigated for three of the samples. The accuracy was estimated by comparison with the results obtained by flameless atomic absorption spectrometry. The relative standard deviation was evaluated from five consecutive analyses of the same sample. The results, summarized in Table 1, show satisfactory agreement between the two methods. The systematically higher results obtained by atomic absorption are partly due to lead contamination in the EDTA reagent used in the Ebert—Jungman procedure.

Non-deaerated samples

The ten different urine samples were analyzed without previous deaeration at a plating time of 16 min. Figure 2 shows the potentiometric stripping curves recorded for a sample containing 3.2 μg Pb l^{-1} (curve I) and for the same sample after a standard addition corresponding to 100 μg Pb l^{-1} (curve II). Curve III of Fig. 2 shows the potentiometric stripping curve recorded for a second sample, containing 5.0 μg l^{-1} lead(II), after an addition corresponding to 100 μg l^{-1} lead(II). Curve IV shows the potentiometric stripping curve recorded for this sample after ten consecutive plating/stripping cycles.

TABLE 1

Comparison between results obtained by potentiometric stripping analysis (p.s.a.) and by graphite-furnace atomic absorption spectrometry (a.a.s.)

Sample No.	P.s.a. (μg l^{-1})	Rel. stand. dev. (n = 5)	A.a.s (μg l^{-1})
1	11.2	0.06	13.6
2	3.8	0.07	4.4
3	6.1	0.07	7.8

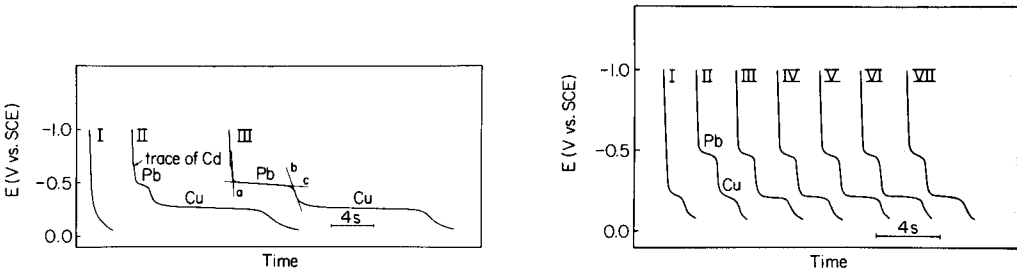

Fig. 1. Potentiometric stripping analysis of a deaerated urine sample containing 2.3 μg Pb l^{-1}. (I) Background recorded after plating at —0.95 V for 5 s; (II) signal recorded after plating for 32 min at —0.95 V; (III) signal recorded after the addition of 10 μg Pb l^{-1} and plating at —0.95 V for 32 min. The distance between the intersection of line *c* with lines *a* and *b* represents the analytical signal.

Fig. 2. Potentiometric stripping analysis of non-deaerated urine samples after the addition of Triton X-100 (10 g l^{-1}). Plating time, 10 min at —0.95 V vs. SCE. Curves I and II were recorded for a sample containing 2.9 μg Pb l^{-1} before (I) and after (II) the addition of 100 μg Pb l^{-1}. Curves III and IV were recorded for a sample containing 5.0 μg Pb l^{-1} after the addition of 100 μg Pb l^{-1}; curve III represents the first stripping and curve IV was recorded after ten consecutive plating/stripping cycles. Curves V—VII were recorded for a sample containing 4.8 μg Pb l^{-1} after addition of 100 μg Pb l^{-1}: (VI) after the addition of albumin (1 g l^{-1}); (VII) after increase of the Triton X-100 concentration from 10 to 30 g l^{-1}.

The results shown in Fig. 2 confirm that the detection limit after plating for 16 min is of the order of 12 μg l^{-1}. It also shows that the mercury film plated onto the glassy carbon electrode can be used for several hours of analysis in urine samples containing Triton X-100. Furthermore, the analytical signals obtained from the ten different urine samples after the addition of 100 μg l^{-1} of lead(II) agreed within 20%. This indicates that a calibration plot could be used in screening tests for high lead levels in urine.

Influence of water-soluble proteins and Triton X-100

It is well-known that proteins interfere with voltammetric electrode processes. In order to investigate their effect on the potentiometric stripping analysis of urine, a non-deaerated sample containing 105 μg Pb l^{-1} was analyzed before (curve V of Fig. 2), and after (curve VI) the addition of albumin (1 g l^{-1}). As can be seen, the addition of albumin has little effect. This is also the case when the total concentration of Triton X-100 is increased. Curve VII was recorded under the same experimental conditions as curve VI except that the Triton X-100 concentration was increased from 10 to 30 g l^{-1}. Both these observations suggest that the rate of transport of metal analytes to the working electrode during plating is affected by surface-active agents in approximately the same way as is the rate of transport of oxidants during oxidative stripping.

Influence of EDTA

Lead poisoning is treated by the calcium—EDTA complex, EDTA thus being present in urine from such patients. In the acidified samples used in this investigation, complex formation between lead(II) and EDTA could, however, be neglected [27]. This was verified experimentally by analyzing a sample of urine before and after the addition of 0.1 M EDTA.

Ultraviolet radiation

An aliquot of a deaerated urine sample was analyzed by plating for 16 min at −0.95 V vs. SCE. The procedure was repeated on a second aliquot after exposure to ultraviolet radiation for 2 h. The results obtained from the two samples, 3.3 and 3.8 $\mu g\ l^{-1}$, respectively, agreed within the standard deviation of the method. This result indicates that unreducible complexes between lead and organic ligands are absent from a normal acidified urine sample.

Analysis for copper and cadmium

Curves I—VII of Fig. 2 clearly indicate the possibility of determination of copper in urine samples. The estimated relative standard deviation is 0.12.

Traces of cadmium, corresponding to approximately 2 $\mu g\ l^{-1}$, are apparent from curves I—III of Fig. 1. Obviously the sensitivity for cadmium in urine samples is approximately one order of magnitude less than, for example, in sea water [28]. Consequently, the sensitivity must be improved if potentiometric stripping analysis is to become a suitable method for routine determinations of cadmium in urine.

Conclusions

Potentiometric stripping analysis would appear to be suitable for the determination of lead in urine, the analysis being performed on deaerated samples for low concentrations of lead and on non-deaerated samples for high concentrations of lead. Before the technique is used in routine analysis for lead in urine, it is, however, desirable that it be tested on urine samples from a wider population, including persons occupationally contaminated by lead.

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DIRECT AND SIMULTANEOUS DETERMINATIONS OF Zn, Cd, Pb, Cu, Sb AND Bi DISSOLVED IN SEA WATER BY DIFFERENTIAL PULSE ANODIC STRIPPING VOLTAMMETRY WITH A HANGING MERCURY DROP ELECTRODE

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SUMMARY

Differential pulse anodic stripping voltammetry with a hanging mercury drop electrode is used for the direct and simultaneous determination of Zn, Cd, Pb, Cu, Sb and Bi at their natural levels in sea water after adjustment to pH 1 and to a 2 M chloride concentration. The optimal instrumental parameters are described. With a plating time of 60 min, the detection limits are about 0.1 ppb for Zn and Cu, 0.01 ppb for Cd and Pb, and 0.05 ppb for Sb and Bi. Relative standard deviations are about 10—15%. Simultaneous determinations of the six metals take about 2 h.

Differential pulse anodic stripping voltammetry receives considerable attention as a convenient technique for the simultaneous determination of heavy metals at the microtrace level. Recent papers have described the direct determination of Zn, Cd, Pb, Cu in sea waters with both the hanging mercury drop electrode [1—6] and film [7—10] mercury electrodes, as well as the possibilities of studying the chemical speciation [11—16].

This paper describes the possibility of determining directly and simultaneously six elements (Zn, Cd, Pb, Cu, Sb and Bi) in sea water by differential pulse anodic stripping voltammetry with the hanging mercury drop electrode. This electrode seems to be the most reliable one for routine analysis. It can be applied over a large potential range with high reproducibility and sensitivity.

EXPERIMENTAL

Apparatus

Voltammograms were recorded with a modular Brucker E 310. A three-electrode system was used: the working electrode was a hanging mercury

drop electrode (Metrohm E 410), and potentials were measured against a saturated calomel reference electrode (SCE) with a platinum spiral as auxiliary electrode.

Analysis for bismuth or for bismuth and copper simultaneously

Very little information is available about the distribution of bismuth in the marine environment. Noddack and Noddack [17] used a spectrographic method. Later, Portmann and Riley [18] used an ion-exchange separation procedure followed by dithizone extraction. Florence [19] determined bismuth in Pacific Ocean water by anodic stripping on a graphite mercury film electrode. The concentrations of dissolved bismuth in sea water were found to lie in the range 0.02—0.2 ppb. The optimal conditions for the proposed method were established as follows.

Electrodeposition potential. The influence of the electrodeposition potential (E_d) on the oxidation current (i_p) was tested on sea water spiked with 3.2 ppb Bi(III). The peak potential was —0.065 V vs. SCE. The results (Table 1) show that a plateau current is obtained for E_d values below —0.150 V. The determination of bismuth in sea water is made possible by applying an initial potential of —0.150 V, which is sufficiently negative for enrichment, while reduction of copper is negligible (Fig. 1a). For more negative E_d values, there is simultaneous reduction of Cu and Bi (Fig. 1b).

Scan rate and pulse amplitude. Figure 2 shows that changes in the scan rate have very little effect on the resolution of the Cu and Bi peak currents

TABLE 1

Influence of E_d on i_p

E_d(V)	—0.100	—0.150	—0.200	—0.250	—0.300	—0.400	—0.500	—0.600
i_p(μA)	0.120	0.210	0.220	0.215	0.220	0.215	0.215	0.220

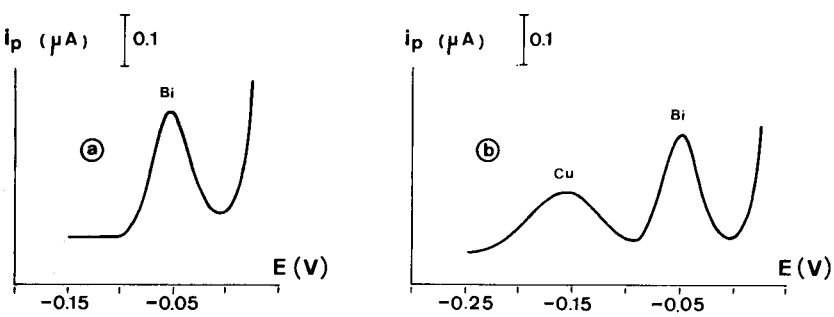


Fig. 1. Voltammograms of Bi (a) and Cu and Bi (b) in sea water spiked with 9 ppb Cu(II) and 3.2 ppb Bi(III). E_d = —0.150 V (curve a) and —0.250 V (curve b); deposition time, 5 min; pulse amplitude, 30 mV; scan rate, 2 mV s^{-1}; pH 2.

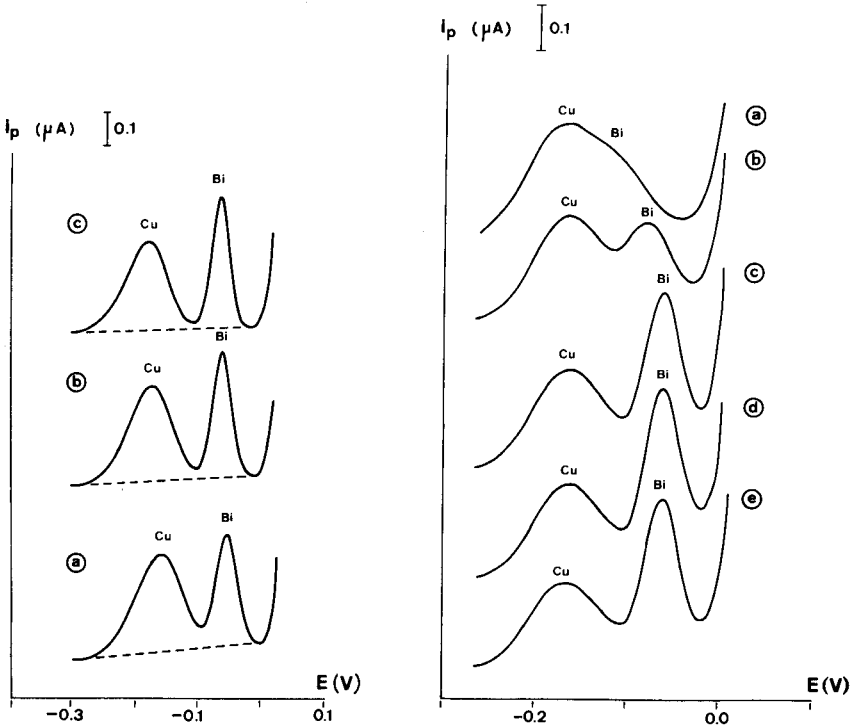


Fig. 2. Voltammograms of sea water spiked with 9 ppb Cu and 5 ppb Bi. Deposition time, 8 min; E_d = —0.300 V; pulse amplitude, 30 mV. Scan rate: (a) 2 mV s^{-1}; (b) 1 mV s^{-1}; (c) 0.5 mV s^{-1}.

Fig. 3. Voltammograms of sea water containing 7 ppb Cu and 5 ppb Bi (after addition). Deposition time, 8 min; E_d = —0.250 V; pulse amplitude, 30 mV; scan rate, 1 mV s^{-1}. pH: 4.1 (a); 3.1 (b); 2 (c); 1.2 (d); 0.6 (e).

TABLE 2

Influence of pulse amplitude (ΔE) on i_p for bismuth(III)

ΔE(mV)	10	20	30	40	50
i_p(μA)	0.100	0.200	0.280	0.340	0.370

between 0.5 and 2 mV s^{-1}. Table 2 shows the effect of changes in pulse amplitude. The voltammograms prove that the resolution between the peaks for Cu and Bi with a pulse amplitude of 50 mV and with scan rates below 1 mV s^{-1} is still good and that the peak currents increase with increasing pulse amplitude.

Linearity of calibration plots. The linearity between i_p and concentration was tested by the standard addition method on natural sea water. Satisfactory linearity was obtained over the concentration range generally found in sea water (Table 3).

TABLE 3

Linearity between i_p and concentration of bismuth

Bi(ppb)	0.3	1.0	1.7	2.4	3.1	3.8
$i_p(\mu A)$	0.015	0.035	0.060	0.080	0.105	0.125

Intermetallic compound formation between Cu and Bi. The formation of an intermetallic compound between Cu and Bi could cause an error in analyses for these metals. This aspect was investigated by comparing the slopes of the i_p—concentration relationships for bismuth in the presence of 1—12 ppb Cu (E_d = —0.400 V), and in the absence of copper (E_d = —0.150 V). The results indicated that there is no intermetallic compound formation in the mercury drop electrode under the conditions used.

Influence of pH on the peak height and peak potential for bismuth. The sea water had been adjusted to pH 0.6 with nitric acid for storage, and the pH was increased by addition of sodium hydroxide (Suprapur Merck). The peak height decreased with increasing pH (Table 4); above pH 3, bismuth(III) is hydrolysed, the peak shifts to more negative potentials and becomes masked by the copper peak (Fig. 3).

Simultaneous analysis for Sb and Bi or Cu, Sb and Bi

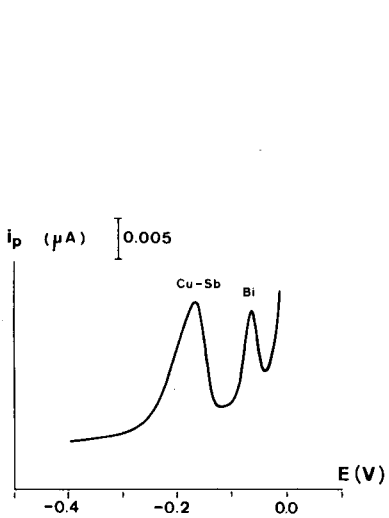
Preliminary experiments showed that the differential pulse anodic stripping peak for antimony is strongly affected by the presence of copper (Fig. 4). To avoid this superposition and to obtain consistent results, a rigorous procedure was evolved. The experiments were carried out on natural sea water to which Sb and Bi were added to raise the concentrations to about 1 ppb. The behaviour of the antimony peak and the Cu—Sb interference were investigated in order to select optimal conditions for simultaneous determinations.

Electrodeposition potential. The effect of E_d on the antimony peak current was tested on purified natural sea water containing 5 ppb Sb after standard addition. Deposition potentials between —0.175 and —0.450 V (vs. SCE) were investigated. The results (Table 5) show that the peak height is strongly affected by the deposition potential down to —0.300 V. It is interesting to note that both Sb and Bi can be deposited at a potential of —0.225 V whereas copper is not reduced during the electrolysis (Fig. 5, curve a); at a potential of —0.350 V all three metals are deposited (curve b).

TABLE 4

Influence of pH on bismuth peak

pH	0.6	1.2	2.0	3.1
$i_p(\mu A)$	0.290	0.290	0.285	0.130
$E_p(V)$	—0.065	—0.065	—0.065	—0.080



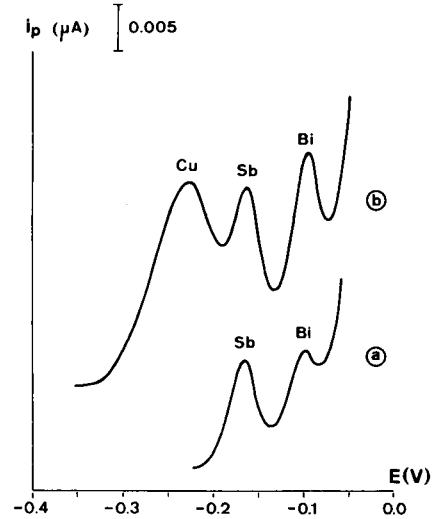


Fig. 4. Voltammogram of natural sea water. Deposition time, 10 min; pulse amplitude, 10 mV; scan rate, 0.5 mV s^{-1}; pH 1 (HCl).

Fig. 5. Voltammograms of natural sea water containing 7 ppb Cu, 0.6 ppb Sb and 0.7 ppb Bi. Deposition time, 40 min; pulse amplitude, 10 mV; scan rate, 0.5 mV s^{-1}; E_d = —0.225 V (curve a) and —0.350 V (curve b); pH 0.5.

TABLE 5

Influence of E_d on i_p for antimony

E_d(V)	—0.175	—0.200	—0.250	—0.300	—0.350	—0.450
i_p(μA)	0.003	0.015	0.020	0.023	0.024	0.024

Scan rate and pulse amplitude. As shown by the results obtained (Fig. 6) with a curve resolver (E 310 Du Pont) the effect of the scan rate is negligible for 0.2 and 0.5 mV s^{-1}, but above this value satisfactory peaks are not obtained. From a practical point of view, a scan rate of 0.5 mV s^{-1} was chosen as an optimum value which gives adequate resolution and a lower analysis time for routine work.

Figure 7 shows that for low pulse amplitudes, the antimony peak current increases linearly with the amplitude. Curve b indicates that $b_{1/2}$ (peak width at half height) increases from 30 mV at an amplitude of 10 mV to 45 mV at an amplitude of 40 mV. To obtain satisfactory selectivity, an amplitude of 10 mV was used, although this gives lower sensitivity.

Intermetallic compound formation between Cu and Sb. As for Cu and Bi, the possible formation of intermetallic compounds was tested by comparison of the calibration curves obtained by electrodeposition of antimony (—0.225 V) and by co-deposition of Cu and Sb in the mercury drop electrode

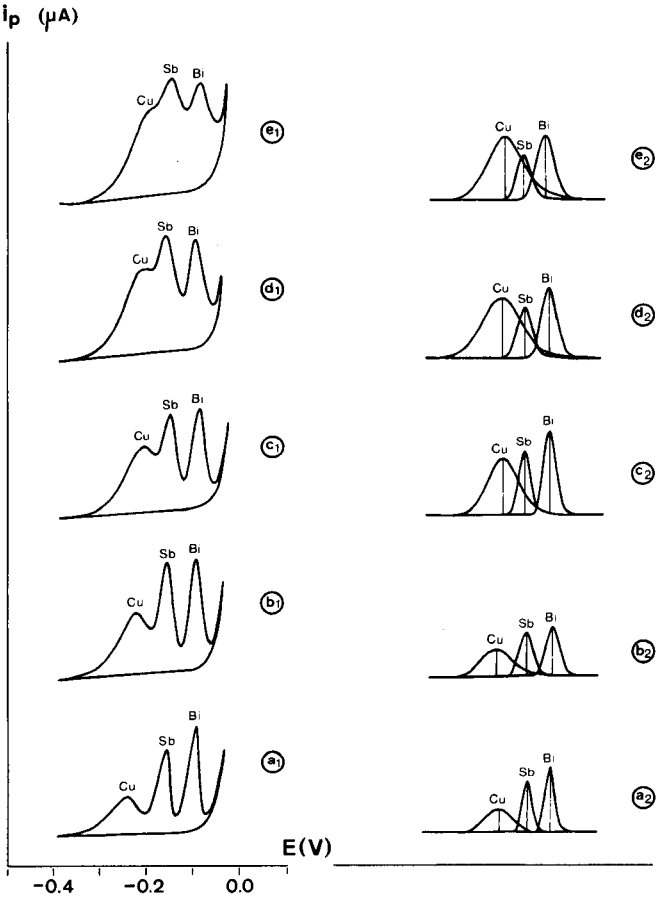


Fig. 6. Voltammograms of sea water at pH 0.5. Deposition time, 15 min; E_d = —0.400 V; pulse amplitude, 10 mV. Scan rate: (a) = 0.2 mV s^{-1}; (b) 0.5 mV s^{-1}; (c) 1.0 mV s^{-1}; (d) 2 mV s^{-1}; (e) 5.0 mV s^{-1}.

(—0.400 V). The concentration of copper reduced on mercury drop electrode had no effect on the antimony peak current, which suggests that no intermetallic compound is formed in the simultaneous determination of Cu and Sb with copper concentrations in the range 1—12 ppb.

Influence of pH on Cu, Sb and Bi peaks. The effect of pH on the resolution of the Cu, Sb and Bi peaks in sea water was studied by addition of hydrochloric or perchloric acid (Suprapur, Merck). Figure 8 shows the curves for Cu, Sb and Bi as a function of pH only ($HClO_4$) and pH plus chloride (HCl). A peak depression and a potential shift can be seen. The potential shift is more apparent with HCl than with $HClO_4$. The best resolution is obtained for 0.3 M hydrogen ion concentraion. The shift is attributed to the complexation of metals with chloride.

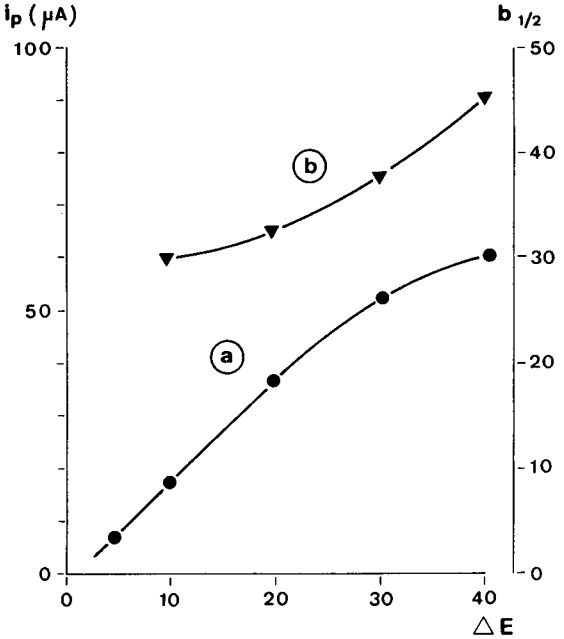


Fig. 7. Influence of pulse amplitude on i_p (a) and $b_{1/2}$ (b).

Effect of the Cu—Sb and Sb—Bi concentration ratios. Antimony in sea water can be detected easily in the presence of a 50-fold amount of copper (Fig. 9, curve a) or a 30-fold amount of bismuth (curve b). Similarly the detection of bismuth is not affected by a 30-fold amount of antimony (curve c). These results indicate that simultaneous determinations are possible.

Simultaneous analyses for Zn, Cd, Pb, Cu, Sb and Bi

In oceanographic analysis, it is frequently desirable to analyse several hundred samples for as many elements as possible by a rapid direct method. Anodic stripping voltammetry has proved to be a remarkable method for determining toxic metals in sea water. However, a major problem in highly acidic solution is that the zinc oxidation peak is masked by the hydrogen reduction peak, and therefore zinc was determined after the pH had been adjusted to 3—4 [11].

With the introduction of the differential pulse method, zinc can be determined in sea water at about pH 1 [20]; the hydrogen reduction wave becomes more negative (Fig. 10, curve 1) and an excellent zinc peak can be obtained in sea water acidified with 0.1 M HCl (Fig. 11).

However, the analysis for Cu, Sb and Bi must be made at about pH 0.5 because the best peak resolution is obtained with 0.3 M HCl. Thus, at first sight it looks impossible to reach the optimum conditions for simultaneous

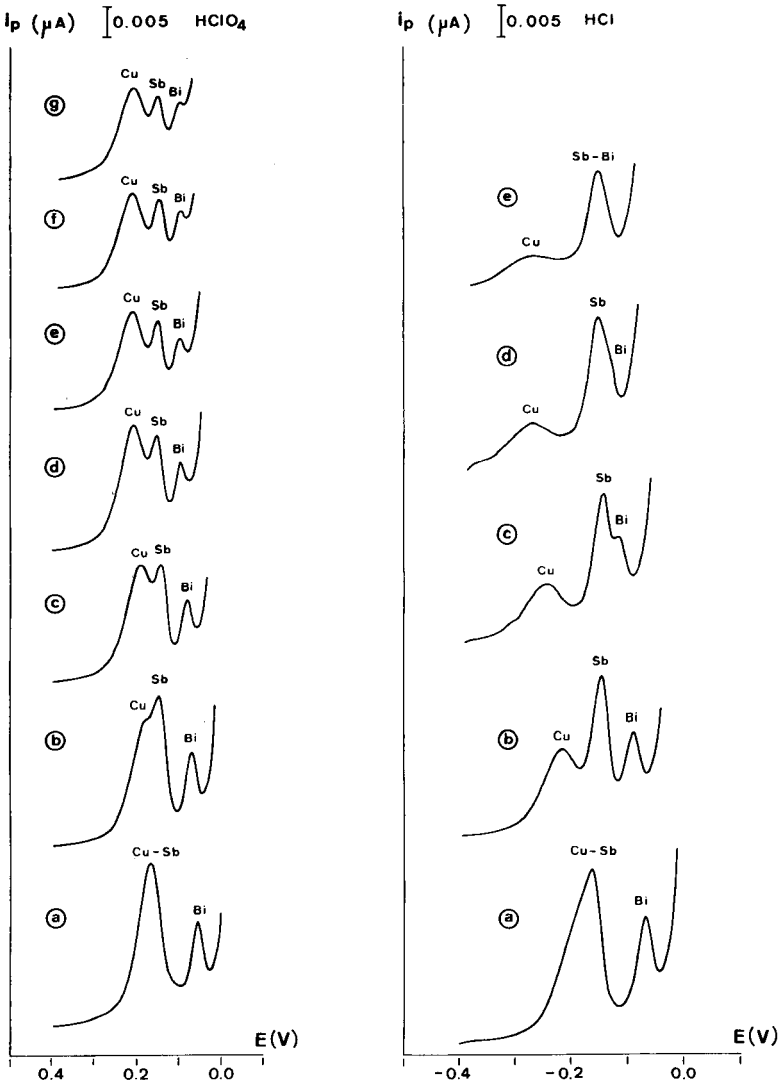


Fig. 8. Influence of $HClO_4$ and HCl on potentials of peak currents of Cu, Sb, Bi and Hg (mercury drop electrode). Natural sea water spiked with 5 ppb Sb and 1 ppb Bi. Deposition time, 10 min; pulse amplitude, 10 mV; scan rate, 0.5 mV s^{-1}; E_d = —0.400 V. Molarity of $HClO_4$: (a) 0.1; (b) 0.4; (c) 1.0; (d) 2.0; (e) 3.0; (f) 4.0; (g) 5.0. Molarity of HCl: (a) 0.1; (b) 0.3; (c) 1.0; (d) 2.0; (e) 3.0.

detection of Zn, Cd, Pb, Cu, Sb and Bi. However, adjustment of the chloride ion concentration allows the difficulty to be overcome.

Influence of chloride concentration in the electrolyte. Figure 12 shows voltammograms of Cu, Sb and Bi in natural sea water at pH 1 (HCl) as a function of chloride concentration. As in the case of acidification by HCl,

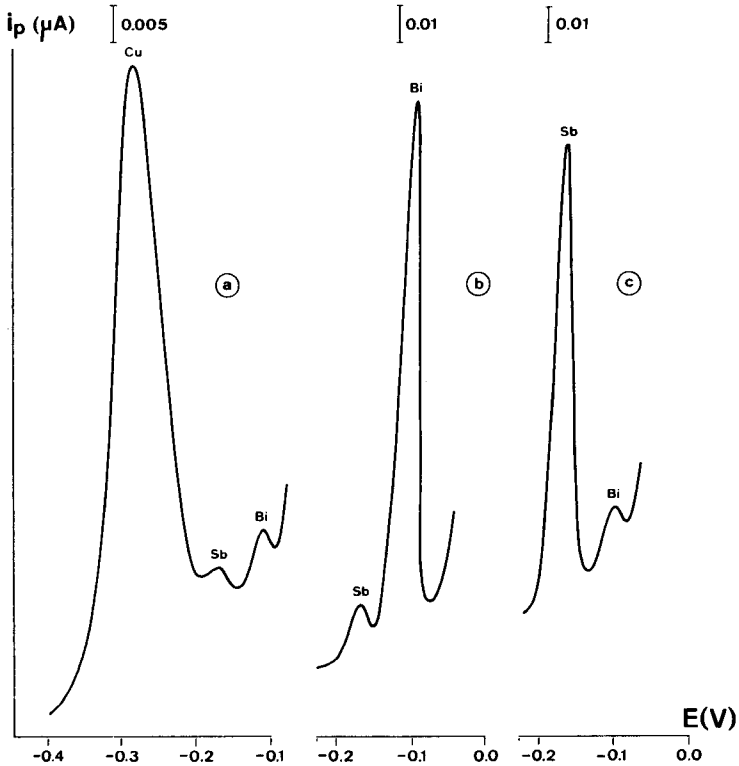


Fig.9. Voltammograms of spiked sea water, at pH 0.5 with a pulse amplitude of 10 mV and scan rate of 0.2 mV s^{-1}. (a) 16 ppb Cu and 0.3 ppb Sb with deposition at —0.400 V for 40 min; (b) 0.6 ppb Sb and 16 ppb Bi with deposition at —0.225 V for 25 min; (c) 16 ppb Sb and 0.6 ppb Bi under the same conditions as (b).

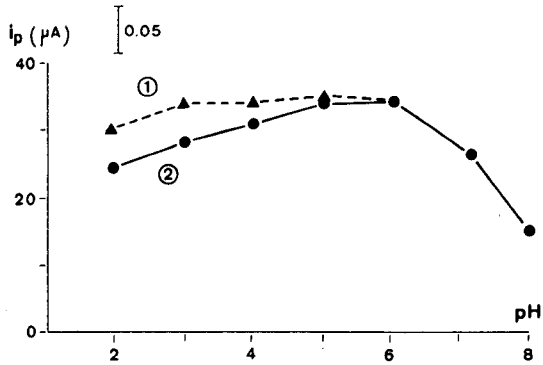


Fig. 10. Relationship between the pH of the electrolyte and peak current for zinc by (1) differential pulse anodic stripping, and (2) d.c. anodic stripping.

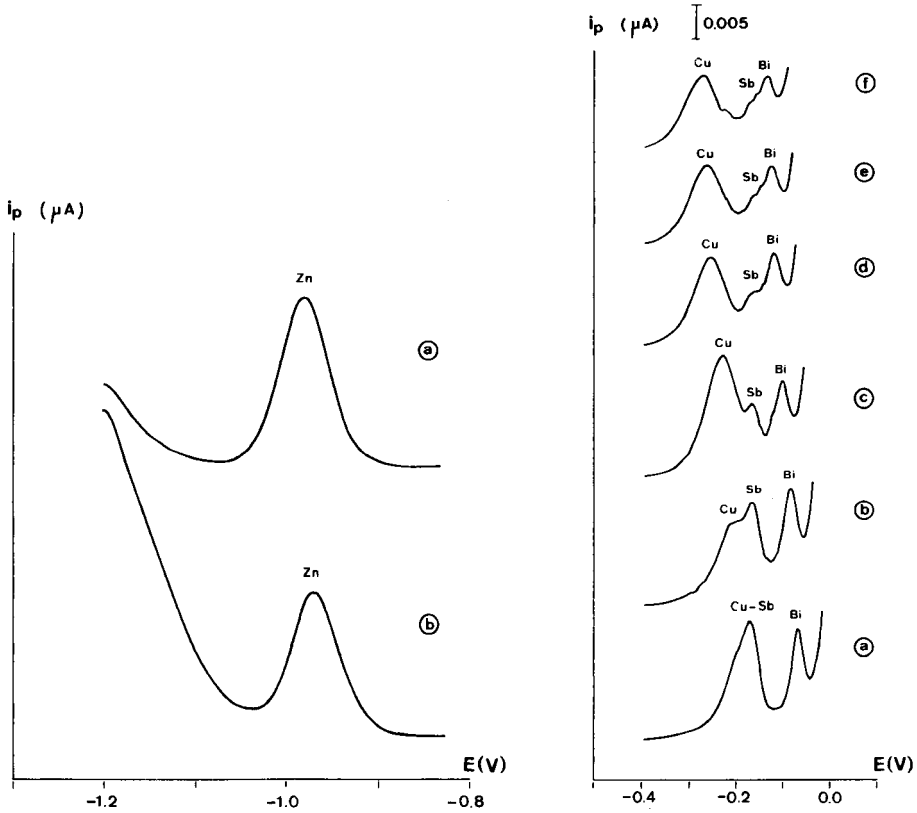


Fig. 11. Voltammograms for zinc in sea water spiked with 15 ppb Zn at pH 3 (curve a) and pH 1 (curve b).

Fig. 12. Voltammograms of natural sea water adjusted to pH 1 (HCl) with chloride added. Electrodeposition and stripping conditions as for Fig. 8. Molarity of chloride: (a) 0.5; (b) 1; (c) 2; (d) 3; (e) 4; (f) 5.

it can be seen that the potentials of the Cu, Sb and Bi peaks are shifted while peak currents decrease slightly with increasing chloride concentration. This effect is less for the antimony peak. A chloride concentration of 2 M in sea water (pH $\approx$ 1) is required for the best resolution (Fig. 12, curve c). The blank value introduced by the addition of Suprapur sodium chloride is negligible.

Test of the linearity of i_p *with concentration.* The variation in peak heights with the concentration was studied by the addition of the metals to sea water samples. Linear relationships were observed for Zn, Cd, Pb, Cu, Sb and Bi (Fig. 13). The first point on each of the calibration curves corresponds to the natural concentration of the metal in the sea water sample. It is interesting to note that the slope of the calibration curves varies from one metal to another. There are various reasons for this variation; for

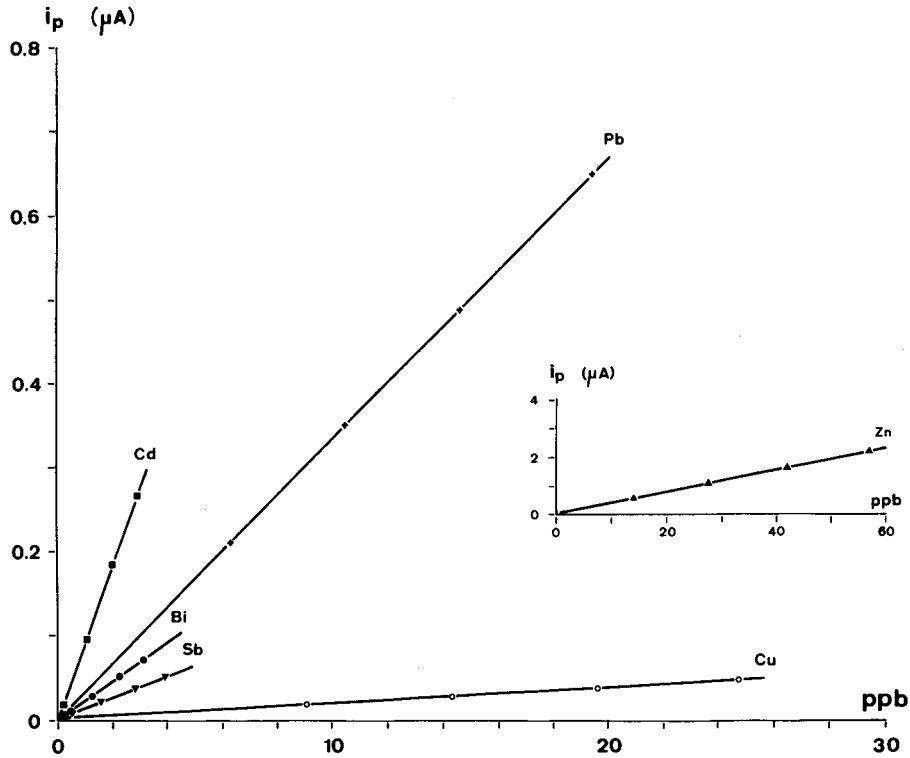


Fig.13. Calibration curves for Zn, Cd, Pb, Cu, Sb and Bi in a sea water sample at pH 1, containing 2 M chloride. Deposition timing, 15 min (Zn, Cd, Pb) and 30 min (Cu, Sb, Bi); pulse amplitude, 35 mV (Zn, Cd, Pb) and 10 mV (Cu, Sb, Bi); scan rate, 2 mV s^{-1} (Zn, Cd, Pb) and 0.5 mV s^{-1} (Cu, Sb, Bi).

example, the solubility of the metal in the mercury and the presence of 2 M chloride may have a marked effect on the slope.

Evaluation of stripping peaks. The measurement of the peak height generally yields results with sufficient precision. Measurements were done by the following procedure: the heights of the Zn, Cd and Pb peaks were measured from the baseline (Fig. 14); the heights of the Cu, Sb and Bi were measured by deconvoluting the overlapping peaks with a curve resolver (Du Pont, E 310) as shown in Fig. 14. This method is very efficient: it is possible to separate the Cu and Sb peaks where the potential difference is only 45 mV.

Precision and detection limits. The standard deviations of the analytical procedure were determined by analysing six aliquots of the same sea water sample. The results are shown in Table 6. The detection limit is strongly dependent on the experimental conditions. In the present case for a 60-min plating time, the detection limits were of the order of 0.1 ppb for Zn and Cu; 0.01 ppb for Cd and Pb, and 0.05 ppb for Sb and Bi.

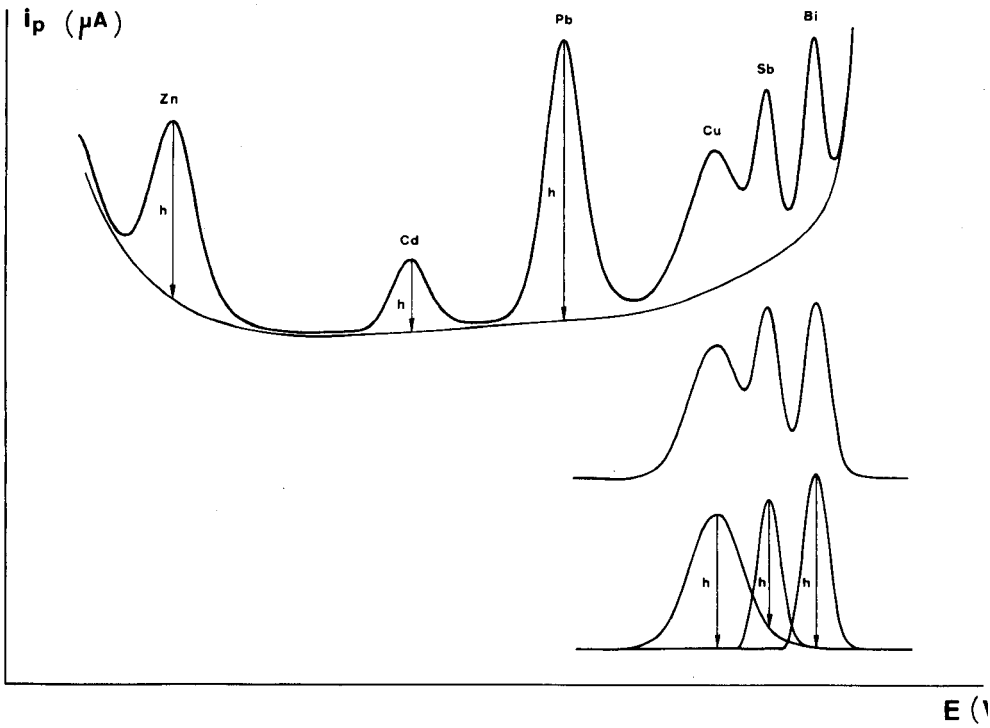


Fig. 14. Evaluation of stripping peaks.

TABLE 6

Precision of method

Metals	Concentration (ppb)	Standard deviation (ppb)
Zn	5.2	0.26
Cd	0.6	0.06
Pb	6.2	0.70
Cu	4.3	0.40
Sb	0.40	0.06
Bi	0.30	0.04

Recommended procedure

The concentrations of metals dissolved in sea water were determined by the method of standard additions under the optimized experimental conditions described above.

The following procedure is recommended: sea water (30 ml) is introduced into the electrolysis cell and adjusted to about pH 1 and to a 2 M NaCl concentration with diluted Suprapur HCl and Suprapur NaCl solutions, respectively. The sample is stirred magnetically (450 rpm) and oxygen

is removed by bubbling pure nitrogen for 30—40 min. Electrolysis is carried out at a potential of —1.200 V (vs. SCE) for 15—20 min while nitrogen is introduced into the space above the surface of the solution. One minute before the end of the electrolysis, the stirrer is stopped and the current—potential curve is then recorded from —1.200 to —0.350 V. The potential is held at —0.350 V with stirring for 15—20 min. At the end of this plating time, the stirring is stopped again and the resulting current—potential curve is recorded down to a potential of 0 V with an appropriate current sensitivity.

RESULTS AND DISCUSSION

The following results concern samples of sea water from the North Sea off the Belgian coast collected at a depth of 3 m, filtered through a 0.8-μm Millipore filter and stored in acid-cleaned polyethylene bottles at —20°C until the analysis is started (Fig. 15). The results shown in Table 7 indicate that the concentrations of Zn and Cd are of the same order of magnitude of those reported by Duinker and Kramer [21]. However, the concentrations of Pb and Cu are 2—3 times higher than those reported by the same authors on samples collected around the same area of the North Sea. In contrast, the present results are generally higher than those reported by Valanta et al. [22] for North Sea water (taken 13 km off the shore of Walckeren, The Netherlands). This difference is probably due mainly to experimental conditions such as the pH of analysis [11, 21] and conditions of filtration [22]. The present results for antimony range from 0.2—0.6 ppb, which is in good agreement with the values (0.18—0.40) found by Portmann and Riley [18] and those found (0.25—0.47) by Gilbert and

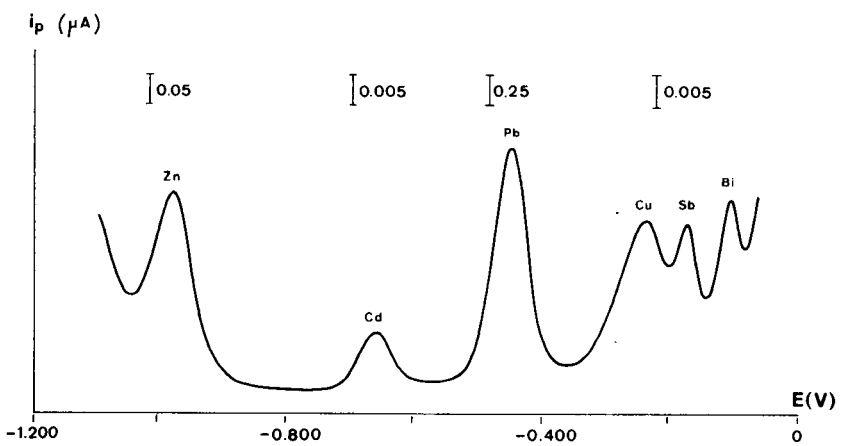


Fig. 15. Voltammograms of Zn, Cd, Pb, Cu, Sb and Bi dissolved in sea water at pH 1 containing 2 M chloride. Deposition times 20 min (Zn, Cd, Pb) and 40 min (Cu, Sb, Bi). Pulse amplitude and scan rate as for Fig. 13.

TABLE 7

Analysis of five samples of North Sea water

Metals	Metal content (ppb)				
	1	2	3	4	5
Zn	7.00	2.66	14.20	22.00	14.00
Cd	0.40	0.30	0.20	0.95	0.30
Pb	1.80	7.44	7.26	3.60	6.38
Cu	2.82	9.70	5.70	8.00	9.12
Sb	0.30	0.45	0.82	0.30	0.42
Bi	0.20	0.68	0.55	0.20	0.28

Hume [23]. The results for bismuth seem to be surprisingly high and this point needs further investigation.

The procedure based on differential pulse anodic stripping voltammetry with a hanging mercury drop electrode is convenient for the direct simultaneous determination of Zn, Cd, Pb, Cu, Sb and Bi in sea water. The optimal values of the experimental parameters are: pulse amplitude 10—35 mV, scan rate 0.5—2 mV s^{-1}, pulse repetition time 0.5 s, stirring rate 450 rpm, deposition time 20—40 min, pH 1 and a chloride concentration of 2 M.

Under these conditions and with the standard addition method, the simultaneous determination of these six metals in sea water takes about 2 h.

The problems of chemical speciation in sea water will be discussed in later papers.

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ENZYMATIC INHIBITION ENTHALPIMETRY
The Analytical Potential of the Inhibitory Action of a Series of Physiologically Active Alkaloids on the Activity of Serum Cholinesterase

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SUMMARY

A kinetic method is described for the enthalpimetric determination of a series of physiologically active alkaloids based on their inhibitory effect on the cholinesterase-catalysed hydrolysis of butyrylcholine iodide. All analyses are done at pH 8.0 and at 25.0°C (short term stability ± 0.002°C). Precision (< 3.0%) data are reported for the determination of physostigmine sulphate ($1.0—4.0 \times 10^{-3}$), quinine sulphate ($1.0 \times 10^{-6}—4.0 \times 10^{-5}$), procaine hydrochloride ($1.0 \times 10^{-5}—2.5 \times 10^{-4}$), atropine sulphate ($5.0 \times 10^{-5}—3.0 \times 10^{-4}$), morphine sulphate ($1.0—8.0 \times 10^{-4}$), codeine phosphate ($3.0 \times 10^{-4}—2.4 \times 10^{-3}$), pilocarpine nitrate ($5.0 \times 10^{-4}—6.0 \times 10^{-3}$) and thiamine hydrochloride ($1.0—5.0 \times 10^{-3}$); the linear response ranges in mol dm^{-3} are given in parentheses. Complete inhibition curves are presented and relative "potency" is inferred. The effects of several interfering inhibitors are discussed.

The velocity (v) of a competitively inhibited enzymatic reaction can be represented by the hyperbolic equation;

$$v = V_{max}[S]/[S] + K_m (1 + [I]/K_i) \quad (1)$$

where [S] is the substrate concentration, V_{max} the maximum velocity, [I] the inhibitor concentration, K_m the Michaelis constant, and K_i the inhibition constant [1].

Inspection of eqn. (1) shows that the rate of a competitively inhibited reaction will decrease with increasing inhibitor concentration, asymptotically approaching an equilibrium value depending on the magnitude of K_i. A plot of % inhibition versus inhibitor concentration is typically rectilinear at low inhibitor concentration [2].

The more powerful reversible inhibitors of cholinesterase-catalysed reactions usually incorporate a quaternary nitrogen atom and an ester group, and are highly methylated [1]. Molecules of this overall structure can compete successfully with choline-type substrates. Accordingly, a number of quaternary ammonium compounds, carbamate derivatives and alkaloids inhibit cholinesterase activity to a greater or lesser extent [3]. Indeed, some of the alkaloids owe their pharmacological activity to an inhibitory mechanism,

but only in a few cases (e.g. physostigmine) has the action been traced to a particular enzyme [4]. Comprehensive lists of reversible competitive inhibitors of cholinesterase activity have been reported elsewhere [3, 5].

Reports concerned with the determination of organophosphorus inhibitors (mainly pesticides) are legion and have been reviewed by Guilbault [2]. However, there have been to date very few methods that have exploited the inhibitory action of alkaloids to analytical advantage. Ellis et al. [6] used a manometric technique for the determination of physostigmine (10^{-6}—10^{-8} mol dm^{-3}) depending on the relation of cholinesterase activity to physostigmine concentration in serum.

In the area of calorimetric analysis, the enzymatic determination of inhibitors has been confined to the analysis of organophosphorus pesticides by means of flow calorimetric techniques employing sophisticated calorimeters of the heat-leak type [7, 8]. The contribution of calorimetric methods to biochemical analysis in general has been discussed comprehensively in recent reviews [9, 10]. The non-enzymatic determination of alkaloids by calorimetric procedures has been dominated by the "catalimetric" approach [11—14] which allows the determination of microgram amounts of alkaloids. These methods are however confined to analyses in non-aqueous systems. Milligram amounts of alkaloids in dosage forms have also been determined by direct thermometric enthalpy titration with tungstosilicic acid [15].

The sensitivity of enzymatic inhibition effects and the universal nature of enthalpimetric methods combine to make an attractive method of analysis. The combination has however not been adequately exploited. An enthalpimetric determination of urease inhibitors has been reported [16]. The kinetic treatment involved in the latter precludes the use of calibration procedures and restricts the technique to non-competitive inhibitors.

The present paper describes the enthalpimetric determination of nano-, micro- and milli-gram amounts of several physiologically active alkaloids by means of their inhibitory effect on the cholinesterase-catalysed hydrolysis of its optimum substrate [17], butyrylcholine iodide, at pH 8.0. The effects of several interfering species are also reported.

The essence of the enthalpimetric approach to the determination of enzyme activities, *EA* [18], is that the rate of evolution (or absorption) of heat during the course of an enzymatic reaction, $\Delta q \Delta t^{-1}$, can be related to *EA* by the equation

$$EA = \Delta H^{-1} \Delta q \Delta t^{-1} 10^{-6} (\text{I.U.}) \qquad (2)$$

where ΔH is the overall molar reaction enthalpy; *EA* is conventionally expressed as the number of micromoles of substrate consumed per minute at a specified pH and temperature. Therefore, under pseudo zero-order conditions the rate of the enzymatic reaction as represented by the rate of change of temperature depends on the amount of enzyme present. The temperature change produced by the enzymatic hydrolysis is amplified by the concurrent protonation of Tris buffer.

The total enthalpy change engendered by the concurrent reactions is -46.1 ± 0.5 kJ mol^{-1} [19]. This value is used here in all calculations involving eqn. (2). All inhibition data are calculated from the equation [2]

$$\% \text{ Inhibition} = [(\text{Rate, uninhibited} - \text{Rate, inhibited})/(\text{Rate, uninhibited})] \times 100 \quad (3)$$

EXPERIMENTAL

Reagents

Distilled water was used throughout. All buffered solutions were made up in 0.2 mol dm^{-3} 2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris), adjusted to pH 8.0 with hydrochloric acid.

Type IV horse serum cholinesterase (E.C. No. 3.1.1.8; nominal activity, 15 I.U. mg^{-1} protein) and 99% butyrylcholine iodide (both from Sigma Chemical Co.) were stored desiccated below 0°C. The following compounds were used without further purification.

Alkaloids (pharmaceutical grade). Physostigmine sulphate (eserine), quinine sulphate, procaine hydrochloride, atropine sulphate, morphine sulphate, codeine phosphate, pilocarpine nitrate, caffeine citrate and thiamine hydrochloride.

Vitamins (Sigma). *p*-Aminobenzoic acid (Vitamin Bx); d-biotin (Vitamin H); folic acid (pteroylglutamic acid, Vitamin Bc); d-pantothenic acid, calcium salt; riboflavin (Vitamin B_2, G); DL-6,8-thioctic acid (α-lipoic acid); niacinamide (Vitamin B_3, B_5, nicotinamide); pyridoxal HCl, pyridoxine HCl (Vitamin B_6) and L-ascorbic acid (Vitamin C). Stock solutions of these compounds were prepared by quantitative dissolution in 0.2 mol dm^{-3} Tris adjusted to pH 8.0 with hydrochloric acid. A more concentrated buffer solution, 0.4 mol dm^{-3}, was used to make stock solutions of the more acidic pyridoxal and pyridoxine hydrochlorides.

The light-sensitive solutions, i.e. of pyridoxal, pyridoxine, folic acid and riboflavin, were prepared as required and stored in foil-wrapped containers.

Stability of solutions

Unadulterated aqueous cholinesterase solutions were observed to decrease in activity by 0.5% when stored for 3 h at 25°C. Addition of bovine albumin (Sigma Chemical Co.) reduces this decrease to a negligible level. Stock enzyme solutions consisted therefore of 25.5 mg of cholinesterase and 5 mg of bovine albumin diluted quantitatively to 50 cm^3 with water.

Aqueous stock solutions of butyrylcholine iodide (0.200 mol dm^{-3}) remained stable at 4°C for up to 6 days. No apparent decomposition of inhibitor solutions (as evidenced by their inhibitory activity) was observed over a period of two weeks. It should be noted that morphine sulphate in dilute solution adsorbs onto glass surfaces and should be prepared as required or kept in "siliconized" vessels [20]. Experiments with papaverine

hydrochloride were precluded because of precipitation of the free base at pH 8.0.

Apparatus

Temperature changes were monitored by means of the imbalance potential of a d.c. Wheatstone bridge circuit incorporating a 10,000-ohm thermistor as one arm of the bridge. Electrical calibration was done by the Joule heating of a 0.25-W carbon resistor coated with thermally-conducting epoxy resin. The details of the instrumentation used have already been described [21].

Substrate injections were made from a gravimetrically-calibrated precision syringe incorporating a Chaney adaptor. Temperature mismatch between substrate and enzyme solutions was avoided by prior immersion of the reaction cell and "loaded" syringe in a thermostatted water bath at 25.0°C.

Preliminary experiments

Clearly, for competitive inhibition, the amount of inhibitor required to cause a certain degree of inhibition is not absolute but depends on the concentration ratio of inhibitor to substrate. The "potency" of the inhibitor increases as the amount of substrate decreases and conversely. Accordingly, a fixed uninhibited control rate must be established at a constant substrate concentration. To minimize fluctuations in reaction rate caused by small variations in substrate concentration, an amount of substrate sufficient to ensure maximum velocity was employed; i.e., the conditions were arranged so that the reaction rate was zero order with respect to the substrate. In practice, the control rate was established by injecting 100 μmol of butyrylcholine iodide solution into 5.0 I.U. of buffered cholinesterase solution. All inhibition data were then calculated by using eqn. (3).

A salient feature of the enthalpimetric technique is that the total heat effect is observed. Preliminary experiments were therefore performed to ascertain whether or not the inhibitors themselves acted as substrates, as they are, by definition, similar in structure to the choline substrates. Moreover, implicit in the derivation of eqn. (3) is the assumption that the enzyme—inhibitor "complex" EI does not break down to products. Consequently a solution of each inhibitor (typically ca. 1×10^{-2} mol dm^{-3}) was injected into a buffered enzyme solution and the temperature change observed. No reaction was observed for any of the inhibitors examined.

The competitive nature of the alkaloidal inhibition of cholinesterase activity was confirmed by the construction of Lineweaver—Burk plots in the usual manner [1]. A typical plot is shown in Fig. 1. It was concluded from the characteristic invariance of V_{max} coupled with increases in K_m that the inhibition is indeed competitive.

The effect of incubation time on inhibition was established in the following manner. Several aliquots of inhibitor, sufficient to cause 20—60% inhibition, were incubated at 25.0°C with the buffered enzyme solution in the reaction cell for a recorded time interval before injection of butyrylcholine

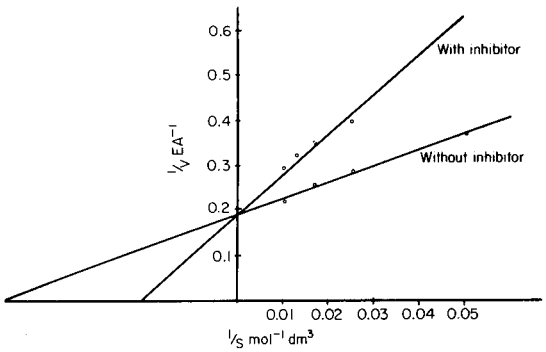

Fig. 1. A typical Lineweaver—Burk double reciprocal plot illustrating the competitive nature of the alkaloid inhibition (by physostigmine) of serum cholinesterase activity.

iodide. Incubation times varied between 20 and 120 min, the minimum incubation time being dictated by the prerequisite of a thermal equilibration period. This procedure, when repeated for all the inhibitors, showed that incubation had no effect on the inhibitory power of the alkaloid within the time limits mentioned.

Determination of analytically useful inhibition range

Before any analytical evaluation was possible, it was necessary to construct a complete inhibition curve. The following procedure was adopted for this purpose and used for all analytical measurements thereafter. Enzyme solution (1.00 cm^3; 5.0 I.U.) was added to 9.00 cm^3 of Tris buffer in the reaction cell; 1.00 cm^3 of a suitable standard solution of inhibitor was added. The cell and its contents were then allowed to come to thermal equilibrium in a thermostatted water bath (25.0°C). On attainment of equilibrium, as evidenced by an isothermal baseline, 0.50 cm^3 of substrate (100 μmol) was injected from a thermostatted precision syringe incorporating a Chaney adaptor. Simultaneously, the resultant temperature change was recorded on a strip-chart potentiometer for a period of 4 min. Typically, heat effects of 0.9—0.5 J were observed during these experiments, representing temperature changes of ca. 0.02—0.01°C. After this time, the signal was attenuated (× 0.5) and a calibration curve recorded by activation of the heating circuitry. Details of the heating circuitry have already been discussed [21]. The reaction rate was calculated in terms of enzyme activity (EA) from eqn. (2) and thence the % inhibition from eqn. (3).

Once the linear range of the plots of % inhibition vs. concentration of inhibitor had been established from the complete inhibition curves and calibration graphs constructed, an identical procedure was used at inhibitor concentrations within this range to determine the accuracy and precision of the method.

RESULTS AND DISCUSSION

A typical enthalpogram/calibration curve sequence is shown in Fig. 2. Complete inhibition curves for all the alkaloids examined are shown in Fig. 3. The order of the inhibitory power of the compounds investigated is physostigmine ⩾ quinine > procaine > atropine > morphine > codeine ≈ thiamine > pilocarpine > caffeine. This trend of inhibitory "potency" is reflected in the minimum detectable amount (defined as the lowest amount

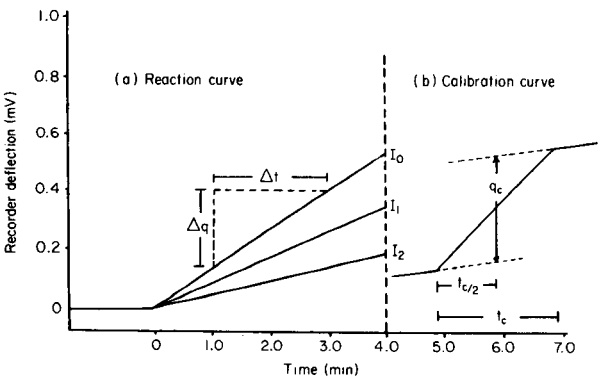

Fig. 2. A typical enthalpogram/joule heating calibration sequence; only one calibration curve is presented. (a) Reaction curves: I_0 = control rate, no inhibitor present; I_1, I_2 = inhibited rate for inhibitor concentrations of $[I]_2$ and $[I]_1$ ($[I]_2 > [I]_1$). (b) Calibration curve: to minimize errors caused by small but finite heat leakages, measurements of the heat effect q_c were standardized at $t_{c/2}$, where t_c is the activation time of the calibration heater. q_c was calculated from the equation $q_{c/J} = V_H V_S t_c / R_S$, where V_H is the voltage across the calibration heater; V_S and R_S are the voltage and resistance, respectively, of a precision resistor in series with the calibration heater.

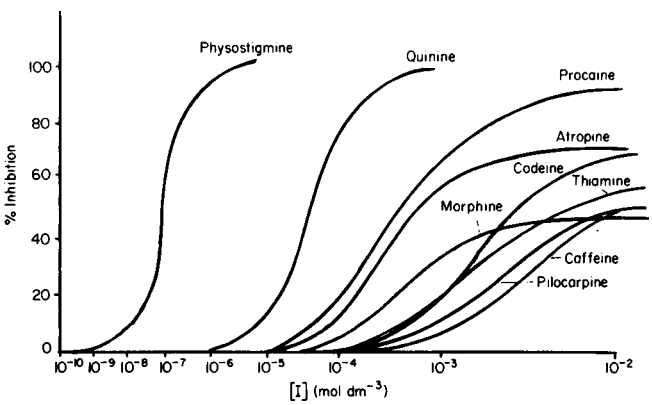

Fig. 3. Inhibition curves (alkaloids). [S] = 8.7 × 10^{-3} mol dm^{-3}, [E] = 5.0 I.U./reaction cell volume.

TABLE 1

Linear ranges and minimum detectable amounts for different alkaloids

Alkaloid	Minimum detectable amount (g)	Linear range (mol dm^{-3})
Physostigmine sulphate	7.4×10^{-6}	$1.0—4.0 \times 10^{-6}$
Quinine sulphate	9.0×10^{-6}	$1.0 \times 10^{-6}—4.0 \times 10^{-5}$
Procaine hydrochloride	3.1×10^{-5}	$1.0 \times 10^{-5}—2.5 \times 10^{-4}$
Atropine sulphate	4.0×10^{-4}	$5.0 \times 10^{-5}—3.0 \times 10^{-4}$
Morphine sulphate	1.1×10^{-3}	$1.0—8.0 \times 10^{-4}$
Codeine phosphate	1.5×10^{-3}	$3.0 \times 10^{-4}—2.4 \times 10^{-3}$
Pilocarpine nitrate	1.6×10^{-3}	$5.0 \times 10^{-4}—6.0 \times 10^{-3}$
Thiamine hydrochloride	3.9×10^{-3}	$1.0—5.0 \times 10^{-3}$

in the linear range) of each compound (Table 1). It should be noted that the inhibition curves apply to the stipulated levels of enzyme activity and substrate concentration only. Consequently, a particular inhibition curve may be displaced along the abscissa in either direction by changing either of these parameters. Figure 3 indicates that it should be possible to determine physostigmine in the presence of other alkaloids by virtue of its considerably greater inhibiting power. Some typical calibration graphs constructed in the analytically useful linear regions of inhibition are shown in Fig. 4. The analytical ranges obtained under the defined conditions are also given in Table 1. Results are not presented for the weakest inhibitor of the series, caffeine citrate; citrate ion itself inhibited the enzymatic reaction. All other critical parameters remaining the same, an 8.7×10^{-2} mol dm^{-3} solution of sodium citrate inhibited cholinesterase activity by 15.7%.

Inhibitor solutions at concentrations from the lower, medium and upper regions of the linear inhibition range were analysed by the procedures

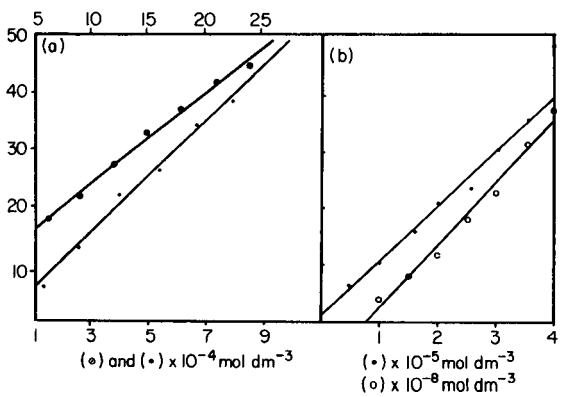

Fig. 4. Typical calibration graphs for (a) morphine sulphate (•) and codeine phosphate (o); (b) quinine sulphate (•) and physostigmine sulphate (o).

described. At each concentration, 5 or 6 determinations were performed and the error (range) and precision (% RSD) were calculated. The results are summarized in Table 2. The precision and error, neither of which exceeds 3%, are clearly analytically acceptable.

Interferences

The cholinesterases are inhibited by more substances, natural and synthetic, than any other group of enzymes [1]. Depending on their concentration in the solution, other cholinesterase inhibitors are potential interferences in the proposed method. Accordingly, some knowledge of the sample composition is essential before application of this technique. In a comprehensive review of cholinesterase inhibitors [3], it has been reported that several vitamins exert an inhibitory effect on cholinesterase-catalysed hydrolyses. The reason for this action is not readily apparent because the usual criteria necessary for competitive inhibition of cholinesterase do not exist in most of

TABLE 2

Error and precision analyses

Alkaloid	Mass taken (g)	Average mass found (g)	Range of mass found (g)	R.s.d. (n = 5 or (%)
Physostigmine sulphate	1.12×10^{-7}	1.13×10^{-7}	$(1.07–1.19) \times 10^{-7}$	0.65
	1.87×10^{-7}	1.84×10^{-7}	$(1.65–2.09) \times 10^{-7}$	2.6
	2.61×10^{-7}	2.56×10^{-7}	$(2.38–2.69) \times 10^{-7}$	2.4
Quinine sulphate	4.50×10^{-5}	4.41×10^{-5}	$(4.14–4.68) \times 10^{-5}$	0.32
	1.80×10^{-4}	1.77×10^{-4}	$(1.59–2.02) \times 10^{-4}$	2.1
	2.70×10^{-4}	2.67×10^{-4}	$(2.61–2.75) \times 10^{-4}$	0.60
Procaine hydrochloride	1.50×10^{-4}	1.59×10^{-4}	$(1.52–1.74) \times 10^{-4}$	0.60
	3.92×10^{-4}	3.83×10^{-4}	$(3.64–4.24) \times 10^{-4}$	1.4
	6.27×10^{-4}	6.25×10^{-4}	$(6.17–6.32) \times 10^{-4}$	0.80
Atropine sulphate	4.00×10^{-4}	4.07×10^{-4}	$(3.20–4.63) \times 10^{-4}$	1.0
	1.20×10^{-3}	1.17×10^{-3}	$(1.09–1.22) \times 10^{-3}$	0.92
	1.60×10^{-3}	1.57×10^{-3}	$(1.51–1.62) \times 10^{-3}$	0.90
Morphine sulphate	2.31×10^{-3}	2.33×10^{-3}	$(2.11–2.46) \times 10^{-3}$	0.37
	4.63×10^{-3}	4.56×10^{-3}	$(4.35–4.71) \times 10^{-3}$	0.51
	6.94×10^{-3}	6.78×10^{-3}	$(6.41–7.09) \times 10^{-3}$	1.1
Codeine phosphate	2.93×10^{-3}	2.85×10^{-3}	$(2.72–3.09) \times 10^{-3}$	0.60
	6.59×10^{-3}	6.69×10^{-3}	$(6.32–7.00) \times 10^{-3}$	1.2
	1.03×10^{-2}	1.02×10^{-2}	$(1.00–1.05) \times 10^{-2}$	1.4
Thiamine hydrochloride	3.88×10^{-3}	3.89×10^{-3}	$(3.18–5.28) \times 10^{-3}$	1.6
	1.16×10^{-2}	1.17×10^{-2}	$(1.11–1.23) \times 10^{-2}$	1.7
	1.94×10^{-2}	1.89×10^{-2}	$(1.84–1.92) \times 10^{-2}$	1.2
Pilocarpine nitrate	3.12×10^{-3}	3.06×10^{-3}	$(2.71–3.90) \times 10^{-3}$	1.4
	9.36×10^{-3}	9.20×10^{-3}	$(8.99–9.45) \times 10^{-3}$	0.83
	1.56×10^{-2}	1.54×10^{-2}	$(1.53–1.58) \times 10^{-2}$	1.1

these compounds. Indeed, the vitamins are classified as a group according to their function, and there is no general structural trend within the group. In order to assess the potential interference of these compounds, the inhibitory or in some cases activating effects of a series of vitamins on cholinesterase activity were examined by enthalpimetric procedures identical to those described for the alkaloids. Complete inhibition data for those compounds with measurable inhibitory action are presented in Fig. 5; thiamine is included in Fig. 3. The inhibition curves of *p*-aminobenzoic acid and pyridoxine hydrochloride are truncated because of solubility and buffer capacity considerations, respectively.

Three of the compounds examined, thioctic acid, d-biotin and ascorbic acid, exerted no inhibitory (or activating) effect on the cholinesterase system up to concentrations of 2.46×10^{-3}, 5.52×10^{-3} and 8.70×10^{-3} mol dm^{-3}, respectively. These concentration limits were imposed by the limited solubility of thioctic acid and d-biotin and the reduction of buffer capacity in more concentrated solutions of ascorbic acid. Riboflavin exerted a 2.7% activating effect, approaching its solubility limit at 4.26×10^{-4} mol dm^{-3}. Folic acid, however, exerted either activating or inhibiting actions, depending on its concentration. A Lineweaver–Burk plot constructed at a folic acid concentration of 5.84×10^{-3} mol dm^{-3} indicated that folic acid acted as a competitive inhibitor. Further experiments, however, showed that at concentrations less than ca. 1×10^{-3} mol dm^{-3}, folic acid activates the cholinesterase system; indeed, 9.3% activation was recorded at a concentration of 7.80×10^{-5} mol dm^{-3}. The latter observation, in vitro, is in agreement with the in vivo results of Davis [22] who reported that serum cholinesterase activity was increased in human subjects by the ingestion of folic acid during the treatment of hyperchromic anaemia. The activating and inhibiting effects of folic acid were not large enough to allow precise inhibition data.

The calcium salt of pantothenic acid activated cholinesterase activity by 19.5% at a concentration of 8.7×10^{-2} mol dm^{-3}. It was concluded that this result was due, at least in part, to activation by Ca^{2+}. In a separate experiment, an identical concentration of calcium chloride produced an increase in activity of 13.8%.

Clearly, the vitamins examined do represent a potential interference to this method of analysis. However, they are in general much less potent inhibitors than the alkaloids and, with the exception of pyridoxal, do not interfere until present in concentrations of at least 10^{-3} mol dm^{-3}. In a separate series of experiments, in which the pH-stat approach was used, the following selected species were also observed to exert an inhibitory action (minimum concentration in mol dm^{-3} in parentheses): F^- (10^{-4}), citrate (10^{-4}), sulphanilamide (10^{-3}), azosulphamide (10^{-3}), chromate (10^{-2}) and sodium barbiturate (10^{-3}).

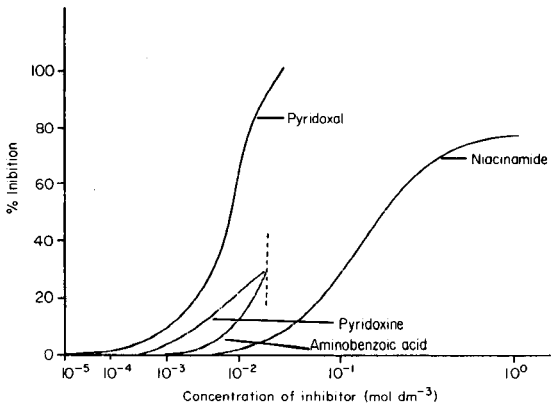

Fig. 5. Inhibition curves for vitamins. [S] = 8.7 × 10^{-3} mol dm^{-3}, [E] = 5.0 I.U./reaction cell volume.

Conclusion

As is the case in most enzymatic inhibition analyses, this technique is subject to limitations imposed by the presence of interfering inhibitors and the limited linear range of the inhibitory effect. The use of enthalpimetric methodology, however, means that, in contrast to many other enzymatic analyses, the primary catalytic reaction can be observed without recourse to complex coupling procedures in order to produce an analytically measurable entity. In this context, the technique provides a convenient method for the compilation of inhibition data for analytical or biochemical use.

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ACIDITY FUNCTIONS OF AQUEOUS FLUORINATED ACID SOLUTIONS BY DIFFERENTIAL PULSE POLAROGRAPHY

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SUMMARY

The acidity functions of aqueous trifluoroacetic and trifluoromethanesulphonic acid mixtures, and aqueous hexafluoropropane-2, 2-diol solutions, have been determined by differential pulse polarography. The apparent shift of the half-wave potential for the ferrocene—ferricinium couple, as the solvent composition is changed, is used to indicate the change in potential of a glass electrode; acidity is measured as the H_{GF} acidity function. The densities of two of these solvent systems as a function of composition are also reported. Trifluoromethanesulphonic acid—water mixtures represent the strongest aqueous acid solvent system so far studied.

Aqueous trifluoroacetic acid (TFA) and hexafluoropropane-2, 2-diol (HFPD) are used in biochemistry as solvents in which the helix-coil transition of proteins may be studied [1, 2]. Aqueous TFA [3, 4] and aqueous trifluoromethanesulphonic acid (TFMSA) are being increasingly used as non-nucleophilic ionizing solvents; the latter is also one of the strongest acids known [5, 6]. A quantitative study of these aqueous fluorinated acid media should enhance their utility as analytical, preparative and general-purpose solvents.

Strehlow and Wendt [7] devised an operational measure of acidity, equivalent to pH, based on a redox equilibrium; in aqueous sulphuric acid this is the redox function R_0 [7]:

$$R_0\,(\mathrm{H}) = F\{E(x) - E(1)\}/RT \ln 10 \qquad (1)$$

where $E(1)$ is the e.m.f. of the cell $Pt|H_2(g, 1\ atm)|H_2SO_4(aq)$ ($a_{H^+} = 1$), $Fec/Fec^+(1{:}1)|Pt$, with the usual symbol definitions; Fec/Fec^+ represents the redox couple ferrocene/ferricinium ion. Janata and Jansen [8] noted possible difficulties in the practical use of this cell, and refined this approach to acidity measurement by introducing a polarographic procedure. The Fec/Fec^+ half-wave potential is used as a reference against which the potential of a lithium-based glass electrode [8—10] or a hydrogen electrode (which has been shown to give the same results [11]) may be measured. The advantage of this redox reference lies in the elimination of undefined junction potentials (e.g. as in a calomel electrode with its salt bridge), or unknown anion activities for

references without liquid junctions. The suitability of ferrocene as a reference has been discussed by several workers [8, 12]; the use of only one indicator compound in determining acidities over a very wide range in non-ideal strong acid or base solutions [8—12] offers considerable advantages over more conventional methods, which need several indicators (see the discussion in [13]).

The measured potential difference E is given [14] by

$$E = (E_{\frac{1}{2}} - E_g) = (E^0_{\frac{1}{2}} - E^0_g) - (RT \ln 10/F)\{\log a_{H^+} + \log (f_{Fec}/f_{Fec^+}) + 0.5 \log (D_{Fec^+}/D_{Fec})\} \quad (2)$$

where E_g refers to the glass electrode and $E_{1/2}$ to the half-wave potential of Fec/Fec$^+$; f represents activity coefficients and D diffusion coefficients. With the usual polarographic assumption that the diffusion coefficient ratio is approximately zero [8], the acidity function H_{GF} can be defined [8, 14] by

$$-H_{GF} = \log a_{H^+} + \log (f_{Fec}/f_{Fec^+}) \quad (3)$$

where GF refers to the electrodes used (Glass, Ferrocene). In this paper, values of this function are reported for the three fluorinated acid solvent systems mentioned, measured by differential pulse polarography.

EXPERIMENTAL

Reagents

Ferrocene (Eastman) was purified by vacuum sublimation (m.p. 174—6°C); TFA (MCB, Aldrich) and TFMSA (MCB) were purified by distillation; HFPD (PCR) and perchloric acid (BDH) were used as supplied. All water used was purified from trace organics and doubly distilled.

Apparatus

A three-electrode PAR Model 170 Electrochemistry System with a PAR Model 9346 Dropping Mercury Electrode Kit was used in this work. The glass indicator electrode (Corning Triple Purpose type 476022) was used in conjunction with the dropping mercury electrode and a platinum auxiliary electrode. A noise filter from the drop timer was applied in all scans. For sensitivities providing a full scale of less than 10 μA, a faraday cage of grounded copper mesh was used to surround the polarographic cell and drop timer, in order to reduce outside electrical interference. Further purification of the nitrogen used proved not to be necessary, but it was passed through a Drechsel bottle filled with an appropriate solvent mixture to saturate the gas with vapour before entering the cell. The cell was thermostatted at 25.0 ± 0.1°C. Solution densities were determined at 25.1°C with an Anton Paar Model DMA02C digital precision density meter.

Solutions

Aqueous TFA. Each solution contained a known weight of acid and water, weighed out on a Mettler H54AR balance. After the polarographic measure-

ments, the solutions were collected and their densities measured. These were compared to a calibration density curve to determine accurately the mole fractions of acids. The remaining solution was then checked by titration with 1 M NaOH.

Aqueous HFPD. A stock solution was prepared by weight; further solutions were obtained by accurate dilution. After polarographic measurements, the solutions were collected and their densities measured. A calibration density curve was constructed, and used to determine molarity.

Aqueous TFMSA. Stock and sample solutions were prepared in the same manner as for HFPD. The solutions were collected after the polarographic measurements and were titrated with 0.5 M NaOH.

Polarographic determinations

A differential pulse scanning mode was used to obtain high sensitivity and accurate peak maxima. The polarographic cell was charged with 5—10 ml of solution and nitrogen gas, pre-saturated with solution vapour, was passed through the solution for about 10 min. This served to deoxygenate and stir the solution, and bring it to temperature equilibrium.

The TFA solutions showed no observable impurities. A few crystals of ferrocene were added 30 s before degassing was complete, to allow time for the test solution to become saturated with ferrocene. The polarographic scan was then made, at scan rates of 1—2 mV s^{-1} and drop times of 1 or 2 s. When the scan was completed, the solution was immediately transferred to another vessel and stored for further concentration determinations. The HFPD and TFMSA solutions were found to contain trace impurities; for these, an additional scan was obtained prior to the addition of ferrocene. The ferrocene peak was then located by eliminating the background given by this first scan. Apart from this, the same procedure as for TFA was followed. Aqueous perchloric acid solutions were used to obtain the constant term ($E^0_{1/2} - E^0_g$) in eqn. (2) above.

RESULTS AND DISCUSSION

Compared to conventional polarography, as used by Janata et al. [8—11], the technique of differential pulse polarography was found to be much easier to use. Ferrocene is practically insoluble, at least in the aqueous region, and the extra signal amplification available in pulse polarography simplified location of the ferrocene wave. The differential mode was used because it is much more convenient to locate a peak maximum than to locate the mid-point of a conventional polarographic wave.

The constant in eqn. (2), ($E^0_{1/2} - E^0_g$), was found to be −285 ± 4 mV for the glass electrode used, which agrees well with Janata and Jansen's value [8]. The measured potential differences were converted to acidities via eqns. (2) and (3), and the hydronium ion activities, as given by the H_{GF} acidity function defined in eqn. (3), are tabulated in Table 1 for aqueous trifluoroacetic

TABLE 1

Hydronium ion activity, as measured by the H_{GF} acidity function, for aqueous solutions of trifluoroacetic acid (TFA) at 25.0 ± 0.1°C

X_{TFA}	M_{TFA}	$-H_{GF}$
0.002	1.325	−0.42
0.060	2.500	0.49
0.098	4.361	1.07
0.199	7.009	2.69
0.288	8.864	3.87
0.397	10.285	5.09
0.479	10.854	6.09
0.598	11.540	7.02
0.702	11.966	7.26
0.790	12.319	7.77
0.891	12.642	8.00
0.998	12.730	8.32

TABLE 2

Hydronium ion activity, as measured by the H_{GF} acidity function, for aqueous solutions of trifluoromethanesulphonic acid (TFMSA) and hexafluoropropane-2, 2-diol (HFPD) at 25.0 ± 0.1°C

M_{TFMSA}	$-H_{GF}$	M_{HFPD}	$-H_{GF}$
0.564	−0.25	0.580	−4.60
1.141	1.18	1.088	−4.55
2.239	1.52	1.668	−4.53
3.298	2.37	2.259	−4.43
4.227	3.76	2.275	−3.72
5.492	5.03	3.304	−2.50
6.494	7.29	3.818	−1.83
7.485	8.76	4.398	−1.05
8.322	11.07	4.901	−0.61
		5.402	−0.37

acid mixtures, and Table 2, for aqueous trifluoromethanesulphonic acid and aqueous hexafluoropropane-2, 2-diol. The densities of aqueous TFA and HFPD, determined in this work to facilitate concentration unit interconversions, are given in Table 3; the values for aqueous TFA compare very well with those previously determined by Fialkov and Zhikharev [15].

The three systems are compared in Fig. 1. As can be seen, the measuremen encompass a range of 16 powers of ten in acidity. Trifluoroacetic acid has been studied more than the other systems; the present measurements are compared with the H_O [16—18] and H_R [18] functions in Fig. 2. The resemblance between H_R and H_{GF} is more noticeable than that between H_{GF} and H_O; similar behaviour has been found for the aqueous sulphuric acid system [8]. Acidity functions have also been measured in aqueous hydrofluo

TABLE 3

Densities of aqueous trifluoroacetic acid mixtures (mole fraction units) and aqueous hexafluoropropane-2, 2-diol mixtures (molarity units) at 25.1 ± 0.1°C (g ml^{-1})

X_{TFA}	ρ	M_{HFPD}	ρ
0.000	0.9972	0.000	0.9972
0.051	1.1561	0.500	1.0384
0.100	1.2252	1.000	1.0798
0.199	1.3289	2.000	1.1600
0.400	1.4324	3.000	1.2367
0.605	1.4702	4.000	1.3223
0.792	1.4795	6.000	1.4776
1.000	1.4812		

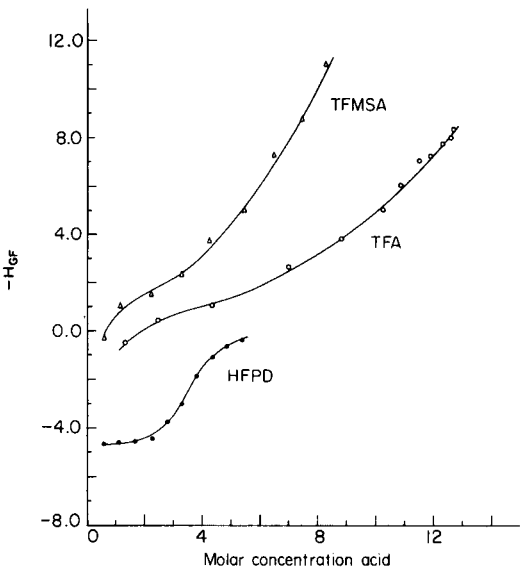

Fig. 1. The variation of acidity as $-H_{GF}$ with acid molarity for aqueous trifluoromethanesulphonic acid (TFMSA), trifluoroacetic acid (TFA) and hexafluoropropane-2, 2-diol (HFPD).

acid systems at acidity levels of the same order of magnitude [19]. TFA is a relatively strong organic acid because of the electron-withdrawing fluorine atoms; this effect also causes the anion to be a poor nucleophile, and apparently inhibits complexation between the anion and ferrocene, such as is believed to occur with acetate ion itself [8]. No evidence for complexation was found in this work, for any of the systems studied.

The HFPD system has been studied at higher concentrations by Mehmet and Roche [20]; their H_O measurements are compared with the present H_{GF}

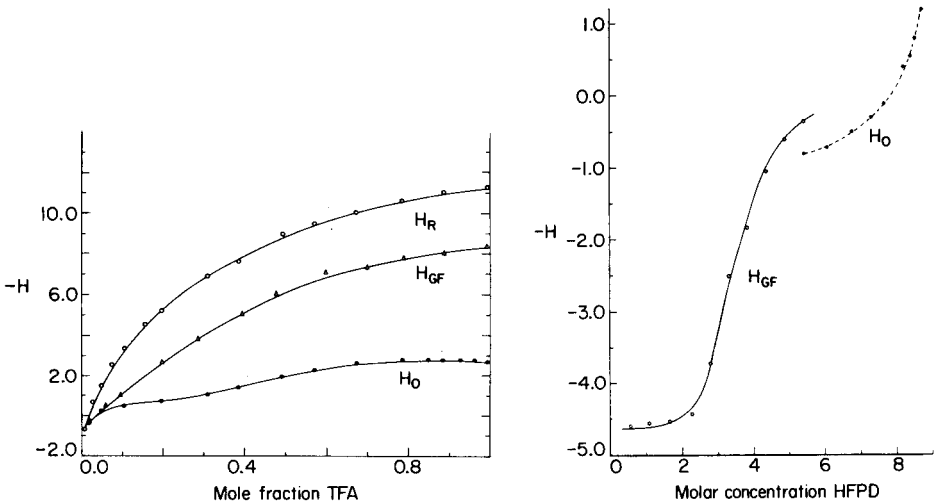

Fig. 2. Acidity functions in the water/trifluoroacetic acid solvent system. H_{GF}, this work; H_0, refs. [16—18]; H_R, ref. [18].

Fig. 3. Acidity functions in aqueous hexafluoropropane-2, 2-diol. H_{GF}, this work; H_0, ref. [20].

data in Fig. 3. As can be seen, they are roughly contiguous. The detailed behaviour of this system appears complex, and further study is indicated. This is the weakest acid system so far studied by this technique [8, 14].

The trifluoromethanesulphonic acid solutions, in contrast, represent the strongest aqueous acid system so far available, stronger even than aqueous perchloric acid. The H_{GF} value of 7.5 M TFMSA, —8.8, is more than two units lower than that for 7.5 M $HClO_4$, —6.7 [8]. Correction from the Fec/Fec^+ standard used here to the tetraethylammonium pentacyanopropenide (TEA^+/PCP^-) standard favoured by organic chemists, which requires additional activity coefficient measurements [14], is unlikely to change this situation, as the activity coefficient variations of Fec and Fec^+ average less than one log unit over wider acidity ranges than those studied here [14]. This solvent system deserves wider study; it appears a highly promising medium for the study of acid-catalysed reactions.

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SPECTROPHOTOMETRIC AND FLUORIMETRIC DETERMINATIONS OF TRICHOTHECENE MYCOTOXINS WITH REAGENTS FOR FORMALDEHYDE

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SUMMARY

Several trichothecene mycotoxins, such as fusarenone-X (F-X) and T-2 toxin (T-2), readily liberate formaldehyde on heating with sulfuric acid. Spectrophotometric and fluorimetric methods for the determination of trichothecenes with reagents for formaldehyde are therefore possible. F-X (or T-2) can be determined in the 50—1000 mg l^{-1} (or 50—1500 mg l^{-1}) range by the chromotropic acid method, in the 30—1200 mg l^{-1} (or 50—2000 mg l^{-1}) range by the phenyl J acid method, and in the 1.25—25 mg l^{-1} (or 2—40 mg l^{-1}) range by the J acid method. Other trichothecenes, neosolaniol, nivalenol, tetraacetylnivalenol, diacetoxyscirpenol and HT-2 toxin, etc. also give positive reactions but trichothecin and dihydronivalenol do not.

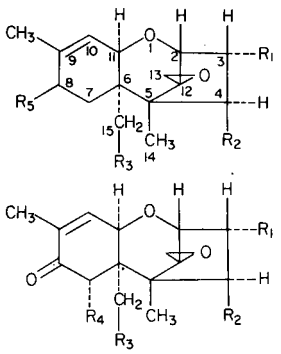
The naturally occurring fungal toxic sesquiterpenoids with a trichothecene nucleus, such as fusarenone-X (F-X), T-2 toxin (T-2), neosolaniol, nivalenol, etc. are among the most interesting mycotoxins in food chemistry because these mycotoxins have developed sporadically in several places in the world, e.g. Akakabi diseases of wheat in Japan and alimentary toxic aleukia in USSR.

Although methods based on gas chromatography [1—3], thin-layer chromatography [4—7], mass spectrometry [8], and enzymatic reactions [9] have been reported for the assay of trichothecene mycotoxins, few methods have utilized a specific color or fluorescence reaction of trichothecenes.

Studies on a fluorimetric determination of F-X and related trichothecenes with ethylenediamine—zirconyl nitrate [10] and a spectrophotometric study of F-X and T-2 with chromotropic acid [11] have already been reported. It seemed desirable to develop the latter method further because of its simplicity and rapidity as well as its high sensitivity for the detection of trichothecenes. The present paper describes three methods based on reagents for formaldehyde (chromotropic acid, phenyl J acid [12] and J acid [13]) for the assay of trichothecene mycotoxins.

TABLE 1

Structures of trichothecene mycotoxins



Mycotoxin	R_1	R_2	R_3	R_5
T-2 toxin[a]	OH	$OCOCH_3$	$OCOCH_3$	$OCOCH_2CH($
HT-2 toxin[b]	OH	OH	$OCOCH_3$	$OCOCH_2CH($
Neosolaniol[a]	OH	$OCOCH_3$	$OCOCH_3$	OH
Diacetoxy-scirpenol[c]	OH	$OCOCH_3$	$OCOCH_3$	H

	R_1	R_2	R_3	R_4
Fusarenone-X[d]	OH	$OCOCH_3$	OH	OH
Nivalenol[e]	OH	OH	OH	OH
Dihydronivalenol[f]	OH	OH	OH	OH
Tetraacetyl-nivalenol[e]	$OCOCH_3$	$OCOCH_3$	$OCOCH_3$	$OCOCH_3$
Trichothecin[b]	H	$OCOCH=CHCH_3$	H	H

[a]Cultured *Fusarium solani*, purified as reported by Ueno et al. [14]. [b]Supplied by Y. Uen (Faculty of Pharmaceutical Sciences, Science University of Tokyo, Japan).[c]Makor Chemic Ltd. (Israel). [d]Cultured *Fusarium nivale*, purified as reported by Ueno et al. [14]. [e]Prepar from fusarenone-X. [f]9,10-Dihydro derivative; supplied by T. Tatsuno (Institute of Physica and Chemical Research, Japan).

EXPERIMENTAL

Chemicals

The sources and structures of the trichothecene mycotoxins used are show in Table 1. The standard sample solutions were used in the concentrations listed in Table 2. Chromotropic acid solution (1%, w/v) was prepared by dissolv its disodium salt in (6 + 1) sulfuric acid solution. Phenyl J acid (recrystallized from water) was used as a 0.3% (w/v) solution in concentrated sulfuric acid. J acid was purified [12] and used as a freshly prepared 0.03% (w/v) solution concentrated sulfuric acid. JIS-Special grade sulfuric acid (Kanto Chemical Co., 96% content) was used. All other chemicals were of analytical grade.

TABLE 2

Preparation of standard solutions

Method	Sample	Concentration range ($\mu g\ ml^{-1}$)	Solvent
Chromotropic acid	F-X	50—1000	Ethanol
	T-2	50—1500	
Phenyl J acid	F-X	30—1200	Dimethyl-formamide
	T-2	50—2000	
J acid	F-X	1.25—25	Ethanol
	T-2	2.00—40	

Measurements

A micro-cell (path length, 10 mm; volume, 1.0 ml) was used in spectrophotometric and fluorimetric measurements. The absorption spectra and absorbances were measured with Hitachi spectrophotometers model 323 and model 101, respectively. The fluorescence spectra and their intensities were measured with a Shimadzu model 510 spectrofluorimeter. The fluorescence intensity was recorded for uncorrected excitation and emission maxima.

Thin-layer chromatography (tlc)

A t.l.c. plate coated with Merck silica gel HF_{254} (0.25 mm thick) was activated at 110°C for 40 min before use. After development with n-hexane—carbon tetrachloride—ethyl acetate (2:10:1) or chloroform—petroleum ether (3:1), each spot could be detected visually.

Standard procedures

Spectrophotometric methods (with chromotropic acid or phenyl J acid). Place an aliquot of sample solution (20 μl for the chromotropic acid method, or 10 μl for the phenyl J acid method), in a 1.5-ml glass-stoppered test tube. Add 1.0 ml of chromotropic acid solution (or 0.2 ml of phenyl J acid solution), and shake the tube. Place the tube in a boiling water bath for 10 min (or for 40 min in the phenyl J acid method). After cooling the solution to room temperature, measure the absorbance of the reaction product at 583 nm for the chromotropic acid method. For the phenyl J acid method, after cooling, dilute with 1.0 ml of methyl cellosolve and measure the absorbance at 655 nm.

Fluorimetric method with J acid. To a 20-μl aliquot of sample solution in a 1.5-ml glass-stoppered test tube, add 0.5 ml of the reagent solution and place the tube in a water bath at 40°C for 15 min. After cooling, measure the fluorescence intensity (λ_{ex}, 468 nm; λ_{em}, 513 nm).

Treat a blank containing only the solvents listed in Table 2 in place of the sample solution in the same way for each method.

RESULTS AND DISCUSSION

Spectrophotometric methods

Various reaction conditions were first examined for the determination of F-X based on reaction with chromotropic acid. Ethanol was chosen as solvent after consideration of both the sample solubility and the low spectrophotometric blanks. A study of the effect of sulfuric acid showed that the absorbance was constant in the range 87.5—95% (v/v) sulfuric acid. A 1% (w/v) chromotropic acid solution was selected because constant absorbance was obtained at concentrations higher than 0.7%.

The relationship between absorbance and reaction time was studied in the temperature range 40—98°C. Constant intensities were obtained after heating at 60°C for 30 min, at 80°C for 20 min, or at 98°C for 5 min (Fig. 1). Accordingly, the color was developed at 98°C for 10 min; the reaction product was then stable for 24 h at room temperature.

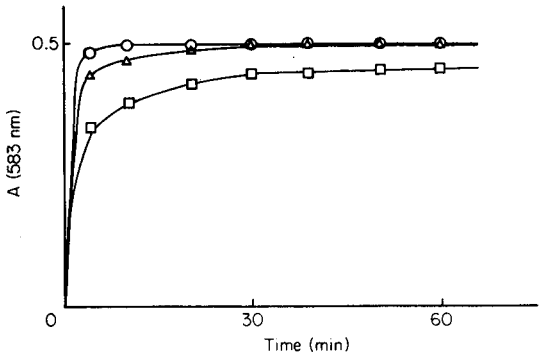


Fig. 1. Effect of reaction time and temperature on the reaction of fusarenone-X (13 μg) with 1% (w/v) chromotropic acid solution: (○) 98°C; (△) 80°C; (□) 60°C.

Next, the optimum conditions for the color reaction between F-X and phenyl J acid were studied. Ethanol, n-propanol and acetonitrile interfered with the reaction, therefore DMF was chosen as the reaction medium (Table 2).The reaction product of F-X was green (λ_{max}, 605 nm), but when methyl cellosolve was used as diluent, the absorption maximum shifted to ca. 650 nm and absorbance was enhanced. Methyl cellosolve was used because it gave a colorless blank and color enhancement, as well as better homogeneity of solution than n-propanol or acetonitrile; other solvents tested gave colored blanks (Table 3).

Variation of the sulfuric acid concentration showed that constant absorbance was obtained at concentrations above 95% (v/v), hence concentrated sulfuric acid was used. A 0.3% (w/v) phenyl J acid solution was chosen

TABLE 3

Effect of diluent on the color reaction between F-X (7.8 μg) and phenyl J acid

Diluent	λ_{max} (nm)	Absorbance	Color	
			Test	Blank
Methyl cellosolve	655	0.675	Blue	none
Ethyl cellosolve	665	0.858	Blue-green	pale brown
n-Butyl cellosolve	665	0.451	Red-brown	brown
Ethylene glycol	—[a]	—	—	—
Diethylene glycol	662	0.510	Blue-green	pale brown
Propylene glycol	—[a]	—	—	—
n-Propanol	665	0.673	Blue	none
Acetonitrile	645	0.680	Blue	none
Phosphoric acid	650	0.527	Blue	none
Dimethylformamide	660	0.614	Blue-green	pink
Sulfuric acid	450	0.252	Blue-green	none
	605	0.232		

[a]Brownish red oil was produced.

because the maximum constant absorbance was observed at concentration above 0.2%. The absorbance showed maximum and constant value after the reaction mixture had been heated in a boiling water bath for 30 min; a reaction time of 40 min was therefore selected.The reaction product was then stable at room temperature for 5 h.

Fluorimetric method

Ethanol gave the best results among the solvents examined (Table 2), and concentrated sulfuric acid gave the maximum fluorescence intensity. Variations in the J acid concentration did not affect the intensity when the concentration exceeded 0.02% (w/v) but high concentrations increased the blanks; accordingly, a 0.03% (w/v) solution was chosen. A study of the influence of reaction time showed that the fluorescence reaction was immediately about 75% complete, because of the heat of dilution when the sample was mixed with the reagent solution at room temperature, but it was necessary to leave the mixture at room temperature for 20 min, or heat at 40°C for 10 min, or heat at 60°C for 5 min, in order to complete the reaction. The blanks were higher when heating was done at 60°C; heating at 40°C for 15 min was therefore selected. The fluorescence obtained was stable for 2 h at room temperature and then increased gradually on prolonged standing.

Calibration curves

The calibration curves for F-X and T-2 by the three proposed methods were straight lines passing through the origin in the concentration ranges shown in Table 4; the reproducibility of the measurements was satisfactory in most cases (Table 4).

TABLE 4

Determinable range, coefficient of variation and reaction yield for F-X and T-2

Method	Sample	Determinable range ($mg\ l^{-1}$)	Coefficient of variation (%)[a]	Reaction yield (%)
Chromotropic acid	F-X	50—1000	1.6 (765) 4.8 (160)	95.1
	T-2	50—1500	1.6 (1060) 2.7 (260)	76.1
Phenyl J acid	F-X	30—1200	2.5 (900) 1.9 (100)	69.5
	T-2	50—2000	1.2 (1500) 4.3 (100)	56.1
J acid	F-X	1.25—25	1.6 (25) 5.7 (5)	48.7
	T-2	2.00—40	0.5 (40) 1.7(10)	36.8

[a]The concentration at which the reproducibility was measured is given in brackets; 10 determinations were done in each case.

Evidence for liberation of formaldehyde from trichothecenes

The absorption and fluorescence spectra of the reaction products between F-X and the three reagents are shown in Fig. 2. These spectra were just the same as those of the products resulting from formaldehyde. Although it is known that intramolecular rearrangement of the trichothecene molecule occurs in the presence of hydrochloric acid or sulfuric acid [15], a reaction in which formaldehyde is released has not been reported previously.

A thin-layer chromatogram for the 2,4-dinitrophenylhydrazones of the reaction products obtained by heating F-X and T-2 with 60% sulfuric acid showed single spots, the R_F values of which were exactly the same as that of the aldehyde derivative. This confirms that the color or fluorescence of the reaction products can be attributed to the reaction between the reagents and the formaldehyde released from trichothecenes.

The total yields of the reactions involved in each method were estimated by comparison of the calibration curves obtained for a standard formaldehyde solution and for the sample solutions; these yields, listed in the last column of Table 4, include the decomposition reaction of trichothecenes with sulfuric acid and the color or fluorescence reaction between the formaldehyde liberated and the detecting reagent in sulfuric acid. The overall yields are highest for the chromotropic acid method and are low for the J acid method. In general, the reaction yields obtained for T-2 are only 75—80% of those for F-X. The differences of the reaction yields in the three methods may be attributed to the variation of the initial reaction conditions which were selected for overall analytical performance.

Other trichothecenes (neosolaniol, nivalenol, tetraacetylnivalenol, diacetoxyscirpenol and HT-2 toxin) gave positive reactions, but dihydronivalenol, in which the double bond at position 9 on the trichothecene nucleus is saturated (see Table 1), and also trichothecin, in which the hydroxyl group at position 15 is replaced by hydrogen, were negative in all these reactions. Accordingly, the double bond at position 9 and the hydroxymethyl group (or its acyl derivatives) at position 6 on the trichothecene nucleus are considered to be the essential functions for liberation of formaldehyde. It has

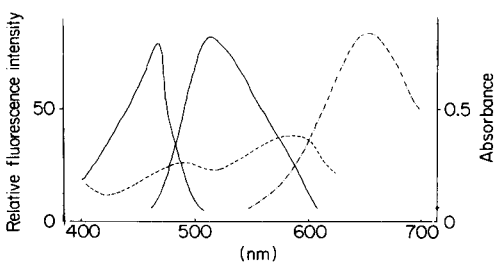


Fig. 2. Absorption, excitation and emission spectra of the reaction product with F-X. (—) J acid method for 0.4 μg F-X; (----) chromotropic acid method for 10 μg F-X; (—·—·—) phenyl J acid method for 10 μg F-X.

been postulated [11] that the bond between positions 6 and 15 is cleaved oxidatively by sulfuric acid so that the hydroxymethyl group is oxidized to formaldehyde. Accordingly, the differences in the reaction yields between F-X and T-2 (Table 4) are considered to reflect the ease of bond cleavage.

Phenols and related aromatics, which easily react with the formaldehyde produced in presence of sulfuric acid, and dextrose, saccharides and anisyl alcohol, which liberate formaldehyde, as well as other interferences on the assay for formaldehyde, will affect the determination of trichothecenes by the proposed methods. Consequently, an appropriate clean-up, e.g. by chromatography, in which the use of a multi-layer column (Na_2SO_4—silica gel—Na_2SO_4—Florisil—Na_2SO_4, [4]) or an active carbon column, may be effective, will be necessary in application of the present methods for agricultural samples. Nevertheless, the present methods are considered to be interesting for determinations of some of the trichothecene mycotoxins.

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