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ANALYTICA CHIMICA ACTA

International journal devoted to all branches of analytical chemistry

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Foreword

In January 1961, Dr. Alison Macdonald became an editor of this Journal. Twenty five years later she is the senior editor, filling the position with distinction, and demanding, as she always has, the highest standards, knowing that this was the best way to promote the reputation of analytical chemistry. Contributors to Analytica Chimica Acta during that time have had many reasons to be grateful to her, for helpful criticism, often forthright comments, but, equally often, characteristic humour and encouragement.

Many of her good friends, including her co-editors and publisher, felt they would wish to mark this 25th anniversary by the publication of a unique issue of this Journal. The readiness of authors to accept invitations to submit manuscripts confirmed this. In particular, it was felt that this was a golden opportunity to survey the changes in the analytical scene that had taken place over the last quarter of a century. Several articles of this type are included, written by groups of authors who themselves were responsible for many of the developments described. In addition, it was considered most appropriate to include articles representing some of the best current analytical research, from eminent scientists and institutions.

The article on flow injection analysis is different from all these. It comprises brief, personal contributions from a host of the most active workers in an area in which Dr. Macdonald has taken a particular, practising interest. It provided the impetus for the rest of the volume, and therefore appears as the first article.

The affection of the analytical community for Alison Macdonald, and its appreciation of her considerable efforts which have enhanced the quality of so many papers and books, were very much in evidence in the greetings enclosed with or included in the original manuscripts submitted for this issue. With one notable exception, these have not been included in the published papers, as we would wish the articles to stand strictly as scientific communications. We hope, therefore, that this volume will serve to present the appreciation and good wishes of the authors, and analytical chemists throughout the world, to Dr. Macdonald, and that it will remind her of the flourishing state of the science which she has done so much to promote.

Harry L. Pardue
J. Thomas Clerc
Alan Townshend
Marc Atkins

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FLOW INJECTION ANALYSIS (FIA) — A PERSONAL VIEW

R. APPELQVIST, G. R. BEECHER, H. BERGAMIN F°, G. DEN BOEF, J. EMNÉUS, ZHAOLUN FANG, L. GORTON, E. H. HANSEN*, P. E. HARE, J. M. HARRIS, J. J. HARROW, N. ISHIBASHI, J. JANATA, G. JOHANSSON, B. KARLBERG, F. J. KRUG, W. E. VAN DER LINDEN, M. D. LUQUE DE CASTRO, G. MARKO-VARGA, J. N. MILLER, H. A. MOTTOLA, H. MÜLLER, G. E. PACEY, C. RILEY, J. RŮŽIČKA*, R. C. SCHOTHORST, K. K. STEWART, A. TOWNSHEND, J. F. TYSON, K. UENO, M. VALCÁRCEL, J. VANDERSLICE, P. J. WORSFOLD, N. YOZA and E. A. G. ZAGATTO

(Received 30th August 1985)

SUMMARY

36 authors from 10 countries express their viewpoints on the merits of flow injection analysis.

Alison Macdonald has contributed invaluably to the development of FIA through critical evaluation of work of all the above authors, by editing manuscripts, and by carefully resolving differences between authors and referees. Therefore Analytica Chimica Acta, together with its Editor, may be regarded as the Alma Mater of FIA. It is our intention to honour (and humour) Dr. Macdonald by publishing this nonorchestrated and, by us, unedited patchwork of individual papers which reflect a rich mixture of applications, modifications and interpretations of FIA, together with a variety of personal opinions. We are most grateful to our colleagues for endorsing this unconventional way of celebrating the 25-year anniversary of our Editor (J. R. and E. H. H.).

A. UNSTABLE REAGENTS IN QUANTITATIVE ANALYSIS? WHY NOT APPLY FIA!

G. DEN BOEF and R. C. SCHOTHORST

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The success of FIA can be attributed to the reproducibility of the operations involved in the system, such as injection, pumping and timing, leading to the possibility of carrying out quantitative analyses without the necessity of completeness of chemical reactions. As a complete chemical reaction is unnecessary, the idea arises that instability of the reagent also might be no

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objection as long as the rate of decomposition is reproducible. This would open possibilities for the application in quantitative analysis of reagents which are impracticable under normal conditions. An example is the application of strongly reducing and oxidizing agents, some of which have been widely used in quantitative analysis, especially in titrimetry.

Table A1 represents a number of half reactions, involving such reagents, and their standard (formal) potentials. The standard potentials involving the reduction of hydrogen ions and the oxidation of water, i.e., the reduction of oxygen, have also been included. From the Table it can be seen that all these oxidizing and reducing agents are thermodynamically unstable towards water in aqueous solution and the reducing agents also towards oxygen. However, many of these reactions are very slow. Reagents like MnO_4^-, Ce^{4+}, Cr^{2+} and V^{2+} are stable in aqueous solution and can be applied in titrimetry in the classical way, although the reducing agents must be protected against oxygen, which makes the storage of standard reagent solutions rather complicated. This eventually led to their disappearance from the analytical scene. Species like silver(II) and uranium(III), the strongest oxidizing and reducing agents mentioned in Table A1, react rapidly with water and for this reason have hardly been applied in titrimetry.

Flow injection analysis can help in making possible the application of these reagents in quantitative analysis, by generating them in flow systems, starting from a stable oxidation state of the same element. Figure A1 represents the simplest flow diagram for this purpose. A solution of a stable compound is introduced into the flowing system. For the generation of uranium(III) this will be a solution of uranium(VI) acetate, for chromium(II), a solution of green chromium(III) chloride and for silver(II), a silver(I) nitrate solution. The solution is pumped through a reactor I which converts

TABLE A1

Half-reactions involving some strongly reducing and oxidizing agents, and their standard potentials

Half-reaction	Potential (V vs. NHE)	Ref.
$Ag^{2+} + e = Ag^{+}$ (1—4 M HNO_3)	+1.93	A1
$Co^{3+} + e = Co^{2+}$ (4 M $HClO_4$)	+1.92	A2
$Mn^{3+} + e = Mn^{2+}$ (7.5 M H_2SO_4)	+1.49	A3
$MnO_4^- + 8H^+ + 5e = Mn^{2+} + 4H_2O$	+1.49	A4
$Ce^{4+} + e = Ce^{3+}$ (1 M H_2SO_4)	+1.44	A4
$O_2 + 4H^+ + 4e = 2H_2O$	+1.23	A4
$2H^+ + 2e = H_2$	0.00	A4
$Mo^{5+} + 2e = Mo^{3+}$ (2 M HCl)	—0.25	A5
$V^{3+} + e = V^{2+}$	—0.26	A6
$W^{5+} + 2e = W^{3+}$ (12 M HCl)	—0.31	A7
$Cr^{3+} + e = Cr^{2+}$	—0.41	A8
$U^{4+} + e = U^{3+}$	—0.61	A8

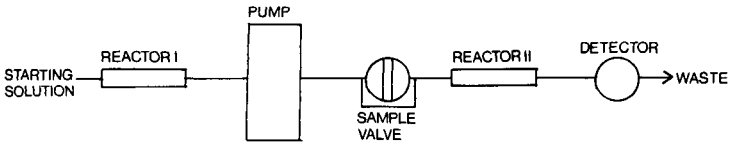


Fig. A1. Simplest manifold for generation and use of reagents in unstable oxidation states.

the starting solution into the unstable species. For the reducing agents a Jones reductor appeared to be very effective and for the oxidizing agents an electrochemical reactor with a working electrode of gold powder kept at +2 V vs. SCE is suitable.

The sample to be analyzed is injected by means of an injection valve. The reaction between the reagent and the analyte occurs in reactor II which may be a coiled tube or a single-bead string reactor. Detection occurs in a flow-through cell. The great advantage of this method is that the reagent only needs to be sufficiently stable during its residence time in the flow system, which may be less than 1 min. The application of uranium(III) [A9] and silver(II) [A10] as well as a number of other reagents appears to be possible. For silver(II) the half-life of the reagent solution under the experimental conditions was measured and appeared to be of the order of 1 min.

Also, for reagents like chromium(II) and vanadium(II), the application of a flowing system has great advantages. Although these reagents are relatively stable towards water, their storage is a problem because they are oxidized by air. When generated in a flow system, oxygen is eliminated from the solution in reactor I and the only thing to be done is to deaerate the sample solution before injection. This results in the simple applicability of these reagents [A11, A12].

The applicability of unstable reagents might not be restricted to these reducing and oxidizing agents, but may well provide similar advantages for other types of reaction and in solvents other than water.

B. DETERMINATION OF STARCH IN A FLOW INJECTION SYSTEM USING IMMOBILIZED ENZYMES AND A MODIFIED ELECTRODE

JENNY EMNÉUS, ROGER APPELQVIST, GYÖRGY MARKO-VARGA, LO GORTON and GILLIS JOHANSSON*

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Starch is a mixture of linear and branched polymers of α-D-glucopyranosyl units compounded together in granules of 2—150 μm diameter. The granules can be broken up after swelling by acid treatment, heat and mechanical pounding and by enzymatic degradation. A fraction with intermediate molecular weight and excess amylose units can be dissolved in water.

Amyloglucosidase splits the 1,6-α-D and the 1,4-α-D-glucosidic bonds, but not phosphorylated terminal residues [B1], which are abundant in, e.g., potato starch. The reaction proceeds with stepwise removal of β-D-glucose units from the non-reducing end of the starch chain in contrast to acid hydrolysis, which occurs by random breakage and which yields a variety of products. The yield of glucose in the amyloglucosidase hydrolysis will increase if the phosphorylated residues are removed by either phosphatase or α-amylase [B1]. The last two enzymes are normally present as impurities in amyloglucosidase preparations and a cessation of the hydrolysis at 90—99.7%, depending on the conditions, is often reported [B1, B2].

Immobilization of enzymes for the hydrolysis of starch and the kinetics of the reactions have been the focus of much interest because of their technological importance. An enzymatic hydrolysis to glucose units with soluble catalysts and fairly long incubation times is often used analytically. The most common method for the next step in the determination of starch components involves the use of glucose oxidase and chromogenic reagent for spectrophotometric detection of the hydrogen peroxide which is formed. Immobilized enzymes for starch determination have been studied by several researchers [B3—B8], either in the form of membranes in enzyme electrodes or in the form of reactors in flow systems.

EXPERIMENTAL

Amyloglucosidase (E.C. 3.2.1.3 from *Aspergillus niger*, Merck, cat. no. 1332, 100 IU mg^{-1}) was dialyzed for 2 h against a buffer. It was immobilized on porous glass (CPG-10, pore size 73 nm, particle size 74—120 μm) with glutaraldehyde, following essentially previously published procedures [B9]. The coupling yield was 45% when 150 mg of enzyme per g of activated glass was taken. The enzyme/glass was filled into a packed-bed reactor (AMG) with a volume of 500 μl (2.4 mm i.d., length 110 mm). A packed-bed enzyme reactor containing immobilized glucose dehydrogenase (GDH) was made as described previously [B10], except that the enzyme had been purified and that the particle diameter (37—74 μm) and pore size (71 nm) had been optimized. The new reactor was more efficient so that glucose could be converted to gluconic acid quantitatively in a smaller reactor (160 μl) than that used before.

The enzyme reactors and a modified electrode were inserted in a manifold as shown in Fig. B1. Various pH values between 3.7 and 4.7 have been reported as optima for the amyloglucosidase reaction. The pH dependence is unusually critical for this enzyme, making it necessary to select different pH values for the two enzyme reactions. The first buffer was a 0.015 M acetate buffer, pH 4.7, and the second a 0.2 M phosphate buffer with 2 mM nicotinamide adenine dinucleotide (NAD^+), to give a pH of 6.0 after mixing. The latter pH is optimum for the combination of the GDH reactor and the modified electrode [B10]. The detection was done amperometrically with the modified electrode kept at 0 mV vs. Ag/AgCl, 0.1 M KCl.

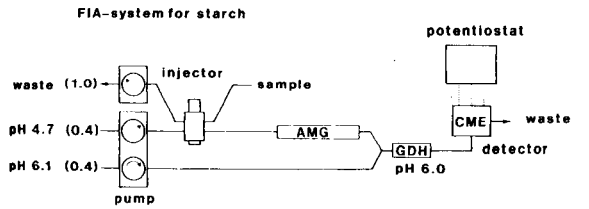


Fig. B1. Manifold for starch determination. AMG, amyloglucosidase; GDH, glucose dehydrogenase, both immobilized on porous glass in packed-bed reactors; CME, chemically modified electrode for NADH oxidation. Injection volume 30 μl, flow rates are in ml min^{-1}.

The water content of soluble potato starch (Sigma, cat. no. S-2630) was 15%, as found by a Karl Fischer titration. Sample solutions were made by adding a weighed amount of starch to boiling water and diluting with buffer to known volumes.

RESULTS AND DISCUSSION

The reaction sequence (Fig. B2) starts by hydrolysis of starch to β-D-glucose units in the AMG reactor. The glucose is oxidized further in the GDH reactor so that an equimolar amount of NADH is obtained. The stepwise reaction in the AMG reactor yields β-D-glucose which may isomerize spontaneously to a mixture of 63% β-D- and 37% α-D-glucose, but the reaction is so slow in the absence of catalysts that it was assumed that all the glucose would be available in the enzymatically active β-D-form at the entrance of the GDH reactor.

Detection (Fig. B2) was by mediated oxidation of NADH at a modified electrode, mounted in a confined wall-jet flow-through cell. The mediator was *N,N*-dimethyl-7-amino-1,2-benzophenoxazine (MB^+) which was adsorbed on the surface of a graphite rod. NADH and MB^+ form a complex which decomposes in a rate-limiting step to NAD^+ and the reduced mediator [B11]. The latter, MBH, was reoxidized amperometrically.

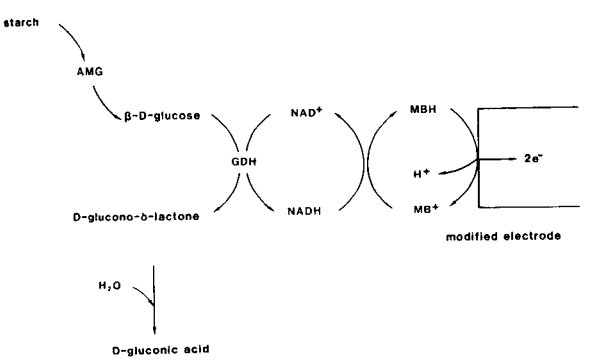


Fig. B2. Reaction scheme for the determination of starch.

Flow injection response comparisons cannot be done with peak height measurements when the diffusion coefficients and thus the dispersion is different for the compounds. The properties of the GDH reactor were therefore evaluated at steady-state by comparing the response of glucose with that of NADH as described before [B10]; it was found that the reaction was quantitative. Starch or glucose was next mixed with the pH 4.7 buffer so that the steady-state responses could be compared (Table B1). The results indicate that 90% of the weight of the starch (after correction for the water content) could be recovered as β-D-glucose. The constancy of the ratio from 0.34 to. 0.90 ml min^{-1}, i.e., for mean residence times of 88 to 33 s in the AMG reactor, indicates that hydrolysis has been essentially completed. The temperature of the AMG reactor was increased to 40°C, but the yield of β-D-glucose remained constant.

Abe et al. [B1] found that a purified enzyme could hydrolyze 60% of purified soluble starch to glucose during the first 30 min and 83% after 12 h. The phosphate of the starch could be recovered in an insoluble fraction. An enzyme containing impurities of α-amylase and phosphatase degraded 98% of the starch. Technical studies of starch solubilization and saccharification with α-amylase and amyloglucosidase indicate a limit of the hydrolysis at 90—95% after 24 h [B12]. A hydrolytic yield of 90% as evidenced by Table B1 may thus represent a limit with the enzyme used. The decrease in the yield above 0.90 ml min^{-1} indicates that the residence times then become too short for completion of the hydrolysis.

The enzyme must operate on each starch molecule as many times as there are glucose units in the chain segment; i.e., well above 300 times if the molecular weight of a linear chain had been 50 000. Hydrolysis at a branching point will result in an increase in the number of chain fragments, each of which can react independently. The overall speed might therefore be expected to increase, although the 1,6-α-D bond is hydrolyzed slower than the 1,4-α-D bond. The number of 1,6 bonds in potato starch is large and a fast multiplication of the number of reacting chains can therefore be expected to occur at the front end of the AMG reactor.

TABLE B1

Ratio of the steady-state response factors for starch and glucose at different flow rates (at the outlet); sample concentrations ca. 0.5 mM

Flow rate (ml min^{-1})	Current ratio starch:β-D-glucose
0.34	0.90
0.68	0.91
0.80	0.88
0.90	0.89
1.02	0.85
1.46	0.84
1.70	0.83

A number of limiting factors, as well as the need for long residence times in reactors, have been reported. The enzyme is inhibited by glucose and a halt at 50% conversion was reported for a hollow-fibre reactor in a recycling system [B13]. The excellent properties of the AMG reactor used in this work may be a result of the low starch concentrations. The upper limit of the method is set by the NAD^+ concentration in the buffer.

Samples of 30 μl of starch solutions were injected into the manifold operated with the flow rates shown in Fig. B1. The peak currents were linearly related to the amount of starch in the samples over almost four decades (0.1—1000 $\mu g\ ml^{-1}$). The log-log calibration plot had a slope of 1.03 (r = 0.9999, 11 points).

The accumulated band broadening including the effect of the large injection volume is shown in Table B2 for samples of glucose and starch. The band width was almost doubled upstream of the buffer merging point because of the smaller flow rate in this part of the manifold. The AMG reactor adds comparatively little broadening for a glucose sample, but more for a starch sample, because of the small diffusion coefficient of the latter. The maximum injection frequency was 23 samples h^{-1} at 1% carryover and the detection limit was 0.1 μg starch ml^{-1}, which is equivalent to 0.7 μM β-D-glucose at 90% hydrolytic yield. No stability problems with the immobilized enzymes were observed during the two months spent on the experiments reported here.

Only one type of sample has been studied so far and no attempts were made to estimate the importance of the purity of the enzyme. Depending on the outcome of such studies it might be possible to select reaction conditions so that a higher yield of glucose is obtained or that some additional information about the chain properties can be obtained with one or more purified enzymes.

CONCLUSIONS

A report of the progress with methods for carbohydrate determination was selected for this special article because it is thought to illustrate the importance of the FIA concept. Enzyme reactors can be designed to process

TABLE B2

Band broadening measured at half peak height when the injector was connected at different positions in the manifold (flow rates as shown in Fig. B1)

Sample introduction	Broadening (s)	
	Glucose	Starch
Before GDH reactor	13	—
After AMG reactor	24	—
Complete manifold	31	38

a sample until the reaction has been completed or equilibrium has been attained [B14]. Each reactor added to a flow injection manifold adds a new step so that a complex reaction scheme can be realized in a controlled way. The reproducibility and convenience of the FIA approach enhances the performance and the reproducibility of enzymatic methods. Similar arguments can be given for the importance of the flow injection concept for the performance of some types of detectors, especially for modified electrodes [B11—B15]. These detectors can give linear responses over 3.5 decades [B10] or up to 6.5 decades of concentration [B15] and are available for dehydrogenase as well as for oxidase catalyzed reactions.

Glucose determinations have often been used as a model in the development of detectors or enzyme reactors. The technique has now matured so that it can be applied to much more complex problems. This is illustrated here by the degradation of a polymer containing different bond types and different monomeric units.

High reproducibility is characteristic of FIA, and indications have started to accumulate to show that this holds true also for manifolds containing enzyme reactors. Recent work on sucrose determination showed that all calibration factors remained unchanged after 17 000 injections if the pump tubing, which were the weakest components, were changed [B16]. The reliability should therefore soon be good enough for use in process control analysis.

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C. POTENTIAL OF THE FLOW INJECTION TECHNIQUE IN SOIL CHEMICAL ANALYSIS

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Since its introduction into the soil chemical laboratories of the Institute of Forestry and Soil Science (I.F.S.S.), Academia Sinica, about eight years ago, FIA has gradually become accepted as one of the most efficient and versatile techniques ever used and tested in these laboratories. More than twenty well-established yet sometimes tedious manual methods have been replaced by automated flow injection procedures (Table C1). These are considered as the basis of a fully automated soil analysis system. For the most frequently determined species such as total nitrogen and phosphorus in soils, adoption of flow injection techniques has increased sample throughput three to four-fold without an increase in the number of technical personnel. Flow injection procedures have generally proved to be more precise and reliable than their manual counterparts and reagent consumption is also considerably lower.

TABLE C1

FIA methods for soil analysis used in the I.F.S.S.

Species determined	Detector[a]	Method/reagent	Measurements per h
Available P	S	molybdenum blue	120
Total P	S	molybdenum blue	120
Total N	S	indophenol blue	120
Total N	S	gas diffusion	100
Organic matter	S	$Na_2Cr_2O_7/H_2SO_4$	120
Available Fe	S	2,2'-dipyridyl	200
Available Zn	S	dithizone/CCl_4 extraction	60
Available B	S	azomethine-H	60
NO_2^--N, NO_3^--N	S	1-naphthylamine-7-sulphonic acid/sulfanilamide reduction to NO_2^- by Cd/Zn column	120
Exchangeable and water-soluble Ca	S	xylenol orange/CTMAB	120
Exchangeable and water-soluble Mg	S	eriochrome black T	120
Water-soluble Mo	S	catalytic reaction, H_2O_2/KI/HCl	90
Water-soluble Cl^-	S	$Hg(SCN)_2/Fe^{3+}$	120
Available Al	S	aluminon	120
CN^-	ISE		120
Water-soluble Cl^-	ISE		120
pH (0.01 M $CaCl_2$)	ISE		120
Available Cu, Zn, Fe, Mn	AAS		500
Available Ca, Mg	AAS	merging zone	240
Extractable Cd, Pb	AAS	on-line ion-exchange preconcentration	60
Total and soluble Se	AAS	hydride generation	200

[a]S, Spectrophotometry; ISE, ion selective electrode; AAS, atomic absorption spectrometry.

The pretreatment of soil samples, including extraction, digestion, filtration etc., cannot be accommodated in a flow injection procedure at present, which might be considered as a limitation of FIA, but the small sample consumption of flow injection systems has brought about considerable changes in the philosophy of soil pretreatment. In the I.F.S.S., instead of handling samples in the conventional way in batches of 6—10 or attempting automated batchwise treatment, large batches of 50—100 samples in much smaller vessels are processed manually by a single operator. For example, soil digestions are completed in 50-ml test tubes which are heated in locally produced far-i.r. heating block digestors capable of holding 60 tubes in a batch. After cooling, the digests, often containing a residue, are diluted to volume in the graduated digestion tubes and left standing overnight. The

supernatant digests are aspirated directly into the sample loop of the flow injection system without filtration, and analyzed. Test tubes are also used for soil extractions. After shaking, the test tubes containing the soil/extractant mixtures are left standing overnight and the extracts are sampled by aspirating either directly or from folded filter paper tubes inserted into the supernatant solution after standing.

The determination of trace elements plays an important role in the evaluation of soil nutrient status, and the merits of FIA in this field deserve special mention. Trace element analyses of soils can be done efficiently and accurately by FIA even when an atomic absorption spectrometer is not available. The wealth of existing spectrophotometric reactions in the literature could be fully exploited to provide reliable FIA procedures for the essential trace elements in soils. The spectrophotometric methods for molybdenum, zinc and boron included in Table C1 represent some of these procedures. As the flow system is essentially closed and mostly built from plastic materials, contamination from glassware or from the laboratory environment is easily controlled. Liquid/liquid extraction, a technique used quite often in trace analysis, can be done readily in FIA by using the systems proposed by Karlberg and Thelander [C1] and Bergamin et al. [C2]. Catalytic and chemiluminescence techniques will also be very useful, whereas on-line ion-exchange preconcentration will extend the capabilities of FIA to sub-μg l^{-1} levels [C3] (see also Section Q).

Various attempts at combining FIA with flame AAS have been very fruitful [C4, C5]. Soil analysis is a field which could benefit much from this combination. In its simplest form, the combination presents an efficient approach for sample introduction which imposes practically no restriction on the salt content of soil extracts. The combination also provides simple and efficient standard addition methods for soils with complex matrices. The application of flow injection techniques to hydride-generation AAS [C6] resulted in the development of a simple and efficient system in the I.F.S.S. capable of determining selenium in soils and plants at sub-μg kg^{-1} levels and sampling frequencies of over 200 h^{-1} [C5]. Using an FIA system with on-line ion-exchange preconcentration, 50 to 100-fold preconcentration has been achieved in analyses for heavy metals in sea water with a sampling rate of 60 h^{-1} [C3]. Recently this system has also been applied to the determination of cadmium and lead in soil extracts [C5], replacing the more sophisticated and less precise graphite furnace AAS method used originally. The development of integrated microconduits might result in extremely compact FIA equipment capable of precise soil analyses in the field [C7].

The benefits of introducing FIA into the soil laboratory can hardly be limited to what has been mentioned above because the technique is still developing rapidly. It has been fully demonstrated in the agricultural laboratories of the Centro de Energia Nuclear na Agricultura, Piracicaba, Brazil, that FIA could be a powerful routine analytical tool (see also Section V).

This has been further proved in the soil laboratories of the I.F.S.S. It would not be surprising if the flow injection analyzer soon becomes one of the most popular pieces of equipment in soil laboratories.

D. FLOW INJECTION ANALYSIS FOR CHEMISTRY UNDER NON-AMBIENT CONDITIONS

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The development of analytical procedures historically has been limited to those which are in large part compatible with the conditions of a laboratory or field environment. This need for compatibility with ambient conditions places rather severe restrictions on the choices of reagents and solvents, the range of temperature and pressure to which the sample may be subjected, and the ultimate performance of the analytical method in terms of reproducibility and lower limit of detection.

Flow injection analysis, developed originally for the automation of analytical procedures, appears to hold considerable promise for expanding the range of conditions under which chemical analysis can be carried out. The basis of the technique is the controlled injection of a precise volume of liquid sample into a continuous stream of reagent or carrier. This concept of sample manipulation in a closed manifold allows the entire analytical procedure, including chemical processing (reaction, extraction, preconcentration), physical processing (dilution, phase separation) and detection to be carried out in an environment isolated from the laboratory. Several recent examples of how this strategy allowed a chemical analysis to proceed under non-ambient conditions will be presented, to provide license for speculation about future applications.

A first example of using flow injection analysis to overcome an incompatibility of the laboratory environment with the conditions required for an analytical method is ultra-trace level determinations. As the sensitivity of laser-based spectroscopic detection approaches the single molecule level [D1, D2], the uncertainty of a determination is no longer limited by the detection step but rather by the probability of contamination during the determination procedure. In this situation, the sensitivity of detection is not compatible with traditional sample manipulation in a typical laboratory environment, where the opportunity for contamination from particulates leads to irreproducibility in the blank. While the ambient environment may be modified through extraordinary measures to reduce the particulate density in the air [D3, D4], the cost of such modification exceeds the resources of most analytical laboratories.

A simple flow injection manifold is capable of providing an ultra-clean, closed environment for physical or chemical processing and detection of

samples for ultra-trace analysis without exposure to contamination from the laboratory. This concept was first tested using laser-excited fluorescence detection of dyes [D5], where detection limits corresponding to a 20-μl injection of a 0.01 $\mu g\ l^{-1}$ solution or 200 fg of analyte were achieved. Laser excitation was also used in thermal lens detection of flow injected samples [D6]. Non-fluorescent analytes producing absorbances as low as 1×10^{-6} could be detected in a carbon tetrachloride carrier. Trace-level spectrophotometry with FIA was also tested using a conventional spectrophotometric detector modified for series differential response in order to stabilize the baseline [D7]. The technique was evaluated with respect to the spectrophotometric determination of iron, chromium and copper, for which the detection limits were 0.7, 0.3, and 1.0 $\mu g\ l^{-1}$, respectively. In all of these applications, the flow injection method provided controlled, high purity conditions for sample manipulation. In addition, the continuous flow of liquid carrier in the manifold efficiently rinsed previous sample injections from the system, thus decreasing carry-over and allowing blank measurements to be made between each sample with minimal consumption of ultrapure reagents.

A second example of using flow injection methods to solve the problem of incompatibility between laboratory and measurement conditions is thermal lens detection in supercritical fluid solvents [D8]. An initial study of the thermo-optical properties of supercritical fluids carried out using batch-type sample handling revealed that a 100-fold increase in sensitivity could be achieved with these solvents compared with normal liquids [D9]. The gain in sensitivity, however, was offset by the practical difficulty of transferring samples into a high pressure cell, filling the cell with a known mass of carbon dioxide or other dense gas, and raising the temperature of the massive cell to a precise value prior to making a measurement. These

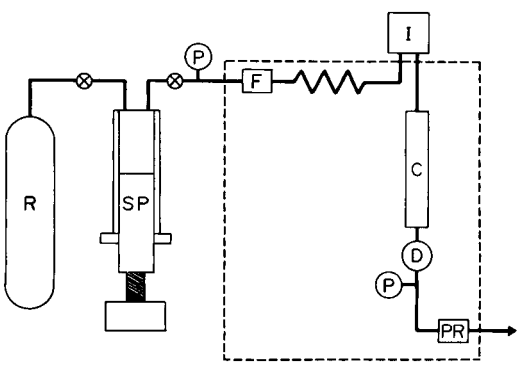


Fig. D1. Diagram of flow-injection manifold for supercritical fluid carrier. The gas reservoir, R, is used to fill the high pressure syringe pump, SP. Pressure gauges, P, monitor the system before the injection valve, I, and after the detection cell, D. Other components within the oven, indicated by the dashed box, include a partial filter, F, chromatographic column, C, and pressure regulator, PR.

time-consuming steps were avoided by the use of a high-pressure flow injection manifold [D8], shown in Fig. D1. Although the components of the instrument were made of materials designed to withstand pressures of several hundred atmospheres, the fundamental design of the manifold was not different from typical FIA systems. The injection valve provided a convenient means of transferring a sample from laboratory conditions to the high pressure, controlled-temperature environment for detection. A short chromatographic column was needed to separate the analyte from the liquid solvent in which it was originally dissolved so that it can be solvated in pure carbon dioxide. This step not only achieved the greatest sensitivity but also allowed sample detection to take place in a region well separated from the large refractive index gradients which accompany the dispersion of the liquid solvent in the less dense supercritical fluid.

Operation of a high-pressure flow manifold for this application was no more difficult than a traditional flow injection system; throughput of samples was somewhat slower (10 h^{-1}) because of the chromatographic separation step. The absorbance detection limit in supercritical carbon dioxide was 2×10^{-7}, with only 50 mW of laser power. The ease with which FIA allows samples to be manipulated into supercritical fluids should provide considerable impetus for further applications of these unique solvents. As most dense gases having favorable critical conditions (critical temperature near to room temperature, critical pressure not excessive) are small molecules with simple electronic structures, these materials are generally u.v. transparent and have relatively few vibrational modes leading to simple i.r. spectra with numerous windows. A recent report [D10] indicated, furthermore, how the unique mass transport properties of supercritical fluids affects their use as solvents for electrochemical measurements.

At present, there are only a few examples of using flow injection methods to overcome the incompatibility between the conditions of a measurement and the environment of the laboratory. These examples demonstrate, however, the relative ease with which one can carry out analytical procedures under non-ambient conditions such as high purity, high pressure or controlled temperature. In each case, the injection valve serves as the interface between the laboratory and measurement environments, while the FIA manifold isolates the two environments from each other. Future applications of this concept might include exploiting high temperature conditions in a high-pressure flow injection manifold to speed up chemical reactions which at near ambient temperature are too slow to be useful in analysis. In addition, isolation of the analysis from the laboratory by FIA could allow safer use of toxic or corrosive reagents in analytical methods, where FIA provides the additional benefit of producing minimal quantities of waste per determination.

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E. HETEROGENEOUS SAMPLES IN FLOW INJECTION ANALYSIS

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Although flow injection systems have been well established for analysis of homogeneous solutions, the analysis of heterogeneous samples such as whole blood and suspensions of soils presents problems. The central assumption of FIA is that the dispersion of the sample is constant and reproducible so that calibrating solutions and unknown samples are treated in the same way.

The consequence of heterogeneity of the sample is that in non-segmented flow systems, such as FIA, the sample is dispersed differently than are the homogeneous calibrating solutions. The presence of solids can lead to two types of error. One is related to the hydrodynamic effect of the solid on the dispersion, and the other to the effect of the excluded volume of the solid on dilution. The occurrence of other sources of error, such as viscosity and the presence of bubbles, can be controlled to some extent by the design of the manifold. It has been shown [E1, E2] that, in principle, complete elimination of all of these problems for heterogeneous samples in non-steady state systems such as FIA is not possible.

Flow injection analysis is primarily a precise dilution technique. For homogeneous samples this dilution is described as the dispersion $D = C_0/C_{max}$, where C_0 is the initial concentration of the sample and C_{max} is the peak concentration in the detector. In heterogeneous samples in which the fraction of the excluded volume of the solid phase is h $(0 < h < 1)$ the effective dispersion is [E1] $D_{eff} = (D - h)/(1 - h) = C_0/C_f$ where C_f is the final peak concentration. Therefore, the addition of another component (solid phase) adds another degree of freedom, and in order to determine the initial concentration C_0 both dispersion D and the excluded volume h must be known. This fact is illustrated in Fig. E1. Consider a sample containing 50% by volume of solid. If the total volume of the sample is doubled, the volume of the liquid phase is increased three times. For example, if a

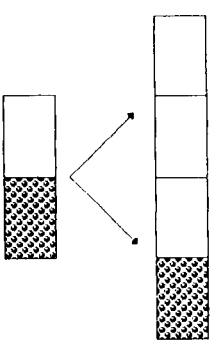


Fig. E1. Dilution diagram for heterogeneous samples. $D = 2$, $h = 50\%$, $D_{eff} = 3$.

blood sample of hematocrit 40% (normal healthy male) is injected in to a FIA manifold with (homogeneous) dispersion $D = 5$ the effective dispersion will be 7.7, which would result in ca. 50% error.

The method of standard addition has been used successfully for elimination of matrix effects. In potentiometric analysis it is known as the Gran Plot technique. The possibility of using the standard addition method as the means of eliminating the problems caused by heterogeneous samples has been investigated [E2]. The manifold used is shown in Fig. E2. It uses the hydrodynamic injection principle [E3] with solenoid valves operated under computer control [E4]. In the first step the sample, S, is loaded by applying vacuum, V. In the next step, a small volume (0.1- 24 μl) of concentrated standard solution, A, is introduced just in front of the sample. Finally, the carrier stream, C, is activated and the sample with the added standard travels through the detector, D, to waste, W. It is important to note that both the initial sample and the added standard undergo the same dispersion in this process and appear as one peak in the detector. It has been shown [E2] that under those conditions the effect of dispersion is eliminated and the initial concentration is obtained from $C_0 = -V_A^0 C_A / V_0$, in which V_A^0 is the negative volume obtained by extrapolation of the Gran Plot, C_A is the concentration of the added standard and V_0 is the sample volume.

In the presence of solids the above equation transforms to $C_0 = -V_A^0 C_A / (1-h)V_0$, again showing no dependence on the dispersion, be it caused by viscosity, the heterogeneity of the sample or by bubbles. Unfortunately, the initial concentration of the sample cannot be evaluated without the prior knowledge of the excluded volume fraction, h. These conclusions have been verified experimentally. It has been shown that FIA is indeed unsuitable, at present, for analysis of heterogeneous samples.

There is, however, an interesting possibility which may circumvent this problem [E5]. If an internal standard (a marker ion), of known concentration in the initial sample (it can be zero), is used in conjunction with the standard addition method, the excluded volume fraction can be determined

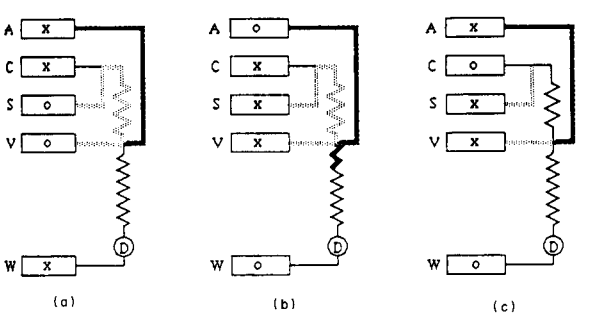


Fig. E2. FIA manifold for standard addition: (a) after loading, before addition; (b) after addition, before injection; (c) after injection. The status of a valve is indicated as: (○) open, (×) closed (for other symbols, see text).

independently. The method would, however, require a separate detector for the internal standard. For example, assume that the marker ion is indium, present at some relatively high concentration in the carrier stream but not in the heterogeneous sample of unknown excluded volume fraction. Owing to the dispersion, a certain concentration of this ion will appear in the sample as it passes through the indium detector. This concentration will be directly proportional to h which can be evaluated independently. The standard addition can be then done with the species of interest as outlined above.

F. FLOW INJECTION ANALYSIS — OR THE ART OF CONTROLLING SAMPLE DISPERSION IN A NARROW TUBE

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Flow injection analysis was introduced in 1974 by Růžička and Hansen [F1]. The complete definition appeared later [F2], based on a combination of sample injection, reproducible timing and controlled dispersion. Sample introduction into nonsegmented streams was, of course, known long before 1974. It was a standard procedure in, for example, liquid chromatography and amino acid analysis. For amino acid analysis the sample supply from a sampler and post-column addition of ninhydrin had been mechanised [F3], and this approach has a great similarity with the nonsegmented, continuous flow systems described by Stewart [F4, F5], with the exception that the separation column was not needed in the latter systems since only one sample component was to be analysed. It was in fact Hare [F6] who pointed out the possibility of using amino acid analyzers for methods where no separation was required and as a consequence he, together with Frantz [F7] constructed a system for the determination of silica. The sample was inserted into a stream of ammonium molybdate solution via a micropipette. There were several other publications before 1974 describing continuous flow analysis based on sample introduction in nonsegmented streams [F8].

The second cornerstone, reproducible timing, was extensively explained by Růžička and Hansen [F1] as they, at this time, realised that neither physical nor chemical equilibrium was required in a continuous flow system in order to carry out a successful analysis.

The third cornerstone, controlled dispersion, is the key issue for understanding and application of FIA, but it has unfortunately been ignored in most historic reviews of nonsegmented, continuous flow analysis. Sample dispersion can be changed in a flow injection system by varying the sample volume, reaction coil length and diameter, flow rates, etc. The purpose of controlling the dispersion is to optimize the chemical reactions taking place between sample and reagents. The fundamental means and methods for controlling the dispersion were first demonstrated by Hansen and Růžička [F9].

Further refinements of the basic FIA concept have proceeded gradually, especially as knowledge about the dispersion process in narrow tubes has increased. Hardware refinements have resulted in a variety of sample injection systems and methods which often have been claimed to surpass each other with respect to performance, reliability, simplicity and price. Exploitation of reproducible timing has led to gradient techniques like stopped-flow [F10] and "titrations" [F11] and two-pump techniques, namely intermittent pumping [F12] and hydrodynamic injection [F13]. However, all gradient techniques rely on the fact that the sample dispersion process can be controlled so that the conditions for the chemical reaction can be optimized.

One practical consequence of the dispersion process is that the sample is diluted in the system. For most FIA systems the dilution factor can conveniently be varied tenfold, simply by changing the injector volume and the number of merging streams and their individual flow rates. By including gradient chambers [F11] or by applying zone sampling [F14] much larger dilution factors can be obtained. Figure F1 shows a manifold where sample splitting has been combined with zone sampling. In Table F1 some results are presented showing the wide dynamic range over which this FIA configuration can be operated. A large relative standard deviation is obtained when the difference between C_2 and the split flow is small. A second practical consequence of the dispersion process is the axial prolongation of the sample zone. This prolongation results in a decrease in the maximum sampling frequency. The sampling frequency is inversely proportional to the standard deviation of the peak (in time units).

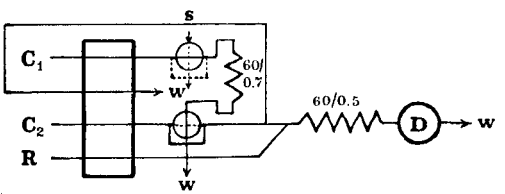


Fig. F1. FIA manifold for zone sampling combined with sample splitting (one-reagent chemistry); w = waste, D = detector, R = reagent; tube dimensions given as length/width (in mm).

TABLE F1

Dilution factor, D, and r.s.d. values for corresponding peak heights as a function of the flow rates defined in Fig. F1

C_2 (ml min^{-1})	Split flow to waste (ml min^{-1})	Time between injections (s)	D	R.s.d. (%)
1.8	0.7	8	50	2.1
1.1	0.7	12	390	2.7
0.7	0.6	15	1360	8.4
0.8	0.6	18	6900	3.6

The theory of dispersion in nonsegmented, continuous flow systems is still incomplete but several conclusions can be drawn and guidelines can be given for practical analytical chemists [F15]. For two-phase flow injection systems, some dispersion studies have been reported recently [F16]. The remaining part of this Section is devoted to dispersion in FIA extraction systems.

A typical FIA extraction system is depicted in Fig. F2. The sample (S) is injected into a carrier (C), the composition of which can be adapted to the sample matrix (pH, ionic strength, etc.). The carrier is merged with a reagent stream (R) and mixed in a coil. In the segmentor this aqueous solution is segmented with an organic phase delivered from a displacement bottle. Extraction takes place in a coil and a membrane separator is used to divert a fraction of the organic phase to the flow cell while the remaining organic phase plus the aqueous phase are expelled to waste via a restriction coil.

A photographic technique was developed by which it was possible to measure and study the film thickness behind a solution segment as a function of the flow velocity and the nature of the organic phase [F16]. An almost linear relationship exists between the film thickness and the flow velocity. For pentanol in a pentanol/water system, the film thickness was estimated to be ca. 0.03 mm at a linear flow velocity of 5 cm s^{-1} (0.7 mm i.d. PTFE tubing). For chloroform the film formed under similar conditions was about 1/8 of the thickness. Since the theory of film formation in liquid/liquid segmented systems had not previously been developed, equations describing the film thickness behind one plug in a gas/liquid segmented system have to be used for a qualitative discussion. Such equations have the form [F17, F18] $d_{film} = \text{const.} \times d_{tube}\ (u\ \eta/\gamma)^a$, where d_{film} is the film thickness (cm), d_{tube} the tube inner diameter (cm), u the flow rate (cm s^{-1}), η the viscosity (poise), γ the surface tension (dyne cm^{-1}) and a an empirical constant (1/2 and 2/3 have been used).

Study of this equation implies that the quotient η/γ could be useful in estimating and comparing film-forming properties for different solvents. In the extraction coil the analyte can leave the aqueous segment and enter the film as illustrated in Fig. F3. At time '1', the aqueous segment having an ellipsoidal shape is approaching a region (dotted, vertical lines) where the analyte concentration can be followed. When the aqueous segment passes across this region (time '2'), the analyte is extracted into the organic film and C_{film}

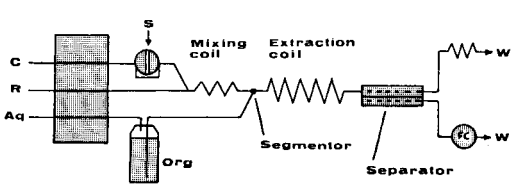


Fig. F2. Extraction system based on FIA; Aq = aqueous, Org = organic, FC = flow cell, W = waste.

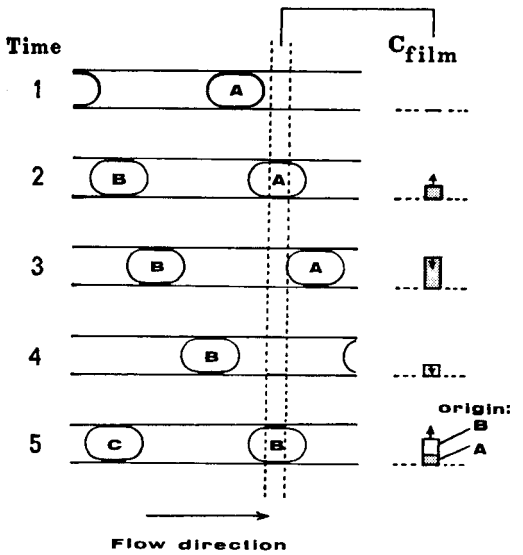


Fig. F3. Stages during analyte extraction into the organic film. The analyte concentration in the film, C_{film}, is illustrated for the tube area within the dotted zone. If C_{film} has not returned to a "zero" value when the water segment, B, arrives (time '4'), axial dispersion occurs.

increases rapidly. When the aqueous segment leaves the observed region, C_{film} starts to decrease because of the diffusion of analyte into the organic segment (time '3'). If C_{film} has not reached its original "zero" value by the time the next aqueous segment arrives (time '4'), the net result will be that analyte material originating from the first aqueous segment appears not only in the next organic segment but also in the second, pursuing organic segment. This leads to undesired dispersion and the analyte concentration profile is subjected to tailing.

Intuitively, the dispersion process within the extraction coil should, according to the arguments raised in discussing Fig. F3, be less significant when the film is thinner. Results indicate that this is true (Fig. F4). Caffeine was extracted from the aqueous phase into chloroform at different flow velocities. As the flow rate increased the film thickness increased. The analyte material became more dispersed and lower peak heights resulted.

The coil material also influences sample dispersion. Tubing materials such as PTFE and polyethylene allow the organic phase to form a film, which improves the conditions for efficient extraction. When, however, the analyte zone appears only in the organic phase, and it is to be transported over a certain distance with minimum dispersion, segmentation of this stream with water is recommended in a coil of glass or steel. The aqueous phase then forms a film on the tube walls thereby encapsulating the organic segments so that no intersegment mass transfer occurs.

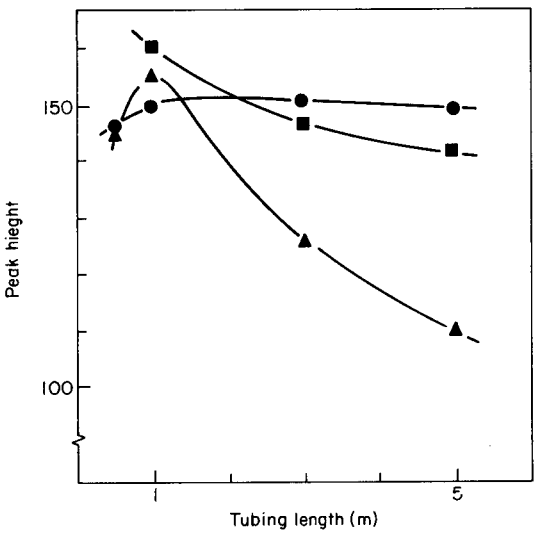


Fig. F4. Flow-injection extraction of 1×10^{-4} M aqueous caffeine into chloroform. Influence of flow velocity on dispersion for flow velocities of: (•) 2.7; (▪) 5.4; (▲) 17.8 cm s^{-1}. Conditions: 0.7 mm i.d. PTFE tubing, segment length 6 mm.

The most critical part of the extraction system, with respect to overall dispersion or dilution of the sample zone, is the phase separator. An almost linear relationship between the peak height and the fraction of organic phase transported to the flow cell was found [F19]. This observation emphasizes the importance of an efficient phase separator. Separators of the membrane type seem to be preferred for flow injection extraction systems. It may be dangerous to give general guidelines for controlling (i.e., minimizing) the sample zone dispersion in an FIA extraction system but the two most important aspects are separator efficiency and the nature and thickness of the film formed in the extraction coil.

The author is indebted to Drs. L. Nord and B. Bubnis for valuable discussions.

G. FLOW INJECTION ANALYSIS — THE INTERMARRIAGE OF ANALYTICAL CHEMISTRY AND CHEMICAL ENGINEERING SCIENCE

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Flow injection analysis has developed into a well-established analytical procedure just like, for instance, a titrimetric or spectrophotometric determination. The practical analytical advantages and limitations have been

explained already in many articles, and also the more fundamental technological aspects including band broadening and mixing have been extensively, if not exhaustively, discussed. In studying the more theoretical aspects advantage was taken of the know-how accumulated in the field of physical transport phenomena as developed by chemical engineering scientists. In this respect there exists a parallel to the development of theoretical concepts in column chromatography.

STANDARDIZATION

With FIA coming to a reasonable degree of maturity, it seems desirable to pay more attention to the standardization of the technique and its concepts. Of course, this has to comprise first of all a definition of FIA: what should be considered to be part of FIA, what should not. For instance, in the author's opinion the determination of viscosity based on an Ostwald viscometer as described by Betteridge et al. [G1] lies beyond the borderline because one of the main characteristics of FIA, i.e., the reproducible residence time of samples of varying concentrations, is not fulfilled. However, apart from this definition the concepts that characterize FIA systems also have to be standardized. It is strongly advisable to aim at the closest association with concepts already available from chemical engineering science in order to gain the maximum possible benefit from the large amount of knowledge gathered in that discipline. Furthermore, it should be indicated what kind of information has to be provided for the unambiguous description of a FIA system. One of the Analytical Committees of the I.U.P.A.C. should consider it its task to start this discussion.

MINIATURIZATION

Reaching maturity does not mean that FIA will not show new and challenging developments. It has been stated before, that FIA offers possibilities whose only limitation is the ingenuity of its users [G2]. Good illustrations can be found in the construction of several modules such as liquid/liquid extraction modules [G3], flow-through enzymatic reactors [G4], dialysis membrane modules [G5] and gas diffusion membrane modules [G6]. The use of such membrane modules will especially contribute to the use of FIA in process analysis applications [G7].

One of the more exciting developments in FIA is certainly miniaturization. A first step in this direction was made by Růžička and Hansen [G8, G9] with the introduction of their integrated microconduit manifolds. However, in their approach, emphasis is laid on diminuation of the external size of the manifold and the integration of the component parts, rather than on miniaturization of channel width etc. Therefore, the improved performance of their manifolds with respect to dispersion has to be traced mainly to the integration of the component parts leaving out band-broadening connections

etc. Since lithographic techniques offer the opportunity of etching on a very small scale, it is worthwhile to examine the possible advantages of decreasing the dimensions of channels, sample injection volume and detector volume.

In the subsequent part of this Section the consequences of scaling down will be discussed in terms of dimensionless numbers as is normal practice in chemical engineering science and chromatography [G10, G11]. Because of the limited format of this contribution two restrictive conditions will be adopted. One is that the residence time (t_v) should remain constant on scaling down to maintain the same degree of chemical conversion. The other is that the pressure drop across the system should not increase on decreasing the tube radius, to avoid problems with the pump, tightness of connections etc.

In Table G1 a survey of the relevant equations is presented. From Eqns. (1) and (2) it is evident that on decreasing R by a factor p both L and $\langle v \rangle$ have to decrease by the same factor in order to keep t_v and ΔP constant. It is interesting to notice that both τ increases and ReSc decreases by p^2, indicating that Taylor flow conditions will become better fulfilled as depicted by the arrow in Fig. G1 [G12, G13]. A decrease of D_L and simultaneous increase of Pe_L is equivalent to saying that there is a decrease in dispersion. This is also reflected by the decrease of σ_t and σ_v, and an increase of the maximum sampling frequency.

The simple conclusion might be drawn from these considerations that miniaturization is very attractive in terms of sampling frequency and reagent

TABLE G1

Equations describing various aspects of an FIA system consisting of tubular conduits; the influence on parameters of scaling down the channel radius by a factor p, assuming t_v and ΔP are constant

Parameter[a]	Influence of scaling down	Special remarks
(1) $t_v = L/\langle v \rangle$	constant	
(2) $\Delta P = 8\eta\langle v \rangle L/(R^2)$	constant	Hagen-Poisseuille
(3) $V_r = \pi R^2 L$	decrease by factor p^3	
(4) $f_v = \langle v \rangle \pi R^2$	decrease by factor p^3	
(5) $\tau = \mathbb{D}t_v/(R^2)$	increase by factor p^2	
(6) $ReSc = 2\langle v \rangle R/\mathbb{D}$	decrease by factor p^2	
(7) $D_L = R^2\langle v \rangle/48\,\mathbb{D}$	decrease by factor p^4	Taylor flow regi
(8) $Pe_L = \langle v \rangle L/D_L$	increase by factor p^2	
(9) $\sigma_t = (2D_L L/\langle v \rangle^3)^{1/2} = (t_v R^2/24\,\mathbb{D})^{1/2}$	decrease by factor p	Taylor flow regi
(10) $\sigma_v = f_v \sigma_t$	decrease by factor p^4	
(11) $f_{max} = 600/\sigma_t$	increase by factor p	

[a] t_v, Residence time; L, channel length; $\langle v \rangle$, average linear flow velocity; ΔP, pressure drop over channel; η, dynamic viscosity; R, channel radius; V_r, channel volume; f_v, volume flow rate; τ, reduced time; $\mathbb{D}$, diffusion coefficient; Re, Reynolds number; Sc, Schmidt number; D_L, longitudinal dispersion coefficient; Pe_L, longitudinal Péclet number; σ_t, standard deviation (in time units); σ_v, standard deviation (in volume units); f_{max}, maximum sampling frequency.

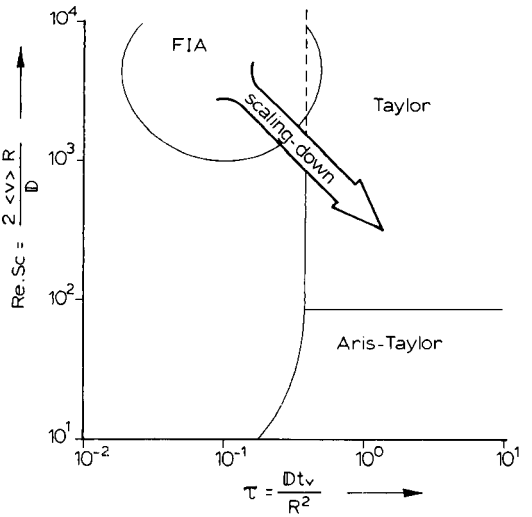


Fig. G1. Influence of scaling down on flow regime.

consumption. One has to realize, however, that the sample volume injected should be several times smaller than V_r and that the detector volume should be less than 0.5 σ_v. This means that a p-fold decrease in channel radius would demand a p^3-fold decrease in sample volume and a p^4-fold decrease in detector volume. It is evident that the detector volume in particular will set limits to the maximum allowable miniaturization, as was previously stated [G14, G15]. As an example it can be estimated that for a channel radius of 0.1 mm, a required residence time of 20 s and a limiting length of the reaction channel of 10 cm, both sample and detector volume should not exceed 1 μl! Therefore, at the present state of the art of not too-expensive flow-through detectors, most of the present FIA systems already operate close to the minimum permitted scale and that the microconduit manifolds as advocated by Růžička and Hansen are of interest more particularly because of the aspect of module integration. According to their experimental results this integration leads to a decrease in band broadening.

H. FLOW INJECTION BIOCHEMICAL ANALYSIS WITH LUMINESCENCE DETECTION: TOWARDS THE SIMPLE DETERMINATION OF SINGLE MOLECULES

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One of the many delights of FIA is the ease with which it can accommodate different detection systems. Practically every conceivable detection procedure

has been investigated, and successful results achieved in almost every case. But the combination of FIA with chemiluminescence (CL) and bioluminescence (BL) detection clearly has special attractions. CL and BL analyses are frequently done on small volumes of aqueous solutions with a requirement for high sampling rates. The precision of the CL or BL intensity invariably depends crucially on the reproducible mixing of the reagents that FIA offers, and the precise control of timing that is another important feature of FIA is also valuable in the detection of the transient CL or BL signals.

Following early applications of FIA with fluorescence detection [H1—H4], the adoption of a luminometer as an FIA detector seemed an obvious step. Most commercially available luminometers, even "automatic" ones, are designed for batch rather than continuous flow operation, so modifications to such equipment [H5] or the construction of a new detector [H6] have invariably been necessary. Key features include the need to mix reagents close to, or even directly in front of, the photodetector to maximise the sensitivity achieved from the rapidly generated luminescence signal, and the precautions necessary to avoid fibre optic effects in the flow tubing; the latter can become crucial when low light levels are being detected.

Among the many applications of CL and BL, perhaps the best known is the luminol reaction [H7]. It is widely used for the determination of hydrogen peroxide, metal ions, haem groups etc. The reagent is readily available, but quantum yields are very low (ca. 0.01). Higher yield BL reactions for the determination of ATP and FMN [H8] utilise costly and labile enzymes. Much interest has recently been expressed in the peroxyoxalate CL system [H5, H9] which allows the excitation of conventional fluorophores via an energy transfer mechanism. The reagents are an oxalate such as bis-[2,4,6-trichlorophenyl]oxalate (TCPO) and hydrogen peroxide. They generate the high energy intermediate dioxetanedione, which excites the fluorophores. The advantages of the system include the relatively high quantum yield of the CL process (ca. 0.25 for TCPO), the ability to excite a wide range of fluorophores (e.g., fluorescein, dansyl labelled amines, aromatic hydrocarbons), the possibility of using an immobilized form of the CL reagent [H10], and rate control by regulating the pH and by the use of various additives. In this section some biochemical FIA applications of the oxalate ester CL system are described.

EXPERIMENTAL

Conventional FIA equipment was used, with tubing internal diameters of 0.5—0.8 mm, and Rheodyne loop injection valves. The flowing stream was impelled by a peristaltic pump, or by using a gas pressure bottle. The latter approach was preferable in view of its freedom from pulsation and decreased bubble formation with peroxide solutions. Two detectors were used. One was a Fluorimet filter fluorimeter (Baird Atomic Ltd.) fitted with a flow-cell (100 μl) and with the light source disabled. The design of such an

instrument is far from ideal for CL and BL studies, yet it proved effective. The second detector was an LKB Model 1250 luminometer modified to accept flow tubing and a coloured filter. TCPO was synthesised as previously described [H5]. Labelled proteins and hormones were prepared by conventional methods.

RESULTS AND DISCUSSION

Preliminary results showed that the detection of a fluorophore via CL excitation gave detection limits several times lower than the photoluminescence detection limits of the same species. This is probably due, at least in part, to the absence of scattered incident light signals in CL systems. Thus fluorescein concentrations of $<10^{-11}$ M could be detected even in the modified fluorimeter, and in the luminometer concentrations of <1 ng ml^{-1} of fluorescamine-labelled thyroid hormones (T3, T4) could be determined in pure solution. A well-known problem in the determination of CL intensities is the quenching effect of proteins; the FIA, system allowed these interferences to be evaluated simply and rapidly.

These results encouraged further studies of CL immunoassays, using fluorescence or enzyme labelling methods, and CL detection. Several different systems have been studied. Figure H1 shows the calibration graph for

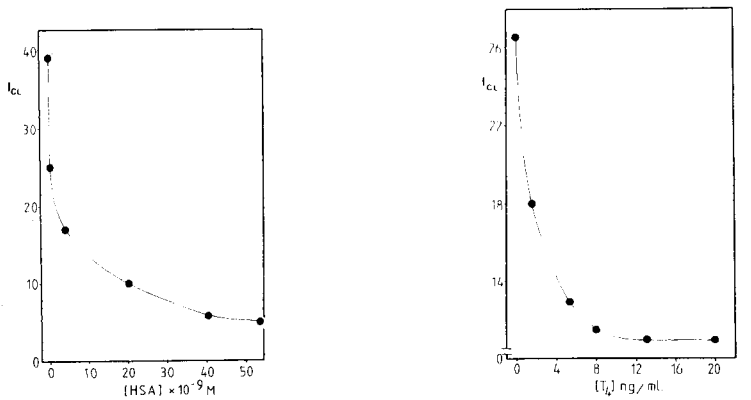


Fig. H1. Immunoassay calibration graph for the determination by FIA of albumin, by CL enhancement of a dansyl-labelled albumin conjugate. The conjugate (28 nM) had a fluorophore:protein ratio of 1:1. Samples also contained TCPO (0.5 mM) anti-albumin antibodies and unlabelled albumin, and were analysed after 2-h incubation periods by injection into a flowing (4.8 ml min^{-1}) stream of 1 g ml^{-1} hydrogen peroxide.

Fig. H2. Immunoassay calibration graph for the determination by FIA of T4 using a fluorescein-T4 conjugate (33.3 nM). Samples also contained TCPO, diluted anti-T4 serum and sample T4. The assay was two-phase, T4 and anti-T4 being pre-incubated for 30 min before addition of labelled T4 and a further 150 min incubation. The incubation mixture (pH 7) was injected into a stream of hydrogen peroxide (1 g ml^{-1}) flowing at 4.8 ml min^{-1}.

a homogeneous immunoassay for human serum albumin. This assay utilizes dansyl-labelled albumin conjugates, whose TCPO-excited CL signal is enhanced twelve-fold in the presence of anti-albumin antibodies. This enhancement (which is not detected in photoluminescence studies) is reversed in the presence of unlabelled (sample) albumin, allowing the detection of the latter in serum samples. The results correlate well with a dye-binding method for albumin determination, and careful choice of buffer solution means that this assay is virtually unaffected by non-specific antibodies and other proteins. Figure H2 shows the results of a luminescence enhancement immunoassay for T4 using the TCPO reaction and an FIA system to determine the concentration of fluorescein-labelled hormone in a homogeneous assay. This assay is very sensitive, though subject to some interferences. These examples illustrate the potential of FIA combined with CL or BL detection for the determination of low concentrations of biologically active molecules.

More recent work [H11] shows that limits of detection as low as 10^{-16} M can be obtained using a photon counting detector for FIA with efficient BL such as that of the firefly luciferase system. Such measurements use, as usual, only small volumes (ca. 10 μl) of sample, and are thus capable of detecting only a few tens or hundreds of molecules in solution. Only small improvements might be necessary to allow single molecules to be detected. It is thus clear that, in this field as in many others, FIA is capable of providing a simple and convenient approach to obtain results that would be hard to achieve by other means.

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J. FLOW INJECTION ANALYSIS: SAMPLE AND REAGENT PROCESSING IN THE CHEMICAL LABORATORY

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DEFINING WITHOUT AN EXCLUSIVE DEFINITION

Historical sketches of the origins of unsegmented continuous-flow analysis have been advanced in the literature [J1—J3]. Continuous-flow procedures, which can broadly be divided into segmented and unsegmented forms, are in essence unique approaches to the automation of the handling of reagents and/or sample components (liquid and/or gaseous) in the chemical laboratory. They entered analytical chemistry by the mid-1950s in the segmented form via clinical chemistry applications [J8]. Conceptually they represented a novel manner of sample and reagent mixing and transport to a detector, with

precedents in industrial process control [J3]. Flow injection analysis belongs to the unsegmented group and it has eluded most attempts to generate an inclusive or exclusive definition. The popularity of segmented continuous-flow systems resulted from the implementation of processing of liquid solutions for spectrophotometric determinations.

Since the main practical impact of FIA resulted from the proof that the same reactions and methods can advantageously be applied in an unsegmented system, it has erroneously conveyed the impression, particularly in its earlier developments, that it also was just an automated way to process liquids for spectrophotometric determinations. This restrictive boundary has, fortunately, almost collapsed but it is still clearly reflected in the marketing of commercial units for FIA applications. Another such boundary, which resulted from the unavoidable comparison with segmented applications, is in the rate of sample processing. In page 7 of the first monograph on FIA by Růžička and Hansen [J2], who are primarily responsible for providing the impetus and recognition enjoyed by FIA today, for instance, the following can be found: "...FIA (is) an automated microchemical technique capable of having a sampling rate of at least 100 determinations per h...". The literature, however, records FIA applications in which the sampling rate is below 100 h^{-1}, some as low as 10 or 20 h^{-1}.

Further examples of the elusiveness of adequate statements bordering on definitions can be found in the same monograph (p. 180). An attempt to define boundaries that single-out FIA from other continuous-flow techniques produced the statement that FIA is based on a combination of sample injection, controlled dispersion and exact timing. However, both chromatographic and air-segmented flow methods, for instance, share these characteristics. In chromatography a sample is injected; by adjusting parameters that will yield a minimum height equivalent to a theoretical plate the dispersion (band broadening) is controlled, and exact timing is needed to interpret retention parameters properly. In air-segmented methods, the sample is aspirated (which can be considered a form of intercalation), 'dispersion' is controlled (i.e., prevented) by air segmentation, and timing requirements are again important. A further qualification by the statement that "... (FIA) permits obtaining a highly reproducible read-out even when the mixing is incomplete, the chemistry does not reach equilibrium, and the signal is transient", is also misleading since segmented-flow procedures can be implemented under conditions resulting in the same characteristics.

The problem of defining FIA is common to all approaches that invite the ingenuity of the practitioners: soon limiting boundaries must be expanded and they eventually crumble. It is safe, however, to state that FIA is a continuous-flow sample/reagent(s) processing approach that avoids air segmentation for dispersion control. It should be noted that chromatography has similar (but many other) characteristics, but it exclusively effects separations while FIA was mainly developed for sample transport and sample/reagent manipulations. As a complication, separation steps have been incorporated into FIA systems, making the conceptual boundaries again diffuse.

This rather broad view of FIA reveals the real impact of FIA in contemporary chemistry: its ubiquity in the automation of chemical reactions, particularly those used in analytical chemistry. Its use permits the processing of a large number of samples with acceptable (often highly competitive) precision and accuracy, a meaningful statistical treatment of data, the elimination of human participation in many routine manipulations, and a better utilization of chemicals [this is particularly increased by the adaptability of immobilized reagents to FIA [J4, J5] and by the amenability of FIA to miniaturization [J6] (see Section G)].

UBIQUITY OF FIA IN THE AUTOMATED LABORATORY

On the premise that all samples reach the laboratory in a container of some kind, the overall scheme of present day automation involves three levels.

First level. The sample in the form that arrives at the laboratory is directly and appropriately transferred to the second level or prior to this is converted to a form compatible with the manipulations in the second level.

Second level. The sample is introduced into a transportation system (carrier stream in FIA), is processed as required (e.g., mixing with reagents, separations and preconcentrations performed on-line, as needed), and detection is realized by physical or chemical means, or both.

Third level. The information contained in the analytical signal is manipulated by the appropriate conversions and algorithms to give the final numerical result conveying the information sought.

FIA can be considered to have impact on the second level, integrating a large variety of on-line analytical manipulations (e.g., preconcentration, matrix isolation, liquid/liquid extraction, dialysis). The first level involves mainly mechanical operations (weighing, crushing, powdering, dissolving etc.) which in the present state of development seems to be better handled by robots [J7]. If the sample is a gas or liquid, however, direct transfer to the second level is possible in most cases. In any event, electro-mechanical manipulations (under computer control) characterize the first level. Dilution, mixing with reagent(s) if necessary, some separation steps, preconcentration, and the actual measurement, are better implemented in the second level, and FIA fits almost ideally at this level. As FIA is electromechanical as well as hydrodynamic, this processing of chemicals can also be directed by computer supervision. The third level is dominated by the mathematics (mostly arithmetic) imposed by the physico-chemical relation of monitored species (parameter) and the analytical signal. It involves the auxiliary electronics needed for signal conditioning and transformation as well as the translation of the information (read-out) in an intelligible form. Again the computer

controls all these operations and, as given, the picture of the totally automated laboratory procedure develops as feasible and affordable.

FLOW INJECTION AS A RESEARCH TOOL

Undoubtedly total automation is only realized in a few instances and the bulk of present-day chemical procedures take advantage of automation to different degrees. Since the recognition of FIA as a competitive approach in the realm of continuous-flow methods, the number of published papers dealing with it in international recognized journals has increased from fewer than ten in 1975 to more than 100 in 1984, with a cumulative count of about 600 publications. The utility of FIA in contemporary analytical chemistry is well documented in these papers which include a few descriptions of fundamental aspects. Less realized seems to be the fact that FIA can be a powerful tool for measuring physicochemical parameters as well as helping in other areas of basic chemical research. To illustrate this point, the use of FIA for obtaining diffusion coefficients, in a rather simple and elegant manner, [J9, J10] can be mentioned. The measurement of catecholamines released from chromaffin cells [J11] represents another application outside the conventional use of FIA. Bovine adrenal medullary cells cultured on treated styrene/divinylbenzene microbeads were used in a low-dispersion flow injection system for the measurement of stimulation/response parameters with electrochemical detection of the released catecholamine.

FIA, as a research tool, should also appeal to chemists engaged in synthesis. Dreaming in this line, one cannot help but see that the process designed to speed up and simplify peptide synthesis, for which Merrifield received the 1984 Nobel Prize in Chemistry [J12] leads itself to automation and implementation by flow injection methodology. The method depends on the covalent attachment of the first amino acid of the chain to a solid polymer and the stepwise addition of each succeeding amino acid unit, until the desired sequence is obtained. The final step is the release of the peptide from the solid support. Most of the necessary chemical steps can be readily done by adapting flow injection techniques, and the remaining ones can be implemented with some ingenuity and craftmanship. The parallelism with the use in FIA of reactors with immobilized reagents is evident. If distillation has been employed in continuous-flow systems [J13], for instance, the refluxing step needed in the modification of the solid support should be accessible. Incorporation of flow injection technology in these cases should result in scaling down the consumption of chemicals with the consequent generation of less chemical waste as well as a decreased cost of the investigation.

CONCLUDING REMARKS

This personal view of FIA extends beyond the recognition of its now undisputed value, to pose a direct challenge to the segmentation idea for

continuous-flow automation of wet chemistry. It tries to place FIA, as a processing scheme, within the context of "totally automated chemistry" in the laboratory as well as to point to flow injection as a valuable research tool outside the analytical subdiscipline. The main limitations seem to lie in the imagination of its practitioners or in the search for a restrictive definition. FIA is rather simple to implement; this simplicity, however, should not obscure the actual complexity of the physicochemical processes taking place from the point of injection to the point of detection [J14—J16]. Conversely, such complexity should not obscure the fact that most FIA systems are easy to assemble and the actual implementation of FIA is not difficult nor expensive. The dominating kinetic features of FIA are also worth noting [J17].

I thank the National Science Foundation for supporting our research on transient signals that directed our attention to FIA.

K. AN FIA SYSTEM USING AN ION-SELECTIVE ELECTRODE AS DETECTOR FOR THE CATALIMETRIC DETERMINATION OF MOLYBDENUM(VI) AND TUNGSTEN(VI)

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This Section describes a flow injection system based on a potentiometric detector. A new type of ion-selective all-solid state flow-through electrode has been developed in this laboratory [K1, K2], based on an Ag_2S/AgI membrane electrode. A catalimetric method is described for the determination of molybdenum(VI) and tungsten(VI). Manipulation of dispersion in the FIA system gives optimal conditions with regard to analysis rate, high sensitivity and reproducibility.

EXPERIMENTAL

Reagents and samples

Analytical reagent grade chemicals and redistilled water were used throughout. Stock and standard solutions were always freshly prepared. The contents of the standard solution of molybdenum(VI) and tungsten(VI) were determined gravimetrically.

Instrumentation

The detector consists of a combination of two ion-selective solid-state electrodes with identical membranes and all-solid internal contacts (Fig. K1). This arrangement enables the potential difference to be measured across the

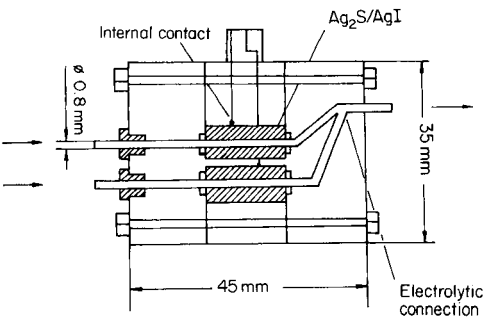


Fig. K1. Flow-through iodide-selective electrode.

membrane, which is the basis of a differential potentiometric method. The electrolytic connection is provided via the tubing junction in the electrode block.

E.m.f. measurements were made with a precision pH-meter type MV 87 (VEB Präcitronic, Dresden) in connection with an Endim chart recorder (VEB Messapparatewerke, Schlotheim, DDR). The samples were injected by means of a 30-μl sample loop. The peristaltic pump used was a DP 2-2 (VEB MLW, Ilmenau, DDR).

DISPERSION IN FIA

Flow injection analysis is based on a combination of sample injection, controlled dispersion and exact timing, which allows a reproducible readout to be obtained in spite of the fact that the mixing between the sample and carrier solution is incomplete, the chemical reactions do not necessarily reach equilibrium and the recorded signal is transient [K3]. The injected sample forms a zone which is transported towards a detector which continuously records the absorbance, electrode potential or other physical parameter which continuously changes as a result of the passage of the sample through the detector flow cell. As the quality of the readout in FIA determines the usefulness of the technique and the quality of the readout depends on the "form" of the travelling sample zone, control of the dispersion of the sample becomes one of the major tasks in FIA [K4]. Dispersion is the result of diffusion and convection in axial and radial directions. The nature and magnitude of the dispersion depend on physical flow parameters, mechanical manifold parameters, the geometry of the reactor and chemical factors.

Růžička et al. [K5] divided dispersion into three ranges: limited, medium and large dispersion; each corresponds to a value range of the practical dispersion parameter, ($D = C_0/C_{max}$), where C_0 is the initial sample concentration and C_{max} the peak height concentration at the detector. In a short, narrow tube the dispersion of the sample zone will be limited (e.g., $D = 2$)

and the sample maximum will appear within seconds after sample injection. Consequently, such a system cannot accommodate assays based on chemical reactions, but it is well suited for transporting the sample to the detector with controlled dispersion and exact timing.

Limited dispersion can also be achieved for flow to such detectors as ion-selective electrodes and atomic absorption spectrometers. Compared to the manual batch techniques, FIA with limited dispersion gives higher sampling rates and better reproducibility.

Medium dispersion (D = 2 to 10) can be applied to attain a wide variety of reaction configurations to develop some detectable entity such as color, fluorescence or electroactivity. Systems with medium dispersion are the most interesting from an analytical viewpoint as they can be applied to a large number of analytical procedures. In this type of determination, sufficient mixing can be achieved by using mixing aids such as a mixing coil.

Finally, large dispersion ($D > 10$) can be utilized to give a substantial degree of mixing between sample and carrier stream to form a well-developed concentration gradient, which is necessary for continuous flow titrations or for investigation of the chemistry at the sample/stream interface [K6].

Any dispersion has a negative effect on sampling frequency and sensitivity. The appropriate degree of dispersion in an FIA system, however, must be a compromise based on consideration of the degree of mixing desired, the sampling interval that can be accepted, the degree of completeness to which the reaction has to proceed and, most critically, the type of analytical read-out used, i.e., peak height or peak width. Examples for such compromise are given in this paper.

RESULTS AND DISCUSSION

Catalimetric determination of molybdenum(VI) and tungsten(VI)

The principle of catalimetric methods of analysis is the measurement of the rate of chemical reactions which are accelerated by homogeneous catalysis. The component to be determined may act as a catalyst or, in indirect methods, as an inhibitor or activator. A chemical reaction whose change in rate is dependent on the catalyst concentration and is used for the determination of the amount of catalyst is called an indicator reaction [K7].

As an indicator reaction for the determination of molybdenum(VI) and tungsten(VI) the well-known reaction between hydrogen peroxide and iodide was chosen.

$$H_2O_2 + 2I^- + 2H^+ \rightarrow 2H_2O + I_2$$

The change in iodide concentration as an indicator substance was determined with the flow-through iodide-selective electrode (Fig. K1). The set-up used in the differential flow-system is shown in Fig. K2. In the channel through L_2 a mixture of hydrogen peroxide and iodide flows. In this channel

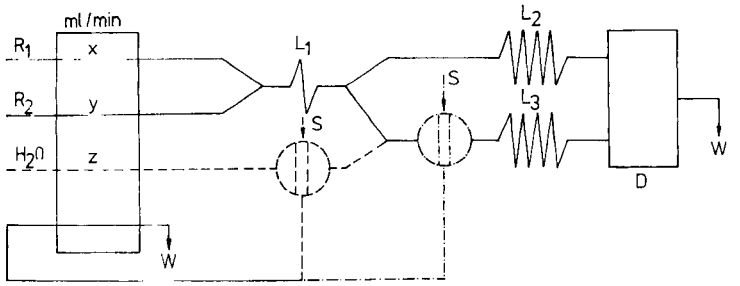


Fig. K2. Manifold for the catalytic determination of molybdenum(VI) and tungsten(VI). For flow parameters see Table K1. (—·—·) Cases I and II: $x = y$, $L_2 = L_3$ (i.d. 0.5 mm). (——) Case III, $x = y = z$, $L_2 = L_3$ (i.d. 0.5 mm). L_1 for all cases is 1 m (i.d. 1.1 mm). R_1 = 0.1 M KNO_3, 0.01 M H_2O_2; R_2 = 0.1 M KNO_3, 1×10^{-3} M KI, 0.4 M H_2SO_4. S = sample, D = detector, W = waste.

only the non-catalytic reaction takes place. In the channel through L_3 both the catalytic and non-catalytic reactions take place because of the presence of a catalyst after injection. Therefore in the latter channel the concentration of iodide will be smaller than that in the former channel and a potential difference will be measured. In this type of determination sufficient mixing must be achieved by using a mixing coil, L_2, L_3 (Fig. K2).

The injection of a catalyst into a mixture of hydrogen peroxide and potassium iodide leads to dispersion resulting from physical mass transport. It also accelerates the decrease in iodide concentration as a result of the chemical reaction described above. The dependence between the chemical reaction and physical dispersion is reciprocal [K4]. Sample/carrier boundaries induce physical dispersion, while the mixing of the sample and carrier solution initiates the chemical reaction. The chemical reaction causes a new concentration gradient, which becomes a driving force for increased dispersion. The signal monitored reflects the result of both processes.

Figure K3 shows the dependence of the peak heights on the concentration of molybdate as catalyst. On the same basis, traces of tungstate may be determined. By choice of different flow conditions (different dispersion) it is possible to realize optimal conditions with regard to detection limit, sensitivity, linearity and sampling rate. The flow conditions and some results for the determination of molybdate and tungstate are listed in Table K1.

Simultaneous determination of molybdenum(VI) and tungsten(VI)

When molybdate and tungstate are present together, the latter may be masked by citrate. Thus the determination of both metal ions is possible. Tungstate is marked with citric acid in the flow system. Without masking the peak obtained represents the catalytic activity of molybdate and tungstate. After reaction with citric acid the peak height is determined only by the concentration of molybdate. The manifold used for masking and measurement, involving a differential flow system, is shown in Fig. K4.

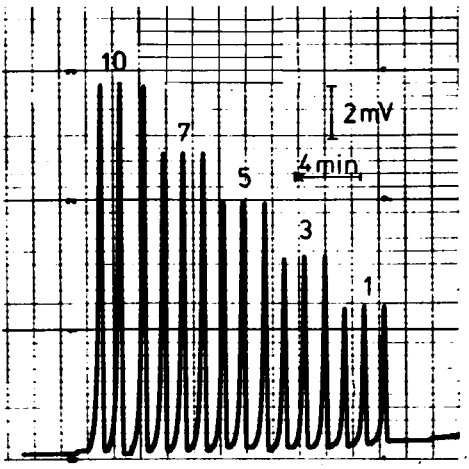


Fig. K3. Readout for the catalytic determination of molybdenum(VI). l = 10 m, t = 1.5 min, D = 10.6.

TABLE K1

Determination of molybdenum(VI) and tungsten(VI) under different flow conditions

Parameter	I	II	III
Flow rate (ml min^{-1})	0.21	1.3	0.12
Mixing coil length (m)	10	10	5
Residence time (min)	8.7	1.5	5.8
Dispersion (D)	7.5	10.6	20
Sample volume (μl)	30[a]	30[a]	30[b]
Results			
Molybdenum			
Limit of detection (μg ml^{-1})[c]	0.09	0.17	0.03
Sensitivity (mV ml μg^{-1})	4.9	1.0	1.4
Blank value (mV)	6.1	4.3	0.0
Working range (μg ml^{-1})	0.09—4	0.17—30	0.03—20
S.d. (mV)	0.09	0.07	0.02
R.s.d. (%)	0.9	1.3	1.5
Sampling rate (h^{-1})	15	60	15
Tungsten			
Limit of detection (μg ml^{-1})[c]	0.12	0.15	
Sensitivity (mV ml μg^{-1})	7.05	1.3	
Blank value (mV)	6.7	4.6	
Working range (μg ml^{-1})	0.12—4	0.15—7	
S.d. (mV)	0.28	0.07	
R.s.d. (%)	2	1	
Sampling rate (h^{-1})	15	60	

[a]Injection into reagents stream. [b]Injection into carrier stream. [c]3 σ Value.

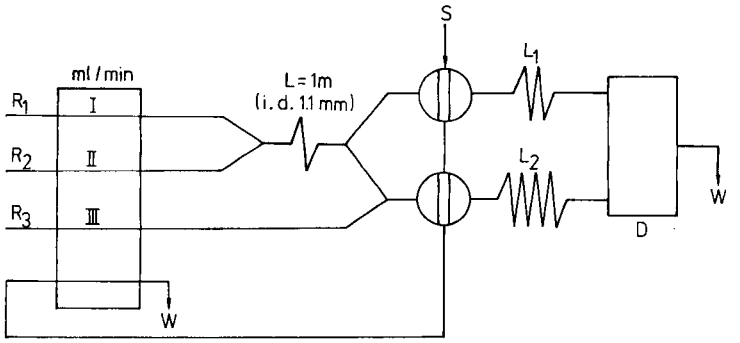


Fig. K4. Manifold for the simultaneous determination of molybdenum(VI) and tungsten(VI). For flow parameters see Table K2. R_1 = 0.1 M KNO_3, 0.01 M H_2O_2; R_2 = 0.1 M KNO_3, 1 × 10^{-3} M KI, 0.4 M H_2SO_4; R_3 = 4 g l^{-1} and 6 g l^{-1} citric acid for cases I and II, respectively. S = sample, D = detector, W = waste.

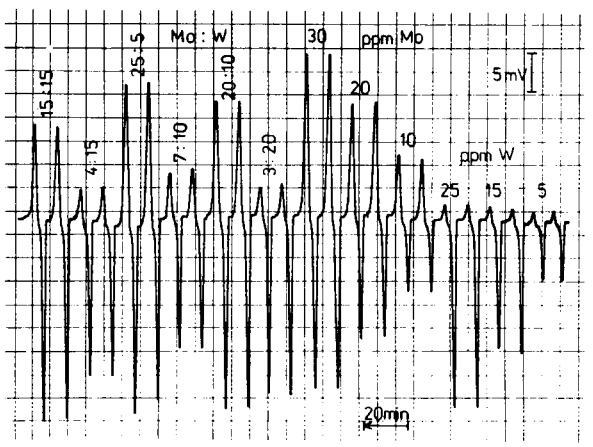


Fig. K5. Readout for the simultaneous determination of molybdenum(VI) and tungsten(VI) at different ratios.

The dependence of the peak heights on concentration for different ratios of molybdate and tungstate is shown in Fig. K5. A series of measurements was made, from which the results are shown in Table K2. By choice of different flow conditions, it is possible simultaneously to determine molybdate and tungstate in the range 0.3—8 μg W ml^{-1}, 0.4—13 μg Mo ml^{-1} and 1.5—27 μg W ml^{-1}; 2—35 μg Mo ml^{-1}, respectively. A system with medium dispersion (D_{max} = 6 and 8 for the channels without and with citrate, respectively, gives high sensitivity and good reproducibility, but the linear range is not great. A system with higher dispersion (D_{max} = 21 and 32, respectively) gives an increased linear working range, but the sensitivity is lower (for more details see [K8]). These examples show that the manipulation of

TABLE K2

Simultaneous determination of molybdenum(VI) and tungsten(VI) under different flow conditions

Parameter	Medium dispersion		Large dispersion	
Flow rates (I = II) (ml min^{-1})	0.21		0.21	
Flow rate (III) (ml min^{-1})	0.06		0.06	
Mixing coil length, L_1 (m)	3.5		0.8	
Mixing coil length, L_2 (m)	7.0		2.4	
Residence time, t_1 (min)	3.75		3.5	
Residence time, t_2 (min)	5.5		8.0	
Coil i.d. (mm)	0.5		1.1	
Dispersion, $D1$	6.2		21.4	
Dispersion, $D2$	8.1		32.5	
Sample volume (μl)	30		30	
Results	Mo	W	Mo	W
Limit of detection (μg ml^{-1})	0.4	0.3	2.0	1.5
Sensitivity$_1$ (mV ml μg^{-1})	1.92	2.31	0.64	0.85
Sensitivity$_2$ (mV ml μg^{-1})	1.81	0.06	0.67	0.02
Blank value$_1$ (mV)	7.0	7.0	3.4	3.4
Blank value$_2$ (mV)	5.1	5.1	1.2	1.2
Calibration range (μg ml^{-1})	0.4—13	0.3—8	2—35	1.5—27
R.s.d. (%)	0.8	4.5	3.4	4.0
Sampling rate (h^{-1})	12	12	6	6

dispersion in an FIA system is useful for optimizing the conditions of analytical procedures with regard to important analytical parameters.

L. SELECTIVITY ENHANCEMENTS OBTAINED BY USING FLOW INJECTION ANALYSIS

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Without question FIA is an excellent technique for the automation of existing analytical methods. Rapid throughput and high reproducibility is now expected of any FIA method. However, FIA promises more. It allows the analytical chemist to utilize the gradients created in the system and to incorporate known separation techniques in order to develop improved, selective methods.

The advantages of FIA allow the selectivity of existing methods to be increased significantly by utilizing knowledge of the chemistry of those methods, while at the same time providing for the possibility of creating new selective methods by utilizing separation techniques in FIA such as gas

diffusion, dialysis, extraction, column separations, as well as kinetic methods. Additional selectivity can be realized by combining these techniques and thereby multiplying the selectivity factors to produce extremely selective methods. Some of the more interesting techniques are those that take advantage of rate differences between the reaction of the reagent with the, analyte and with an interferent. Selectivity enhancements and increased sensitivity can be realized by incorporating kinetic discrimination and/or kinetic enhancement into the system, concentrations or conditions used for a given method.

This section evaluates the advantages that FIA contributes to newly developed methods for determination of chlorine dioxide. Both kinetic discrimination and gas diffusion separation are combined to produce selective methods for the determination of chlorine dioxide in recently disinfected water.

EXPERIMENTAL

All FIA experiments were carried out with the Tecator 5020 Flow Injection Analyzer with Chemifolds I—V. The detectors used were the Tecator 5023 spectrophotometer for visible-region measurements, the ISCO V4 flow-through spectrophotometer for u.v. measurements, and a spiral T-cell mounted on a GCA McPherson photomultiplier housing unit with a Keithley 601 or 617 electrometer, to monitor the chemiluminescent system.

All reagents were at least analytical grade. The chlorine dioxide samples were prepared according to established procedures [L1]. All water was prepared by passing it through a Barnstead ion-exchange system followed by a Barnstead double-distillation system.

RESULTS AND DISCUSSION

Chlorine dioxide will have two major uses, both for disinfection. It will be used as an alternative to chlorine in the disinfection of waste water and drinking water. In this case large amounts of inorganic and organic material will be present in the sample. Chlorine may well be used in conjunction with chlorine dioxide. The second use is as a replacement of ethylene oxide in sterilization processes. The analytical needs for these systems are selective measurements of sub-mg l^{-1} of chlorine dioxide. Until the work described here, no method met this requirement.

Two techniques that can easily be incorporated into an FIA system are kinetic discrimination and gas diffusion. In kinetic discrimination the differences in the rates of reactions of the reagent with the analyte and with the interferents are exploited. In batch chemistry measurements are made at equilibrium, so any kinetic difference is not apparent. However, under FIA conditions species reacting at different rates do so reproducibly but with different apparent sensitivities. These differences can be exploited to produce maximum selectivity for the analyte.

By incorporating gas diffusion into an FIA system, several selectivity factors can be exploited. The most obvious is that by using teflon microporous membranes, aqueous solutions do not wet the membrane surface. Therefore, ionic interferents will not pass through the membrane, thereby eliminating that source of interference. The second less obvious advantage is the difference between gases in terms of their permeability through the membrane. Although gas diffusion is based on physical properties such as vapor pressure [L2], the membrane used adds some selectivity to the system. As an example, although the vapor pressure of chlorine gas is 3 times that of chlorine dioxide the selectivity between them, based on the teflon membrane only, is 3:1 in favor of chlorine dioxide [L3]. When these selectivity factors are combined with those for kinetic discrimination, the resulting selectivities can be very impressive.

The first example is the determination of chlorine dioxide using gas diffusion FIA and u.v.-visible spectrophotometric detection [L3]. The gas diffusion eliminates both ionic and organic materials that absorb in the u.v. region. When the membrane selectivity factor of 3.1 is multiplied by the selectivity factor based on the molar absorptivities of the two species at the wavelength used (175 in favour of ClO_2), the total selectivity factor is 542. When this is enhanced by the masking effect of oxalic acid on chlorine [L3], the overall factor is >5500. This method could not have been used for batch determinations of chlorine dioxide in water because of the problems with the interfering matrix.

For the chemiluminescent determination of chlorine dioxide with luminol, the differences in the signal produced by chlorine dioxide and chlorine are time and pH dependent. Chlorine dioxide reacts extremely quickly with luminol while chlorine reacts slower, with more sustained emission. FIA can be used to control the chemistry and timing in such a way as to minimize the signal for chlorine. There is little difference in signal between the species at pH 0—10. However, at pH 13, chlorine is present as hypochlorite, which does not react well with luminol. Fortunately, at this pH, chlorine dioxide produces its maximum signal. The observed selectivity factor is >500. This procedure could not be carried out in a batch system, because chlorine dioxide decomposes rapidly at pH 13. Therefore, detection must be rapid after the addition of pH 13 luminol solution.

For real samples, inorganic and organic interferents can again be minimized by a gas diffusion cell. The overall selectivity factor is >1500.

This is but one example of how FIA can improve the selectivity of a given analytical determination. Not only can existing methods be improved but new methods that could not have been developed without FIA can be utilized. This raises an interesting question. How many of the older chemical methods were discarded because of non-selective behavior or difficult handling under batch methodology? A careful inspection of this chemistry is in order. It is possible that under FIA conditions these reagents may have another useful life.

M. WHAT FLOW-INJECTION ANALYSIS OFFERS THE CLINICAL CHEMIST

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For many years the basic tool in the clinical laboratory has been a multi-channel analyser which has carried out its full range of tests on each and every sample presented to it. More recently, a combination of financial stringency and legislation has created a need for selective analysers which carry out on each sample only such tests as are specifically requested. This requires a system in which individual channels can remain on standby, often for prolonged periods, but are ready for instant use when required. Thus the simple and reliable continuous segmented-flow analyser which cannot be started and stopped quickly is excluded. For a while it appeared that the future lay with the complex discrete analyser, but the invention of FIA has completely altered this picture by providing the simplicity of flow analysis in a machine which can be turned on and off at a moment's notice.

Most clinical chemists have no longer the time or the inclination to carry out their own development work. They require of the manufacturer, as well as a machine, a full working system complete with analytical methods and data handling. Such systems are becoming prohibitively expensive, but FIA offers the hope that the inexpensive package will return.

There are special problems in applying flow injection to the analyses used in clinical chemistry. These are caused mainly by the small size and physical peculiarities of the sample, but also to a lesser degree by the requirements of some of the analytical methods used. Tests are carried out on blood plasma or serum; the sample is always small and strict economy must be used. By virtue of their protein content (about 7% by weight) serum and plasma are sticky and viscous and tend to adhere to probes and reactor tubes. The samples vary in viscosity. These factors seem to offer formidable problems in sample measurement. Some of the reactions used in clinical chemistry also present problems. For example many of the reactions are inconveniently slow, requiring the reaction mixture to be halted in the system. Also, some of the reagents are extremely expensive, prohibiting their use as a carrier fluid.

Of the commonly used sampling methods, the one which gave the technique its name, that is, injection of the sample by a syringe is the only method which does not waste sample to a prohibitive degree. It has been used by Rocks and co-workers [M1, M2] for the measurement of various metals, using atomic absorption spectrometric detection. This particular application is acceptable to the clinical chemist, since it replaces manual sample preparation and presentation; it would be less acceptable for the modest range of spectrophotometric analyses which make up 90% of the clinical chemist's work load. These are too numerous to be dealt with

manually in any part. The alternative sampling methods, for example, use of injection valves [M3], hydrodynamic sampling [M4] and gas-propelled systems using pinch valves [M5], permit complete automation but waste a portion of the sample. For this reason, Riley et al. developed an alternative system which they call controlled dispersion flow analysis [M6]. In this system the peristaltic pump which propels the sample and reagent is driven by a stepping motor controlled by a microcomputer. The sample probe is moveable and normally rests in reagent. When sampling is to take place the pump is stopped, the probe is transferred to the sample container and the pump rotates through a predetermined angle, drawing up a precisely measured volume of sample. The probe is then returned to the reagent container and the pump restarted so that the slug of sample is propelled by the stream of reagent. This has proved to be remarkably effective. No sample is wasted and the actual volumes used have proved to be small. The most striking example is the estimation of albumin which has been carried out on 250-nl samples with a coefficient of variation of 2%.

This approach has been widened to include the sampling of reagent. Two probes are provided; these normally rest in distilled water which acts as the carrier fluid. When sampling takes place one probe moves to the sample container and one to a vessel containing reagent. The slugs of sample and reagent are, of course, propelled by distilled water and are arranged to merge downstream of the pump. This use of merging zones [M7] has permitted extreme economy in the use of reagents. The relatively large volume of distilled water which passes through the system appears to have two beneficial effects. Firstly, by rinsing the sample probe and pipeline, it eliminates sample interaction (carry-over) and secondly, by achieving early dilution of the sample, it has overcome the problem of varying viscosity.

The remaining major problem, namely that of the test requiring a relatively long incubation, has not been entirely overcome. For fairly fast reactions, the kinetic technique described by Růžička and Hansen [M8] has been very effective, especially for enzyme measurement, but there remains a small group which require incubation for inconveniently long periods. It has proved possible to arrest multiple samples one behind the other in the same pipeline but the most achieved by the present author is three in a 3-m reactor; this requires strict limitation of the sample volume. Recently the use of multiple reactors (suggested by Růžička and Hansen [M9]) has been studied in this laboratory. This arrangement appears promising but forces unwelcome complications on an otherwise simple system.

Apart from its practical value as a means of developing simple and reliable selective analysers, FIA has proved to be a fascinating study in its own right. The unexpected is always just around the corner and these surprises are making appreciable changes in the understanding of the behaviour of liquids moving in small-bore pipes. Even if FIA was of no practical value it would be difficult to restrain one's interest in the phenomena involved.

N. PEAK DIMENSIONS AND FLOW INJECTION TITRATIONS

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Only one paper was published on FIA [N1] throughout 1975, while nowadays FIA articles appear in the literature at a frequency of more than one publication every second day [N2]. Also the concept of what FIA is has changed dramatically over the past decade, as the method has undergone the transformation from merely being a new concept of rapidly conducted serial assays to constituting a generalized approach of solution handling in the chemical laboratory. Thus, as reiterated in detail elsewhere [N2—N4], FIA, besides being a tool for fast serial analysis, has also become: (a) an impulse response technique where the sample is a species situated in the carrier stream and bombarded by zones of reagent solutions, (b) a new tool for continuous monitoring where the sample source is the monitored process, and (c) a new vehicle for conducting physicochemical measurements, where the species under study replaces what originally was viewed as a sample. Such a broad image of FIA has led to the open-ended description of "FIA being a measurement technique based on information gathering from a concentration gradient formed while a well-defined zone of a fluid disperses into a continuous nonsegmented stream of a carrier" [N2]. This definition, though not complete, is internally consistent since it describes the unique feature of FIA, i.e., the exploitation of a matrix of concentrations vs. time (Fig. N1), which matrix is, at time zero, initiated by injection, then formed by controlled dispersion of the originally homogeneous zone into the carrier

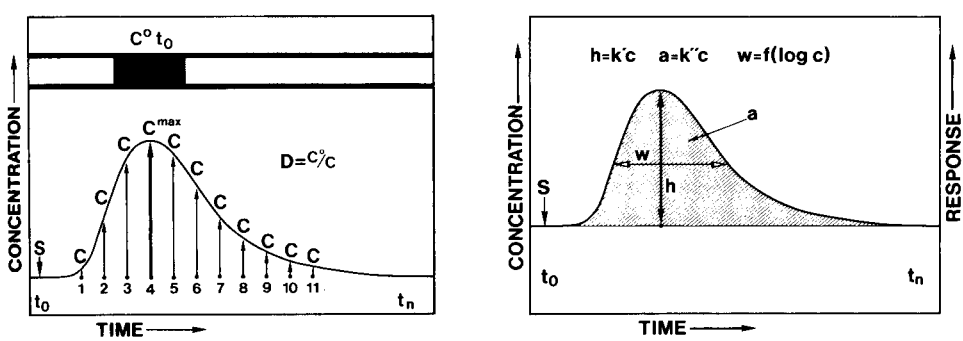


Fig. N1. Dispersed sample zone of original concentration, C^0, injected at position S. To each concentration, C, along the gradient there corresponds a specific value of the dispersion coefficient, D, each of which can be related to a fixed delay time, t_i, elapsed from the moment of injection (t_0).

Fig. N2. Peak height (h), peak width (w) and peak area (a) are all correlated to concentration (C) but in different manners.

stream, thus yielding a concentration gradient from which readouts, obtained through exactly reproducible timing, form the basis for a variety of FIA methods.

In the absence of chemical reactions and when the detector used responds linearly and instantly to the injected species, it does not make much difference whether peak height (h), peak width (w) or peak area (a) are being measured, since they all yield useful information (Fig. N2), though the concentration of the injected material is related to each of these parameters in a different manner. When, however, a sensed species is produced (or consumed) by a chemical reaction between the injected material and the component(s) of the carrier stream, then the shape of the response curve differs from that obtained by measurement of the concentration gradient alone, and therefore the choice of the measurement parameter has to be considered separately for each case.

Peak height. This is the most frequently measured peak dimension, because it is directly related to the detector response, such as absorbance, potential and current, and via these to the concentration of analyte. Besides taking the readout at one point, e.g. at a fixed wavelength, readouts throughout an entire spectrum may be recorded for multicomponent assays. Additionally, not only the peak height at the apex (corresponding to C^{max}), but also at any other section of the concentration gradient may be selected as the source of a readout (Fig. N3), allowing an expedient choice of the concentration range that can be accommodated by the detector [N5], or selection of the optimum analyte/reagent ratio for a given assay [N6], or, for merging zones, selection of appropriate ratios of analyte, standard or interferent [N7] permitting convenient studies of the influence of these variables. Note that FIA gradient techniques utilizing this 'vertical' readout are related to the detector response as obtained for different concentrations, expressed

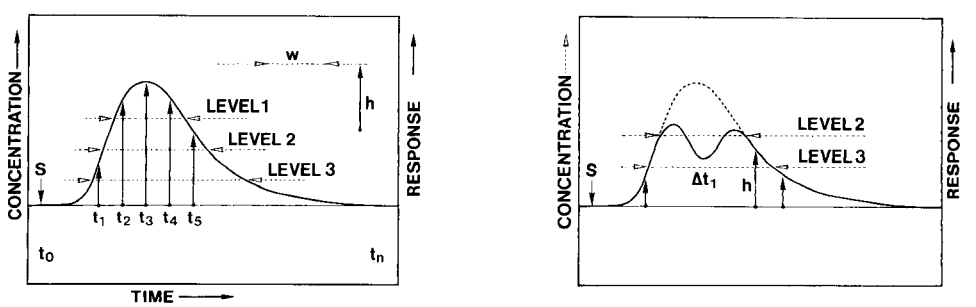


Fig. N3. Peak height (h) and peak width (w) readouts may be taken at such a time delay (t_i) or detector response level where the concentration ratio between the reagent and injected sample material is optimum.

Fig. N4. Readout of dispersed sample zone where the centre of the sample zone is lacking reagent resulting in the formation of a double peak. Useful vertical (h) and horizontal (Δt) readouts are only obtained close to the zone boundaries.

and related to the originally injected sample concentration C^0 via the D-value (Fig. N1). The *peak area*, however, cannot be related to spectra or to gradient readouts since it inherently has an integral character.

Peak width. Being horizontal information, the peak width is not directly related to the detector response and therefore does not yield multicomponent information based on spectra. Instead, the peak width (Fig. N3) is related to time, or more precisely to a time span Δt (or to a peak width measured in any other practical units). Depending on the flow geometry $\Delta t = k' \log C$ (for a one-mixed-tank system, i.e., $n = 1$) or $\Delta t = f(\log C)$ (for a different flow model [N8, N9]). Similarly to the peak height measurement, which can be executed at different delay times by "slicing the peak vertically", the peak width may also be "sliced horizontally" by choosing the level at which the detector will trigger the readout of Δt. While the requirement on the linearity of the detector response may be relaxed for peak width measurements, the requirement for constancy of flow rate of the carrier stream (and quality of the means of propelling the liquids) are as stringent as for peak height measurements. Because of the logarithmic concentration dependence, peak width measurements allow a wide concentration range of analyte to be covered using a detector of limited range, a unique advantage first pointed out by Pardue and Fields [N10, N11].

Lack of reagent in the centre of the sample zone will result in the absence of product to be sensed, and is therefore manifested by a trough in the centre of the recorded peak [N12] (Fig. N4). For those elements of fluid where $C_A > C_R$, i.e., $C_A^0 > C_R^0$ $(D - 1)$, neither h nor Δt reflect the concentration (C_A) of the analyte (C_R is the reagent concentration, and D is the dispersion), yet those levels (for peak widths) and those time delays (for peak heights) corresponding to elements of fluids having sufficiently high D values (i.e., those situated outside the trough) do yield useful horizontal or vertical read-outs. Obviously, the overall increase in D and/or in C_R will decrease or eliminate the trough and thus extend the range of useful read-outs.

FIA titrations. These are a special case of peak width measurements in that they rely on the use of an indicator, which allows the peak width read-out to be located at that level where, at the interface between the sample zone and the carrier, the equivalence condition has been met. For this purpose the carrier stream consists of a titrant, with an indicator added, while the injected sample is being titrated [N8, N9]. An increase in analyte concentration results in an increase of the peak width, since $\Delta t = k' \log C_A$ (for $n = 1$). Note that FIA titrations are a special case of peak width measurements characterized by the use of a titrant in the carrier stream, absence of a stoichiometric excess of the titrant in the centre of the dispersed sample zone, resulting in two equivalence points (A and B, Fig. N5) at the sample zone/carrier stream interfaces, and the use of an indicator (or other means of detection) for identifying the two equivalence points.

It is important to realize that the two equivalence points A and B coincide with the position of those two elements of fluid, within the entire range of

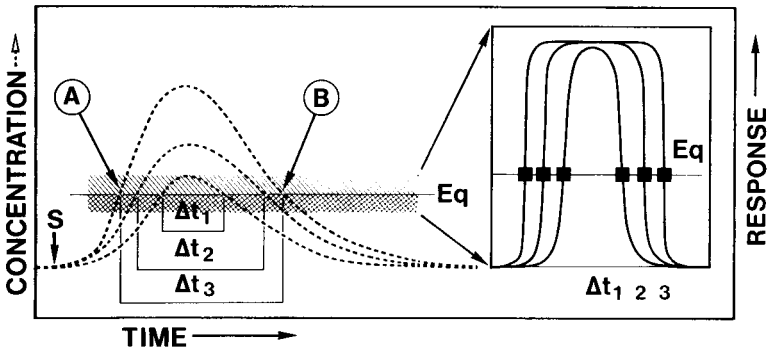


Fig. N5. Limited response range of an indicator selected to allow the horizontal readout of Δt at equivalence level Eq (left) yielding the response curves characteristic of FIA titrations (right). A and B indicate the equivalence points.

those forming the concentration gradient, where equivalence is met. This is a microscopic view of the dispersed zone, where each vertical (and hypothetical) slice is inspected individually so that the horizontal readout (Δt) is obtained. It is only macroscopically, however, that FIA titrations do not fulfill the IUPAC definition of an equivalence condition to be met, since the entire area of the peak corresponds to a larger mass of analyte than is equivalent to the mass of titrant contained within the carrier stream pumped during the interval Δt (if it were the case Δt would be zero!). Again, it was Pardue and Fields [N10, N11] who correctly pointed out this difference between classical batch and FIA titrations. Their thorough analysis also demonstrated the value of peak width measurements in the absence of any titrant in the carrier stream, using an indicator alone. As no equivalence between sample and titrant is sought in such a case, the trigger level may be chosen arbitrarily since the indicator or indicating device serves only as a means of detecting peak boundaries. On the other hand, greater stability of the detector output is required for peak width measurements than for FIA titrations, because in the latter case the abrupt change of signal provided via the indicator would tolerate moderate variations of the detector trigger level. To conclude, it may be said that all FIA titrations are based on peak width measurements, but not all peak width measurements are FIA titrations.

Since the FIA response curve is a result of two parameters, the physical process of dispersion and of the chemical process of formation of a sensed species, the response curve and the concentration gradient may be quite different (cf. Figs. N3 and N5). As the dispersion coefficient $D = C^0/C$ is related to the physical process only, its value is independent of chemical processes. Therefore the introduction of the term "chemical dispersion" [N13], designed to reflect the difference between the concentration gradient and the response curve, is unfortunate since this difference, reflecting chemical kinetics, sample reagent ratios and detector selectivity at a particular

wavelength (or potential), is too complex to be described adequately by a single number. In other words, while all species originating from the same sample injection have identical dispersion coefficients (D values) in a given dispersed element of the sample zone, they would appear to have different "chemical dispersions" depending on the detection mode and chemical reactions involved. Thus, for instance, for the titration of potassium hydroxide with hydrochloric acid (cf. Fig. N5), potassium monitored by flame photometry would appear to have a different "chemical dispersion" (shown as dotted concentration lines) than hydroxide ions, while hydroxide ions would appear to have different "chemical dispersions" if the pH change were monitored by a glass electrode or by acid/base indicator and a colorimeter (full lines on the readout shown at the right)!

It was thought to be of importance to raise these points because:

(a) there has been a recent increase of interest in FIA titrations and peak width measurements for the purpose of continuous monitoring of industrial processes [N14];

(b) there is a large number of classical titrations which may be performed in the FIA mode [N15];

(c) there is some unfortunate confusion in the literature both as to the merits of peak height, peak width and peak area measurements and to the principles of FIA titrations [N13, N16].

(d) it follows from the above that the use of a mixing chamber, or of a gradient tube [N9], is only a means of diluting the sample material and of "stretching out" the concentration gradient thereby increasing the Δt readout. Both FIA titrations and peak width measurements may, however, be done without gradient devices, the price being an empirical concentration vs. time relationship and the need to use means of rapid data collection, with an advantage being higher sampling frequency and less reagent consumption;

(e) the authors feel that they owe a response to important work [N10, N11], which has revealed several novel aspects of FIA titrations and peak width measurements.

In addition it ought to be mentioned that although all the above considerations were made for a sample injected into a reagent stream they would equally well apply to reversed FIA, i.e., when reagent is injected into a carrier stream of analyte. Reversed FIA titrations and peak width measurements, though yet to be explored, will undoubtedly find use as a tool for continuous monitoring.

Finally, it may be summarized that there are numerous ways in which useful information can be extracted from a dispersed sample zone, which is a highly reproducible matrix of concentrations as a function of time. Different response curves then yield additional dimensions to this source of information.

P. FLOW INJECTION ANALYSIS — A PERSONAL VIEW FROM THE UNITED STATES

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In the early 1970s one of us (KKS) was studying the naturally occurring trypsin inhibitors found in plant material. These studies involved preparative chromatography and required a large number of trypsin inhibitor assays, often in excess of 200 per day. Although manual assays had been developed, an experienced analyst could only conduct 30—40 assays per day. The r.s.d. of these assays was typically 10 to 20%, which made it difficult to locate enzyme inhibition activity in the early isolation steps. There were only a few automated tools available for routine enzyme assay. These included the Technicon AutoAnalyzer and a few very expensive automated assay systems which were mainly used in large clinical centers and industrial laboratories. Because of laboratory budget constraints, the trypsin inhibitor assay was automated using the Technicon system [P1]. Although this assay system had a greater throughput and generated results with better precision than the manual method, even greater throughput and precision were required to accommodate the fractions that resulted from preparative chromatography. A better assay approach was needed.

Extensive discussion began between KKS and GRB on how to automate enzyme assays inexpensively with a throughput of at least 100 samples h^{-1} and an r.s.d. of 1%. PEH was included in these discussions because it appeared that many of the concepts that were incorporated into the micro-flow system of his very sensitive amino acid analyzer [P2] could be adapted to an automated assay system. As a result, an automatic sampling assay system was developed that married the sampling aspects of Skeggs' continuous flow analyzer with the analytical advantages of the micro-flow system inherent in Hare's amino acid analyzer. The critical component that brought these systems together was a special stream sampling valve. In the fall of 1972 the first automated, air-pressure pumped, two-stream FIA system was successfully tested in the Geophysical Laboratory, Washington. It was found, however, that such pressure-pumped double stream systems were erratic and the precision varied widely. Eventually an automated one-stream system was developed [P3—P5] (Fig. P1). Not until the development of depulsed positive displacement pump systems [P6] was it possible for reliable multi-stream FIA systems to be designed. Meanwhile PEH and his co-

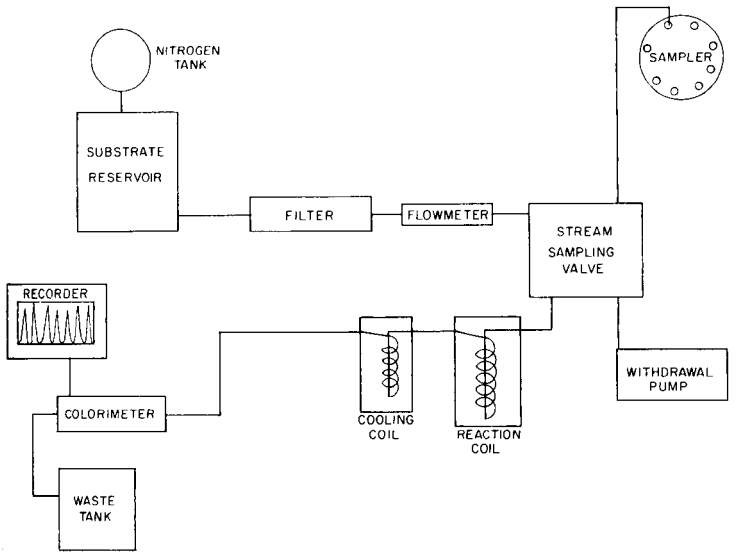


Fig. P1. Schematic diagram of the first automated flow injection analyzer. (Reprinted, with permission, from [P5].)

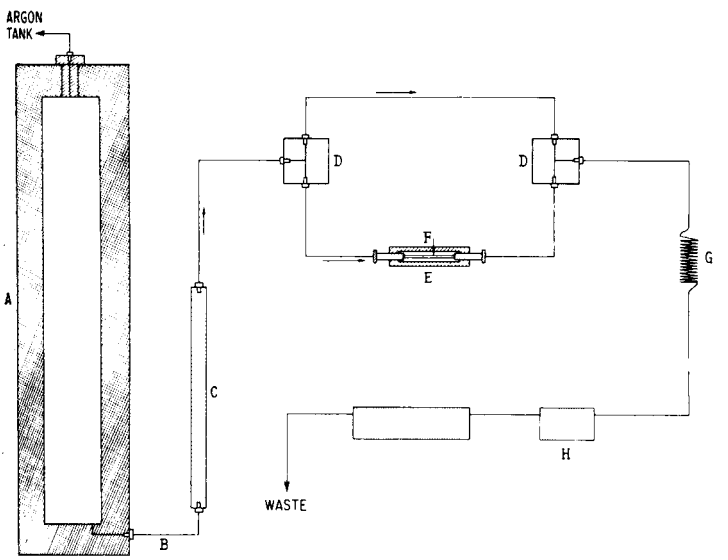


Fig. P2. Schematic diagram of the flow injection analyzer of Frantz and Hare [P7]. (A) Reagent reservoir; (B) 32-gage teflon tubing; (C) flow-meter; (D) 3-way valve; (E) sample injector; (F) "Voluette" micropipette; (G) 40-ft coil of 32-gage teflon tubing; (H) 6-mm colorimetric cell; (I) 60-mm colorimetric cell. (Figure reprinted with permission of the Director, Geophysical Laboratory, The Carnegie Institute.)

workers had developed a manual injection FIA system for the assay of sub-μg amounts of silica [P7] (Fig. P2). These early experiments are believed to be the first on FIA in the U.S.A. The early work was done in isolation, and it was not until the 1975 Pittsburgh Conference that the authors became aware of the concurrent work being done in Europe and South America. Several overviews of the early history of FIA have been published [P8, P9]. It is not necessary to discuss the interplay of the various participants here.

Once the early stages of this work had been completed, it became apparent that such a technique had the potential of being useful in many different areas and the present authors, like others, began to develop a variety of applications for various FIA systems. Table P1 contains a listing of the FIA applications developed by us in collaboration with a variety of other researchers. It was also realized that FIA was well-suited for computer control and data acquisition and several such systems were developed and published [P17, P18].

In the earlier phases, FIA systems were viewed as being in apparent conflict with the accepted theories of the behavior of solute concentrations in flowing streams [P18]. It was evident that full acceptance of FIA methods by the analytical community could not occur unless a solid theoretical foundation was established. As it turned out, the solution of the convection-diffusion equation which controls behavior in capillary streams under laminar flow condition had already been addressed adequately by Gil and co-workers in the U.S.A. [P19—P23] and by Bate and co-workers in Great Britain [P24, P25]. Numerical methods of these workers were extended by Vanderslice and co-workers [P26, P27] to generate equations applicable to regions of experimental interest in FIA. The calculations successfully predicted peak shapes, appearance times and baseline-to-baseline times of the analyte peaks in simple systems, thus lending credence to the fact that laminar flow conditions persist in such systems.

When laminar flow conditions are preserved in FIA systems the theoretical expressions enable molecular parameters such as diffusion coefficients to be generated easily and quickly [P26, P27]. This enabled FIA to be recognized as more than an analytical tool, but also a means of measuring parameters important at the molecular level. For example, the equations relating observed experimental parameters with the diffusion coefficient of the solute

TABLE P1

Application of FIA to analyses

Application	Ref.	Application	Ref.
Enzymes	P3—P5	Phosphate	P14
Atomic absorption	P10	Sugars	P15
Clinical — serum albumin	P11	Scale expansion	P16
Clinical — biuret	P12	Diffusion coefficients	P26, P27
Pseudo-titrations	P13	Rate constants, first-order	P29

[P26, P27] have been used to advantage by Gehrhardt and Adams [P28] to determine the diffusion coefficients for a series of neurotransmitters, a task that would be tedious and difficult by any other method.

The convection/diffusion equation with chemical reaction in a laminar stream can only be solved exactly for a reaction obeying first order kinetics. Thus, flow injection systems are ideal for use in determining first-order rate constants, as has already been demonstrated [P29].

It is sometimes helpful in the design of FIA systems to have some idea about the behavior of the solute segment as it moves downstream. Vanderslice, et al. addressed this problem by numerically integrating the convection/diffusion equation at different times after injection [P30]. The dependence of the segment shape on instrument parameters and molecular parameters has significant implications for the design of FIA systems when working at the more sophisticated levels of molecular parameter determinations [P30].

This Section has deliberately not covered all the field or all the workers and their contributions. What has been attempted in this article is to show how the present authors viewed FIA and its development in their laboratory. The work started as a response to existing problems. The underlying concept was that of the automation of wet chemical measurements. Originally, there was no thought of developing a new type of automation. That came about as a result of the interplay of different points of view on how to solve current problems. Once the new concepts were developed then it was a natural sequence to develop other applications, to adopt the system for computerized control and data acquisition, and to apply basic physical and chemical theories.

Prologue. Flow injection analysis has almost become an accepted technique. There are many research papers covering many different features of FIA. The technique appears to have enormous potential for use in many different situations. However, analysts have been slow to adopt FIA as a routine technique. It is now the job of those of us who work in the field to remove the final barriers to the acceptance of FIA as an automated tool for analysis.

We wish to acknowledge the collaboration of a number of investigators who contributed to the development and applications of FIA in our laboratories. These include Wayne Wolf, A. Gregory Rosenfeld, Darla Higgs, James F. Brown, Bruce M. Golden, John Savory, Michael Wills, Brian W. Renoe, Charlotte E. Schideler, Gregory Furrow, and David Okamoto.

Q. ION-EXCHANGE MINICOLUMNS

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One of the great advantages of flow injection technology is the ease with which additional components can be added to the system, to achieve a

particular analytical objective, without adversely affecting, to any marked extent, the original analytical measurement. An excellent example is the inclusion of a small column (3 mm diam. × 2 cm long) of an ion-exchange resin into a flow injection manifold, which can be used in a number of ways. One of the simplest is to remove interferences. When calcium is determined by atomic absorption spectrometry, using flow injection as a means of sample transport to the spectrometer, incorporation of an anion-exchange minicolumn down-line from the injection value removes anions which interfere (sulphate, phosphate etc.) thus eliminating the need to add expensive suppressing agents [Q1].

A number of metal ions interfere in the chemiluminescence determination of hydrazine by oxidation with hypochlorite. Zinc, Cd^{2+}, Al^{3+} and Ni^{2+} enhance the emission, and Co^{2+}, Cu^{2+}, Mn^{2+} and Cr^{3+} depress it. Incorporation of minicolumns of Dowex A-1 chelating resin into the reagent lines, however, removes all interfering metals and completely prevents any chemiluminescence. Controlled introduction of traces of Zn^{2+}, Cd^{2+}, Ni^{2+}, or, most effectively, Al^{3+}, into the solution after the resin column restores the emission, and allows highly selective determination of hydrazine ($\geqslant 10^{-8}$ M) to be made [Q2].

Chelating resins have also been used in a flow injection system to concentrate metal ions. When sufficient metal ions have been accumulated, a suitable solvent carries the metals to an appropriate detector [Q3, Q4]. Lead and cadmium, for example, were accumulated from sea water on a column of Chelex-100, and subsequently eluted with 2 M nitric acid for atomic absorption spectrometric detection. Reversed flow of the acid through the column results in less dispersion of the eluted sample, and a limit of detection of 10 μg Pb l^{-1} was achieved. The whole operation has been completely automated [Q3]. A similar system using 8-quinolinol immobilized on porous glass has also been described, and applied to the determination of copper in tap water by atomic absorption spectroscopy [Q4]. A sequential system, involving alternate pumping through two identical columns, gives greater sample throughput [Q5, Q6].

Anion-exchange minicolumns have been applied to the spectrophotometric determination of anions which are otherwise difficult to determine spectrophotometrically. If the resin is used in its thiocyanate form, passage of other anions (Cl^-, Br^-, I^-, SO_4^{2-}, NO_3^- etc.) displaces an equivalent amount of thiocyanate, which is determined on the basis of its red iron(III) complex [Q7]. A similar system based on the phosphate form of the resin can also be used, with determination of displaced phosphate as molybdenum blue. Mixtures (e.g., Cl^- and NO_3^-) can also be determined by splitting the injected sample, so that part of it passes through a silver reductor column, where chloride is retained, before passing through the thiocyanate column [Q7].

Finally, it is possible to elute successively ions retained on an ion-exchange minicolumn. Zinc and cadmium collected as their chloro complexes on Amberlite IRA-400 could successively be eluted with 2 M sodium

hydroxide and 1 M nitric acid, respectively, both 0.5 M in sodium chloride. The eluted metals were detected by their depressive effect on the cobalt-catalyzed chemiluminescence of luminol [Q8]. Detection limits were 5 ng of zinc and 3 ng of cadmium. From such a process, it is not a large leap to liquid chromatography in its various forms.

R. FLOW INJECTION TECHNIQUES FOR EXTENDING THE WORKING RANGE OF ATOMIC ABSORPTION SPECTROMETRY AND U.V.-VISIBLE SPECTROPHOTOMETRY

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Nearly all of the chemical manipulations required for conventional u.v.-visible and atomic absorption spectrometries can be done by FIA techniques [R1]. In general, these are simpler, safer, faster and cheaper than the conventional procedures. Most FIA applications concern solution u.v.-visible spectrophotometry [R2] though flow injection techniques have been applied to AAS [R3]. This section examines the ways in which flow injection techniques can extend the working range of AAS and solution u.v.-visible spectrophotometry (SS). Any such development will also have potential for a calibration strategy.

CONTROLLED DISPERSION

The factors which control dispersion in FIA are well known, if not as yet well understood. Some of the controlling factors, such as the extent of coiling, the nature of the injection process, the number of discontinuities in the tubing, and the type of flow cell are either not variable in practical situations or are only capable of providing a limited range of dispersion or both. From a practical view-point, the volume injected, tube dimensions, flow rate and timing of selected operations are important, together with the appropriate manifold design. Most variable dispersion manifolds designed primarily to dilute the sample have been used for flame AAS. The preferred approach for SS appears to be the time-based measurements described below.

Volume injected. The volume injected is normally readily changed (by changing the external loop of the valve), but not readily varied from one injection to another. Large dispersions may be achieved; up to 100 (dispersion being defined as the dilution factor of the injected material at the point of measurement) at the peak maximum [R4]. Thus, the working range is increased by the same factor at either end. It is possible to vary the volume reproducibly from one injection to another by controlled timing, either of the partial injection of the contents of a large loop, or of the aspiration of

the sample in valveless 'controlled dispersion analysis' [R5]. Injection of a portion of the dispersed sample zone into another carrier stream (zone sampling) produces dispersions at the peak maximum of up to 130 [R6]. The timing of this second injection may also be controlled.

Carrier tube dimensions. A range of values may be obtained by arranging a number of flow lines in parallel. The injected material is switched down the most appropriate line. When this technique, with dispersions at peak maxima values ranging from 2.52 to 14.9 [R7], is used a working range of 1—40 $\mu g\ ml^{-1}$ can be expanded to 2.5—600 $\mu g\ ml^{-1}$. One of the problems is that with fixed volume injection, a large dispersion means a large peak width.

Flow rate. This parameter does not provide a sufficient range of dispersions in a single line manifold, but may be used in conjunction with a merging stream configuration [R8]. The total flow rate will vary, which can cause problems with atomic absorption detectors. This can be offset by splitting the flow just prior to the detector.

TIME-BASED MEASUREMENTS

The factors which give rise to reproducible peak heights also provide reproducible peak shapes. Measurement along the rise or tail of the peak gives a variety of dispersions. In addition to the greater dilution, zones on the rise or tail usually have a greater reagent-to-sample ratio, which may be of benefit. Time-based methods also apply when the peak maximum is 'off scale'.

Peak tail. Measurements on this part of the peak have been used to provide 'electronic dilution' [R9]. This requires accurate and precise control over the delay period between injection and measurement. In conjunction with 'stopped-flow', it provides a basis for adjusting the 'initial' conditions of kinetic methods [R10].

Peak rise. Under normal FIA conditions, the peak rise is too fast to yield usable measurements. However, if it is spread out by the deliberate insertion of a gradient-forming device (such as a well-stirred mixing chamber), points on the rise curve can form the basis of a calibration technique [R11]. The time at which the mixing chamber effluent had the same absorbance as the sample is substituted into the known concentration/time equation.

Peak width. This is the most widely used of the time-based methods. Although first introduced as a flow injection 'titration' technique [R12], the concept has wider applications and the more general term, 'peak width' is used here rather than 'variable-time kinetic method' [R13]. All peak width methods allow the conventional calibration range to be extended upwards for several orders of magnitude [R14, R15]. This aspect, strangely enough, is hardly ever mentioned.

The situation where there is no reagent in the carrier stream applies to AAS and to the measurement of preformed coloured products. To derive a relationship between peak width and concentration, some assumptions have to be made about peak shape. So far, only exponential peak shapes have been

considered, for which the peak width at any height is directly proportional to a simple logarithmic function of the concentration injected [R14].

When a reagent is present in the carrier stream, in either a single-line manifold or a merged reagent-stream manifold, the peak width will bear the same relationship to concentration as the situation above, provided that the reagent concentration does not fall below that equivalent to the sample concentration at any point on the dispersed profile.

When the sample concentration exceeds this reagent concentration over a portion of the profile (this can always be achieved in a single-line manifold by injection of a large enough volume), there are points on the rise and tail at which the concentrations of sample and reagent are in their stoichiometric ratios. These are the 'equivalence points' of the flow injection 'titration'. An approximate expression for the time between them has been derived for the single-line manifold [R12, R16] and the merging stream manifold [R12]. Again, this is a logarithmic function. The practical difficulties of locating the equivalence points have not been discussed in any detail and, despite the additional simplification introduced, good correlations are obtained between the width at any height and the logarithm of the concentration [R17].

Double peaks. A new peak-width method has been proposed [R18] in which the separation between the double peaks produced when the reagent

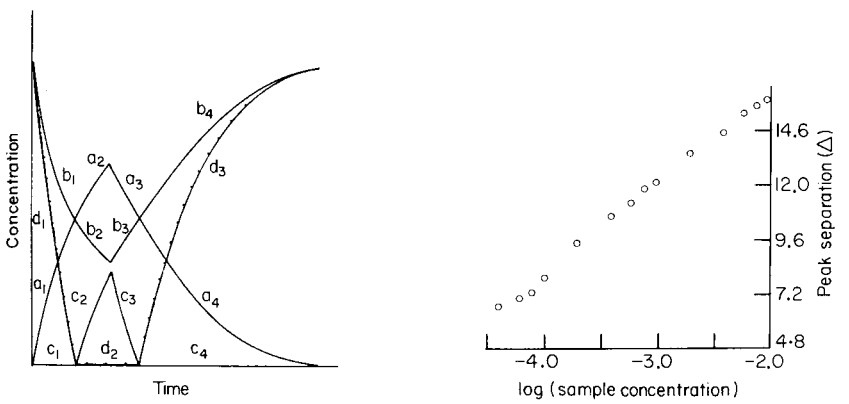


Fig. R1. Formation of double peaks in a single-line manifold. Curves a_1, a_2, a_3, a_4 and b_1, b_2, b_3, b_4 represent the physical dispersion of the sample and reagent respectively. Curves a_1, b_2, b_3, a_4, c_1, c_2, c_3 and d_1, d_2, d_3, represent the product, sample and reagent profiles when reaction occurs. It is assumed that the rate of the product-forming reaction is fast compared with the mixing process and that the thermodynamics ensure 'complete' reaction across the profile. This ensures that the product concentration profile (which is what, in practice, is measured) is an accurate representation of the dispersed sample or reagent profile, depending on which is in excess.

Fig. R2. Plot of peak separation against the logarithm of the injected sample concentration. The carrier stream was 1×10^{-4} M 1,10-phenanthroline flowing at 2.5 ml min^{-1} through a tube 35 cm long. The volume injected was 370 μl and the wavelength 508 nm. A least-squares linear regression gave: slope 3.92, intercept 23.6, standard deviation of residuals 0.197 and correlation coefficient 0.998.

'runs out' is related to the logarithm of the concentration. The basis of this is shown in Fig. R1, and the results for the determination of iron(II) over the range 4×10^{-5} to 1×10^{-2} M are given in Fig. R2. This method allows the time values of the equivalence points readily to be located. The peak heights are also related to concentration and permit extension of the lower end of the calibration down to 4×10^{-6} M.

CONCLUSION

Flow injection methods have more to offer than being just a more convenient way of carrying out existing analytical methods. In addition to opening up new reactions for analytical exploitation and a number of possibilities for manipulating sample and reagent concentrations, the availability of reproducible concentration gradients offers entirely new ways of obtaining analytical information. The challenge to analytical chemists is to discover them!

S. FLOW INJECTION ANALYSIS: AN AVAILABLE TOOL FOR SOLVING ANALYTICAL PROBLEMS

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As in other scientific areas, the development of analytical chemistry has been marked by the appearance at certain moments of concepts, techniques and methods which have revolutionized the progress of this discipline, making possible its consolidation and increasing its social importance. Flow injection analysis may be considered as an important milestone which, since its appearance in the mid-1970s [S1, S2] has changed the orientation of a great many research teams and is already being introduced into the laboratory as an extremely useful analytical tool.

There are five fundamental reasons for the impact and expansion of FIA within the analytical community. They are: the great simplicity of FIA configurations; the possibility of manipulating manifolds in an easy way to develop suitable designs to overcome different shortcomings, the only limitation being the imagination of its users; the low cost of the different components of an FIA configuration; the high sampling frequency, although, as recently demonstrated [S3], only 20% of the methods described so far have frequencies above 120 h^{-1}; and the surprisingly good reproducibility of the measurements (sceptics are only convinced through experimental demonstration).

The basic difference between FIA and other automatic methods (either discrete or continuous) lies in the possibility of designing home-made configurations and carrying out determinations with manual injection, which are interesting for solving shortcomings affecting determinations in which the

number of samples to be analyzed is not very large. Nevertheless, the partial or total automation of an FIA manifold is not difficult.

Some of the aspects in which the use of this technique has solved problems or has represented an important improvement with respect to existing methodologies are briefly discussed below. When it is necessary to process a large number of samples, as in clinical analysis, FIA can easily be automated to compete with other continuous flow methods. FIA allows standard addition methods to be applied readily [S4, S5] as well as other less frequent calibration methods such as electronic calibration [S6] and exponential dilution [S7]. When a decrease of sample and/or reagent consumption is required owing to inaccessibility (body fluids), high price (enzymes) or disturbance to the detection system (samples with a high salt content in AAS [S8]), FIA offers remarkable advantages over other methodologies by the use of modes such as merging zones [S9, S10] and integrated microconduits [S11, S12].

FIA may be adapted to analytical systems with a large variety of reaction rates. Fast reactions can be handled by normal FIA modes, whilst for slow reactions, modes such as zone trapping [S13] and stopped-flow in the detector cell [S14] or in a reactor [S15] can be used. The study and exploitation of unstable chemical systems may also be adequately carried out by this FIA. Thus, determinations involving reagents which are altered through contact with the atmosphere (such as strong reductants [S16]), systems which yield unstable monitored products [S17, S18] or measurement of the acid-base constants of easily hydrolyzable substances [S19] can be tackled by FIA.

When the analyte concentration is outside the analytical range for the method in question, FIA permits the application of amplification or direct dilution methods with the aid of a microprocessor [S20], by multidetection with a single detector in a closed system [S21] or by using a multidetector [S22]. The development of kinetic methods by FIA has remarkable advantages over conventional methodologies, allowing both stopped-flow and differential-kinetic methods [S23] to be implemented. The possibility of carrying out simultaneous determinations of several analytes in the same sample by FIA is in its infancy but is very promising.

It has been widely demonstrated that the development of a method by FIA involves a substantial lessening of interference effects [S24] compared to the manual alternative; this is probably due to the kinetic nature of the measurements. It has been irrefutably proved that the on-line incorporation of continuous separation techniques such as liquid/liquid extraction [S25], ion-exchange [S26], dialysis [S27] or distillation [S28] is very useful for pre-concentration operations and/or interference removal.

FIA is a suitable technique for determining physicochemical parameters such as acid-base [S19] and complex stability constants [S25], refractive index [S30] and viscosity [S31]. In its reversed mode, it is fully adaptable to industrial process control [S32, S33]. The potential of FIA is demonstrated by the large variety of areas covered by its applications.

The number of papers so far published on FIA (>800) clearly indicates that this technique is in the process of consolidation. Nevertheless, many of these papers are oriented to the adaptation of earlier manual methods in which the use of FIA represents only a minor advantage. Research concerning this technique must be directed towards the development of new modes, improvement of troublesome analytical methodologies and consolidation of its theoretical foundations.

A surprising aspect of FIA is the small extent of commercialization, in contrast with its clear advantages. It is difficult to establish the causes of this. Finally, to think that FIA can act as the analytical panacea would be a great error (perhaps induced by the enthusiasm which it generates among those working on it) and by the lack of an overall view of current analytical chemistry. There are established techniques such as gas and liquid chromatography, nuclear magnetic resonance, etc., with which FIA cannot compete to solve certain types of problem, but to which it can certainly be of great help.

T. FLOW INJECTION TECHNIQUES FOR MONITORING BIOCHEMICALLY SELECTIVE INTERACTIONS

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Flow injection analysis has a number of well documented attractions for a wide range of applications (as described in previous Sections). Those of particular interest for the study of biochemically selective interactions include: the small (<30 μl) sample and reagent volumes required in a merging zones manifold for use with expensive biochemical reagents; the ability to perform stopped-flow experiments to enhance the sensitivity of relatively slow reactions and to overcome matrix interferences; the compatability of on-line reactors (e.g., immobilized enzymes) and on-line sample treatment (e.g., dialysis); rapid and reproducible mixing of sample and reagent for fast transient reactions, e.g., chemiluminescent and bioluminescent reactions, and ease of automation and simplicity of operation.

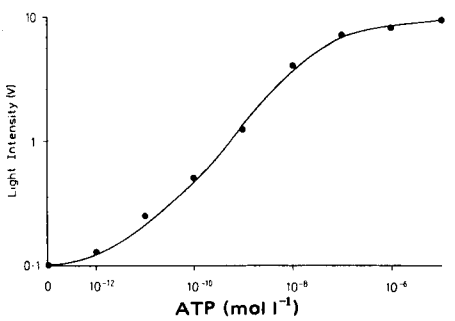
The most common biochemical reactions monitored by FIA are enzymatic assays, where the selective reagent is either in solution [T1] or is immobilized in open tubular [T2] or packed reactors [T3]. Recently the use of enzymes in FIA has been extended to bioluminescent reactions using partially purified firefly (*photinus pyralis*) tails. The enzyme (firefly luciferase) and the substrate (luciferin) require ATP as a co-factor to generate a transient emission of green light (562 nm). The intensity of emission is directly related to the ATP concentration and a detection limit of 10^{-17} mol has been reported using a conventional batch analyser [T4]. An

FIA procedure for the determination of ATP [T5], using soluble enzyme, had a reported detection limit of 10^{-14} mol and a linear range of 10^{-8}—10^{-4} M with a sample throughput of 200 h^{-1}. The manifold used was a merging-zones type with a purpose-built detector [T6].

The limit of detection and the general applicability of the above procedure can be improved by immobilization of firefly luciferase on a suitable support in a glass coil adjacent to the detector [T7]. Using this approach a detection limit of 3×10^{-17} mol of ATP was obtained and the enzyme was stable for 1—2 weeks at room temperature. A typical calibration graph for ATP is given in Fig. T1. The manifold and experimental conditions can readily be changed to allow the determination of other species, e.g. creatine phosphokinase and creatine phosphate, by coupling with the ATP reaction. This technique can therefore be used for the rapid, selective and highly sensitive determination of any species involved in an ATP-producing reaction. The use of bacterial luciferase would enable similar procedures to be developed for NADH.

FIA procedures that use immunological reagents are much less frequently encountered than those using enzymes. To date an energy-transfer immunoassay [T8], a homogeneous enzymatic fluorescence immunoassay [T9] and immunoturbidimetric methods [T10, T11] have been reported. The immunoturbidimetric technique is a rapid (40 samples h^{-1}) and cheap (<1 μl antiserum per assay) merging zones, stopped-flow procedure that is applicable to the determination of most clinically significant serum proteins and certain therapeutic drugs. A typical calibration graph for the determination of human serum immunoglobulin (IgG) using polyclonal goat anti-human IgG is shown in Fig. T2. It shows the antibody excess region of the immunoprecipitin response curve.

Interesting areas for future development in the use of FIA for studying biochemically selective interactions include the wider use of immobilised reagents, bioluminescence reactions in general, the application of dynamic



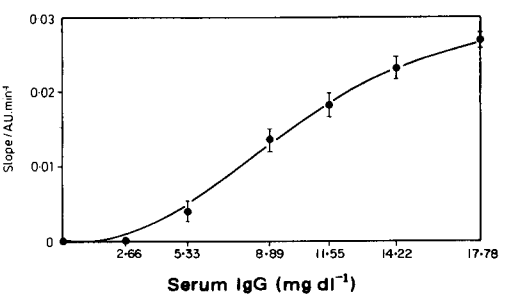


Fig. T1. A typical log-log calibration graph for ATP determination using immobilized firefly luciferase.

Fig. T2. Calibration graph for human IgG standards. The error bars indicate the range of 10 pooled results for each standard.

detection systems, e.g., diode array spectrophotometry, for multi-parameter analysis, the general availability of monoclonal antibodies and advances in automation and instrument design.

U. FLOW INJECTION ANALYSIS IN JAPAN

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It is surprising that, according to the FIA bibliography produced by Tecator [U1], there are as many as 728 FIA practitioners, who published at least one paper on FIA in the last decade. More surprising is that 23% of these were from Japan. FIA activity in Japan continues to grow. This communication is to report briefly such growth in activity, with emphasis on recent developments.

FIA ASSOCIATION

The Japanese Association for Flow Injection Analysis (Chairman, Nobuhiko Ishibashi) started with about 250 members in January 1984 and celebrated the first FIA symposium in "FIAland Kyushu", as coined by Professor Toei in his congratulatory address. The second FIA symposium in Osaka (November 1984, Professor T. Kojima), the third in Nagoya (June 1985, Professor M. Nakagawa) and the fourth in Chiba (December 1985, Professor R. Kuroda), followed. In each one-day FIA meeting, co-sponsored by The Japan Society for Analytical Chemistry, about 20 FIA lectures were presented. The FIA association publishes its own journal, the Journal of Flow Injection Analysis.

RESEARCH AND DEVELOPMENT

A recent survey has shown that about 700 scientists work with FIA in Japan, about 400 in academic work and 300 in routine analysis. Their published papers on FIA are estimated to amount to 200. This is too many to discuss individually in this section, therefore, only marked differences between Japan and other countries are described.

The preferential use of reciprocating pumps (plunger pumps) in Japan

seems to be unique. In contrast to the widespread use of peristaltic pumps in Europe, most Japanese FIA practitioners in academic laboratories tended to initiate their FIA work with reciprocating pumps. These were relatively expensive, but it was easy to control flow rates and they were suitable for doing FIA experiments at higher temperatures (>100°C) or at high pressure (>5 kg cm^{-2}). One of the main reasons of using reciprocating pumps was that many analytical chemists at that time were already familiar with chromatographic components that were readily available for constructing post-column reaction detection systems in HPLC. Accordingly, several manufacturers accepted the concept of a high-pressure flow injection system and constructed their FIA systems with reciprocating pumps.

FIA systems with reciprocating pumps are useful for manifolds used for preconcentrating metal ions, for reducing nitrate or for extraction procedures. Such FIA systems are also welcomed by chromatographers who wish to construct post-column reaction detection systems. One of the new trends in Japan in this respect is to design a FIA system that can be used interchangeably as a chromatographic system useful for both separation and detection, which will in turn promote the development of medium-pressure liquid chromatography.

The first generation of FIA procedures used reactions that had been shown to be useful in batch experiments. However, such an approach limits the versatility of FIA. In the second generation, which is just coming, the use of analytical reactions which do not proceed well in a beaker, but proceed rapidly in a reaction coil, presents a new challenge. The use of platinum metal as a catalyst in the PTFE reaction coil maintained at 160°C may be regarded as one solution to such a challenge. In the reaction coil, polyphosphates can easily be decomposed and detected, which could not be done at 160°C in an open beaker.

V. TEN YEARS OF FLOW INJECTION ANALYSIS IN A BRAZILIAN LABORATORY

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When FIA was conceived [V1], it was important to show and demonstrate that it was a useful technique for performing chemical assays, which at that time had mainly been automated by segmented flow systems. It is interesting to evaluate the impact of FIA in the last 10 years, which coincides with 10 years of FIA in Brazil.

FIA can be evaluated from an academic and a practical viewpoint. The academic impact can be demonstrated by the publishing activity, which is reflected in more than 800 papers [V2]. It has been shown that there are

no limits for automating procedures involving different reactions and different techniques such as gas diffusion, liquid/liquid extraction, ion-exchange and kinetic measurements. The versatility of FIA is probably the main reason why it has become so widely accepted all over the world. The practical impact, which is rather impressive, is related to the problems encountered in a routine analytical laboratory for large scale analysis of many samples. Besides the simplicity of the FIA set-up, the versatility of the technique is also a great asset here.

Both impacts are best illustrated by the Brazilian experience, as seen at the Analytical Chemistry Department of CENA-USP, where in 1975 the first paper [V1] was written. Since then FIA has become the most important tool used in this laboratory, in conjunction with various detectors such as spectrophotometers, atomic absorption and emission spectrometers and ion-selective electrodes for the routine large-scale determination of inorganic species in several materials. Consequently, more than 2 million determinations of 20 different chemical species have been carried out by FIA in natural and sewage waters, plant digests, soil extracts, soil solutions, rocks, minerals, fertilizers, alloys and ethanol. Details can be found in a recent publication [V3].

To achieve this goal, new approaches and processes as well as new FIA configurations were developed and used to solve analytical problems encountered. Amongst the important developments were the use of confluent streams for improving sensitivity [V4], the merging zones approach either for minimizing reagent consumption [V5] or improving baseline stability [V6], promoting standard addition [V7, V8], allowing simultaneous determinations [V9] and simulating solutions with different compositions [V10]. Other problems such as out-of-range samples were solved either by employing the zone sampling process [V11] or by gradient exploitation [V12]. Improving sampling rates by using intermittent flows [V13], and the zone sampling process [V14] for slow reactions were also realized. Indeed, for each task it was possible to find new and simple solutions.

As a result of this experience, other laboratories have become interested in FIA as a means of solving their own problems or applying the same procedures for routine large scale analysis of waters, plant, soils, fertilizers and alloys. Nowadays, more than 20 private industries in Brazil use such systems.

The impact of FIA should not be regarded merely as a technique which provides only high sampling-rates, low reagent and sample consumptions, good precision and low cost, because these are inherent characteristics of FIA systems. More than anything else the most important feature of flow injection analysis is its versatility.

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TWENTY-FIVE YEARS OF ANALYTICAL ATOMIC SPECTROSCOPY

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SUMMARY

Developments in atomic spectrometry are traced over the twenty-five years from 1960—1985. Although a few other methods are briefly mentioned, greatest emphasis is placed on emission, absorption and fluorescence techniques. Two emission approaches are considered in detail and are based on the high-voltage spark and on the inductively-coupled plasma, respectively. Atomic absorption progress is followed from its earliest introduction, through the trial-and-error improvement of instrumentation, to the recent availability of fully automated commercial systems. Finally, the youngest of the methods, fluorescence spectrometry, is described, its strengths and weaknesses reviewed, and its future potential assessed.

At the outset, it is appropriate to define here what is meant by atomic spectrometry. In this group of techniques, a sample is decomposed as extensively as possible into its constituent atoms. The atoms so formed, be they neutral or charged, can then be probed with a variety of methods, emission, absorption and fluorescence being the most common. The resulting signals contain the signature of the original sample and can be used to determine, both qualitatively and quantitatively, the elemental composition of that sample.

Like many other activities in analytical chemistry, the field of atomic spectrometry traces its roots back to developments in physics. Elsewhere in this article are documented some of the early findings that led ultimately to the analytical tools that are in widespread use today. The identification of elemental absorption lines in the solar spectrum by Fraunhofer led naturally

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to the further understanding of atomic behavior and to the cataloging of additional spectral lines. However, it awaited the pioneering work of Bunsen and Kirchhoff to show that such characteristics could be used for identification of elements. Instrumental developments by Lundegardh, Hasler, Walsh, Alkemade and others all set the stage for the explosive progress which is documented in this paper.

It is interesting that before 1960, the early limit of this overview, only one technique for elemental analysis was generally applied. That method, emission spectroscopy, usually involved d.c. arcs, high-voltage sparks or chemical flames. Since that time, these early sources have been discarded, modified, changed and challenged. Also, competitive techniques such as atomic absorption spectrometry (a.a.s.), introduced just before that boundary date, have assumed a large fraction of the work load originally carried by emission methods. Interestingly, emission techniques, particularly using relatively new excitation sources such as the inductively-coupled plasma (i.c.p.), are again in the forefront of atomic spectrometry. Yet, for many applications, the established emission or absorption or the newer fluorescence methods prove superior.

Because of space limitations, the scope of this overview has been necessarily restricted. Progress and trends in emission, absorption and fluorescence spectrometry will be examined in some detail. However, newer explorations into methods such as coherent forward-scattering spectrometry, laser-enhanced ionization spectrometry, photoacoustic spectrometry, and the use of novel emission sources will not be considered. In addition, except for a brief discussion of i.c.p. mass spectrometry, the use of ion detection from atomic sources will not be reviewed.

This paper has been made up from contributions of the five authors. As a result, some variations in style and approach will be noted. Also, no attempt has been made to achieve complete agreement on assessments made by individual contributors. Accordingly, it is appropriate to attribute sections prepared by individuals. First, recent advances in the classical technique of high-voltage spark spectrometry are examined by Alexander Scheeline. Next, the modern alternative to such classic methods of emission analysis is offered by Stanley Greenfield, holder of the original patent in i.c.p.-based methods. Atomic absorption spectrometry, the oldest technique historically and perhaps still the most widely used, is covered by Walter Slavin. Finally, atomic and ionic fluorescence spectrometry are reviewed by Nick Omenetto.

DEVELOPMENT OF SPARK DISCHARGE METHODS FOR SPECTROCHEMICAL ANALYSIS

The period 1960—1985 has seen substantial progress in the understanding of and improvement in the application of, the spark discharge for direct analysis of conductive solids. This progress is the result of developments in instrumentation and insight into the fundamental behavior of the spark.

Hardware developments and theoretical progress have been closely linked. As better instrumentation has allowed more sophisticated measurements, a deeper insight into the mechanisms active in the spark has resulted, and consequently still further instrumental improvements have been made. Many of the salient points discussed here have previously been reviewed in greater detail [1—3].

Throughout this period, the spark has been regarded as a tool for quantitation of the composition of alloys. Gradually, the precision of measurement under the most favorable conditions has improved from a few percent to a few tenths of a percent. Rapidity and simplicity of analysis have improved dramatically. Most emission spectrochemical measurements made in 1960 were made with photographic detection, while most quantitative work is now performed photoelectrically. This is the consequence not only of improved sensing electronics and accessibility of computers, but also of improved spark source designs [4—7] which minimize electrical interference. As the background contribution to observed data has been decreased by temporal and spatial gating [8—12], the linear working range has been substantially improved. Techniques for using the spark to vaporize solid samples with use of other discharges (typically an i.c.p.) to excite analyte vapor have led to reductions in the effect of the sample matrix (metallurgy, elemental composition, and physical properties) on determinations [13—16]. Sample heterogeneity has been shown to be a major contributor to imprecision, which previously had been attributed to the spark rather than the analyte [17]. Remaining problems include an inadequate quantitative description of the excitation and sampling processes in the spark, incomplete knowledge of speciation and energy transfer during the spark, and limited ability to control the nonlinearity of working curves at high analyte concentrations. Developments in multichannel detection of emission signals [18, 19] may soon render this latter difficulty irrelevant.

Hardware developments

In 1960, the only electronically-triggerable spark source was that based on the design of Bardocz [20]. All sources (whether electronically triggered or passively fired) gave rise to substantial radiofrequency (r.f.) bursts at the onset of discharge. While a variety of current waveforms could be produced [21], drift in source electrical behavior was of substantial concern. This drift was caused in part by resistive heating of components and aging of control-gap electrodes. Two developments in the design of such triggered relaxation oscillator sources, which have found substantial application [22], are the use of a diode rather than resistive damping to maintain unipolarity of the discharge [23], and the use of a hydrogen thyratron as a series switch to provide low-jitter triggering [6]. Quantitative waveform simulation to facilitate parameter selection has been reported [24]. Spark breakdown, triggered by buildup of a r.f. standing wave in a quarter-wave resonant cavity [4, 5] has produced discharges of greater positional stability than those triggered by transient gap

overvoltage [25]. In addition, such discharges permit the use of low-voltage current injection, independent of the triggering mechanism [26]. The sampling behavior of wandering discharges is vastly different from that of positionally stable discharges, especially when the signal is integrated over a large number of sparks [27].

Progress in manufacturing techniques, optical design, electronics and computation have led to nearly universal acceptance of the direct-reading spectrometer as the dispersion and detection device of choice for spark emission spectrochemical analyses. This system allows simultaneous multiline monitoring, background monitoring and subtraction, and the selection of a wide range of lines at the time when the focal curve of a given instrument is designed. Some systems also have an auxiliary scanning monochromator for flexibility. Photomultipliers are the transducers of choice. Whereas early direct readers used sequential manual readout of a capacitor voltage to reveal how much light had reached a particular photomultiplier, it is now typical for an inexpensive microcomputer to collect all data and interpret it in terms of concentration with little user intervention. Array-detector technology which combines the need for high resolution with wide wavelength coverage [28] is just beginning to appear. To date, no commercial system has incorporated the use of μs time resolution or sub-mm spatial resolution as discussed in the next section. The impressive improvement in signal-to-background ratios which such techniques provide strongly suggests that future systems will incorporate these features.

Most work in the use of the spark as a sampling device, with another discharge being employed for excitation, has yet to appear in print. What is clear is that linear working curves over many orders of magnitude can be achieved using the spark only for sampling, and that detection limits are better than when the spark is used for both sampling and excitation and without temporal or spatial gating [13—16]. A direct comparison of working curves obtained with state-of-the-art temporal gating of the spark emission [12] with those obtained by separated sampling and excitation has not been made.

Scientific developments

Although quantitation is the goal of most spectrochemical methods, most advances in understanding the spark have occurred through new qualitative observations. Emitted light is the easiest diagnostic feature to use in studying the spark, because it is both conveniently available and is the source of information historically used for quantitation. Non-emitting processes might, however, be important in controlling emission or in affecting the analytical signal. At least one of the dual-discharge (spark sampling/pulsed r.f. excitation) [13] approaches to improved analysis is a direct outgrowth of observations by Schlieren photography of the aftermath of a spark [29].

Sparks are spatially heterogeneous in both axial and radial dimensions. Spatial resolution has been profitably used to observe the spectrochemical processes in various regions of the spark. The space charges near the electrodes

are the location of sampling (the cathode being the primarily sampled electrode), the discharge core is the region of current conduction, and a cylindrical shell around the current-conducting channel is the region of maximum analytically-useful emission. Even after current cessation, the physical structure of the discharge continues to develop. A toroidal cloud [30] evolves from the discharge vapor, positioned at the minimum in the electric field potential gradient between the spark electrodes [31]. This cloud is ionic [32] and at least a portion of its content is sample-electrode vapor [33].

The optical thickness of the central spark channel has not been determined, but it can be seen to emit a continuum during periods of increasing current. There are thus two spatial regions in which light absorption can occur: the current-carrying channel (discharge core) and the surroundings of the spark (outside the cylindrical emitting shell). In the latter region, free atoms or ions might be translationally cold, but still present in sufficient quantity to cause self-reversal of emission lines. The time between sparks thus affects the shapes of working curves in several ways. Shorter interspark times result in better positional stability, lower breakdown voltages, higher electrode melting and erosion rates, and decreased signal-to-background ratios [34]. Increased repetition rate also results in enhanced absorption by atomic vapor, followed by fluorescence after current cessation [35]. Although theory has been developed for deconvoluting the effect of absorption on laterally observed spatial profiles [36, 37], there is a lack of experimental data collected by linearly responding detectors.

Time resolution of spark emission data has been employed as a diagnostic tool since 1901 [38]. The processes involved in breakdown for an argon spark forming in air have been described with 10-ns resolution [39]. The presence of metastable argon was demonstrated by doping the argon with nitrogen, which displayed spectral enhancements consistent with resonant interactions with the metastable energy levels. A pair of classic studies [11, 40] mapped the radial structure and emission propagation in the spark as a function of space and time, and revealed the temporal and spatial trends which later resulted in the generation of linearized, background-decreased working curves [10, 12]. Some progress has been made comparing the population of ground and excited-state electrode vapor with use of time-resolved absorption measurements [41]. Measurements of electron concentration are necessary to adequately compute the degree of ionization of the plasma. It has been demonstrated that classical line-broadening approaches to such measurements are inadequate in a system which is as heterogeneous as the spark [42], and an alternative procedure using Thomson (laser) scattering has been proposed [43].

An unresolved dilemma is the degree to which specific collisional pathways or microchemical, fluid dynamic, or thermodynamic processes affect emitted radiation. Magnesium emission appears to be strongly related to interactions with argon metastable atoms [33]. The order of appearance of ionic emission in mid-gap is a function of the initial polarity of the sample electrode; an

initially cathodic sample will give rise to ionic emission before neutral emission, whereas an initially anodic sample which becomes cathodic during a bipolar spark can be seen to emit neutral radiation before ionic radiation [44]. As these events occur at similar current levels, it is difficult to reconcile them on the basis of temperature or some other equilibrium parameter. Some calculations indicate that the time for an atmospheric-pressure argon system to relax to an equilibrium distribution is longer than the duration of the spark, leading to an excess of ionization at any particular current compared to that which would be observed in a d.c. arc at the same current [45]. Condensation is one mechanism by which free atoms and ions can be removed from the discharge vapor. Although there is ample evidence of particulate formation [16, 46, 47], the details of formation mechanisms and the matrix dependence of those mechanisms have yet to be elucidated.

Many of the problems associated with spark-based determinations can be circumvented with the use of positionally stable discharges, spatial and temporal masking to remove background, and the simultaneous observation of a variety of lines for each element of interest in order to avoid the effects of self-reversal. Microstructure of the sample [17] remains a source of irreproducibility in signals, so that appropriate interpretation might reveal chemical information about the sample. Differentiation between acid-soluble and insoluble aluminum in steel samples has been successfully demonstrated [48, 49]. Irreproducibility in point-by-point determinations using a laser microprobe has been shown to be qualitatively similar to the irreproducibility experienced using ordinary spark methods [50]. An interesting contrast to the nonlinear working curves obtained from spark sampling of solids with simultaneous excitation is the linearity of working curves possible when desolvated aerosols are injected into a short-duration spark [51]. The use of signal fluctuations and sampling variations as indicators of metal composition and structure remains to be fully implemented. The use of separate sampling and excitation discharges might be a means of circumventing the generation of some of this information, but it is not clear that avoiding complexity in data leads to "better" analysis.

Implications for the future

There is a large amount of technical information on the best ways to use spark spectral data which has yet to be incorporated into any commercial analytical instrument. With developments in mass spectral detection for elemental analysis, array detectors and separate devices for sampling and excitation, it is unclear what new developments can be expected in the marketplace in the near future. Spark-source mass spectrometry has been used for elemental analysis for many years, yet spark sampling at atmospheric pressure with mass spectral detection is not routinely used for bulk metal analyses. Simply sorting out the optimal ways to use currently available schemes will require considerable effort. Nevertheless, there are several areas of fundamental science which can be investigated to clarify how the spark samples

and excites electrode material and which might then be useful for revealing the meaning of "matrix".

The mechanism of sampling is poorly understood. Heating by ion bombardment, field emission at small projections from the cathode, and spattering of liquid metal droplets all play some role [52]. A common description of initial sampling is that a micro-explosion occurs, although it is unclear what this term means or implies for discharge behavior. Some recent magnetohydrodynamic calculations are promising in showing vapor trajectories and causes for cathode-spot wander [53].

There are substantial numbers of calculations in fluid dynamics which could be useful in demonstrating the adequacy of current descriptions of the discharge. It is unclear whether such calculations will be made in the near future, or, in the absence of such efforts, what new insights they might stimulate. Plasmas are an example of a mathematically "stiff" system, i.e., a system where summations of very rapid processes lead to long-term results. Statistical mechanics can describe the equilibrium behavior of such systems, presuming that the system has time to relax to equilibrium. This assumption is not met in the spark, as the current changes continually on a time scale that is short with respect to (for example) ionization equilibration. To the degree that steady-state or equilibrium assumptions cannot be made, the mathematical description of fundamental spark processes becomes a massive undertaking, with spatial scales from nm to mm simultaneously being relevant, and time scales from ns to ms also requiring simultaneous consideration. Under these circumstances, the mathematics might be intractable not only because of the amount of computation required, but also because of a lack of algorithms which can work stably over such wide ranges of scale.

Although much information indicates that the primary cause of apparent matrix variation in working curves is the sampling differences among alloys and the heterogeneity of various samples, there may still be secondary interactions between the spark plasma and analyte species which can give rise to variations in excitation efficiency. It is not yet known if the nature of the analyte can affect such fundamental discharge parameters as electron concentration and temperature. Condensation processes are of substantial importance to all spark-based techniques. Measurements of particulate growth as a function of space, time and sample composition are as yet rudimentary.

Atomic absorption is rarely measured in the spark, yet the absorbing atom cloud surrounding the discharge may potentially be useful for such determinations. Measurements of refractive-index gradients have been of major importance in elucidating the structures and material flows produced by the spark, yet no quantitative methods making use of these measurements have evolved. The spark remains a deceptively simple device with substantial unexploited analytical utility. As hardware and understanding continue to evolve, the repertoire of measurements made with this discharge as a major component can be expected to continue to expand.

TWENTY-FIVE YEARS OF INDUCTIVELY-COUPLED PLASMA SPECTROSCOPY

Doubtless there are many ways of writing a piece such as this. One approach is the review, the who did what and when style. Another is the "what has changed in the last two and a half decades" gambit. Of the two the latter would appear to offer the best chance of something fruitful arising from the exercise. However, the author is aware that one change which has taken place is a vast increase in the number of papers on the topic of the inductively-coupled plasma (i.c.p.) which reach the scientific journals each year. Once to be counted on the fingers of one hand, now to be weighed! Perhaps the only excuse for adding to this plethora of paper is to write about the changes which have taken place and to use the space allotted to attempt to separate the important from that which is not, to highlight the main thrust of the progress in the field and hopefully to suggest areas in which effort could be concentrated beneficially.

The early work on the i.c.p. was done using generators designed for plastic welding and induction heating. These generators had Colpitts oscillators with tuned-anode/tuned-grid circuits in which the work coil was an integral part of the tank circuit without the intervention of a decoupling amplifier. They became known as free-running generators because any change in the impedence of the plasma resulted in a slight change of frequency of oscillation with no loss of power. Later workers tended to use crystal controlled oscillators, especially in the U.S.A. Here the frequency of oscillation is controlled by the piezoelectric effect of a quartz or Rochelle salt crystal. Because the frequency is held constant within very tight limits, any change in the impedence of the plasma will result in a loss of power, unless an impedence matching network with automatic tuning is incorporated. There is no believable evidence to suggest that constancy of frequency is important to plasma spectroscopy, but constancy of power most certainly is. Both crystal-controlled and free-running generators perform their task well and, providing they comply with national regulations concerning external radiations, are both suitable for plasma work. The Colpitts oscillator which has been discussed is inherently power stable [54], and is cheaper to construct than its crystal-controlled counterpart. The main improvement in generator design has been in the stability of power output which now has a precision better than that of the nebulizer and other ancillary equipment which constitute the major source of noise in the plasma system. Solid-state generators based on static inverter circuits have not "caught on" as power sources for plasma spectroscopy, possibly because they are expensive to produce. However, solid state analogues of the crystal-controlled oscillator are now available commercially, specifically for use in plasma spectroscopy.

The idea that oscillator frequency played a major role in the formation of an annular plasma [55] was shown to be without too much foundation [56], velocity of injector gas stream being of greater importance. However, there is evidence that frequency does have a role to play in other properties of the i.c.p. Thus, in one experiment [57] an increase in frequency from 5 to 40 MHz gave lower excitation and ionization temperatures and lower electron

number densities. In addition there was an appreciable improvement in limits of detection resulting from the decreased continuum background, and improved background stability which gave a better signal to noise ratio.

Well into the 1970s the author was at the center of a controversy concerning the optimum power to use in an i.c.p. for atomic emission spectrometry. A good many apples were compared with oranges during this period! For instance, plots of power vs. emission intensity were plentiful. These purported to show that the best detection limits were obtained at low power and that these degraded with increasing power. A plot of the contours of detection limit on height of observation and power axes may have given a different result and a fully optimized system yet another. However, all was finally resolved [58]. Power density was the parameter that controlled detection limits, not power. Thus workers with the Fassel torches operating at low power were able to obtain detection limits which required rather higher power to produce in a large Greenfield torch. Hence, the conflicting evidence which appeared in the literature.

Of generators, it might be said, they have changed very little over the past 25 years. Basically, there are still two types, the free-running and the crystal-controlled. Power stability is a little better with more automatic control and there would appear to be a move in the direction of higher frequency. The power rating is now known to depend upon the choice of torch and possibly the application.

The large-diameter plasma torch, which became known as the Greenfield torch [59], has also changed very little since its inception. Quite early on, the integral nebulizer was abandoned because of the delicacy of its construction and the tulip-shaped inner tube was replaced by a straight-sided version. The small-diameter torch known as the Fassel torch went through a period of evolution from constructions giving spheroidal plasmas [60, 61] to versions giving annular plasmas [62, 63], but like the Greenfield torch, it has withstood the test of time. Of these two torches, the Fassel version is more generally used mainly because it requires less power and less argon gas to run. However, it is more critical of construction; the plasma is easily extinguished by the introduction of air, it has a limit of about 2% for total solids and the injector easily becomes coated with carbon if organic solvents are introduced. On the other hand, the Greenfield torch, with appropriate power, is easily constructed and has wide manufacturing tolerances, is not extinguished by air or other gases, will run for long periods with saturated solutions [64] and organic solvents are completely dissociated as evidenced by the absence of the Swan bands. There have been many claims to have produced improved versions of these two torches but there are few signs that those who have to use them in earnest have been persuaded of the validity of these claims. It has already been stated that small torches have to be made with some accuracy and although demountable torches are attractive in principle, and at least one instrument manufacturer supplies them as a standard fitting, one senses that there exists a suspicion that these torches are not sufficiently rigid to maintain their fine tolerance.

Mini-plasma torches [65—67] are also attractive in principle because of their low gas consumption and because they raise the possibility of using small solid-state generators. Again there exists the fear that, much as the Fassel torch is more tricky to use than the Greenfield torch, the mini-versions may be more so. Also, because of the lower heat content it is possible that matrix effects may be greatly increased [68]. It can be said, therefore, that plasma torches have not changed very much in the last two and a half decades so far as the user is concerned.

Very much more is known about pneumatic nebulizers and their spray chambers [69—72] than was so a decade ago. However, for all this increased knowledge there has not been any great increase in the efficiency of these devices. Most of the sample ends up down the drain and there is little, if any, evidence to suggest that this state of affairs is likely to change in the forseeable future. Of these devices, the concentric [73], cross-flow [74], high-pressure [75] and Babington [76] type nebulizers, it is the last which is the most interesting, as it offers the possibility of nebulizing solutions of high solute content, and also particulates. In addition, it is said that these nebulizers retain all the properties of the other types, viz. high sensitivity, good precision and relatively fast wash-out times. The experience of the author is that there is some justification for these claims.

It has been known for some time that ultrasonic nebulizers are more efficient than are pneumatic nebulizers [77] and it can only be concluded that the reason why these nebulizers are not used to any great extent is because of their high cost and complexity, and the increased memory effects which sometimes occur. The use of a sheath gas is said to decrease these memory effects [78]. There has been, however, a recent resurgence of interest in these nebulizers with the advent of the use of i.c.p.s in atomic fluorescence spectroscopy [79].

Because of the ease with which gases can be introduced into the plasma, it was inevitable that the determination of hydride-forming elements in their gaseous form [80] would become popular. However, the chemistry of these systems, especially for mixtures of elements, is not as easy to contrive as is the introduction of the hydride.

Flow injection [81] as a means of introducing samples to the nebulizer has not as yet captured the imagination of many workers in i.c.p. spectrometry. But the ease with which samples can be diluted, concentrated, generally manipulated so as to give an increased throughput, and the possibility of single-standard calibration must surely find many applications [82].

The area which offers the most scope for the exercise of ingenuity is that of non-pneumatic methods of sample transport and in particular those methods that offer a general solution to the problem of introducing solids to the plasma. About 1% of the sample, which is taken up by a pneumatic nebulizer, eventually reaches the plasma (including a lot of unwanted water) at a velocity which is governed by the amount of argon gas required for the nebulizer to work efficiently. In the case of the Babington and ultra-

sonic nebulizers there is a certain independence of operation but the transport efficiency is similar for the Babington and only slightly better for the ultrasonic nebulizer compared with the pneumatic version, and, as has been stated, it is difficult to see any major improvement in design occurring in the near future. For all this there is a great deal of time and talent being expended on what, the author insists, are fundamentally inefficient devices. In the last 25 years there has been just one attempt to continuously introduce solutions to the plasma without liquid aerosol formation and this was by the use of a device called a graphite yarn atomizer [83]. In this system the sample was evaporated onto a moving graphite thread from which it was volatilized by induction heating into the plasma. Just why this atomizer failed to catch the imagination of the workers in the i.c.p. field, even if only to the extent of promoting investigations along similar or superior lines, is something of a mystery. Attempts to introduce solutions non-continuously to the plasma have included filament vaporization [84] and electrothermal graphite-rod atomizers [85], but these techniques are certainly not in general use.

The sporadic attempts over the last quarter of a century to devise a general method of introducing solids to plasmas have been singularly unsuccessful. The main difficulties in the way of success are heterogeneity of composition, density and particle size, together with impaction and selective volatilization, depending upon whether the sample is powdered or a solid lump. The methods which have been tried with varying degrees of success and often with selected matrices have included pneumatic nebulizers [86, 87], swirl cups [88, 89], fluidized beds [89], graphite rods [90], arc/spark chambers [91, 92], laser ablation [93], laser/tape [94] and spark/tape [95]. Ultrasonic nebulization has been applied to molten metals [96]. Mention should be made, perhaps, of the introduction of powders into plasmas as slurries [86, 87, 97] because this technique has had a limited success with some matrices. Here then, lies a golden opportunity for someone to make a considerable advance in i.c.p. atomic-emission spectrometry (a.e.s.) by devising a rapid method of transporting solid samples into a plasma which is of general applicability.

Although the normal uptake rate of a pneumatic nebulizer is 2—3 ml min^{-1}, it has been found that μl quantities can be introduced into the plasma through a conventional nebulizer [98, 99]. More recently [100] very small samples have been introduced into an atomic-absorption flame by means of a technique known as controlled dispersion. This technique, which is akin to flow injection, involves a peristaltic pump operated by a stepping motor together with a solution "dipper". With the dipper in one mode, solvent is pumped to the nebulizer. The pump is stopped and the dipper is moved to the sample position. After a pre-determined number of steps, in which a known volume of sample is drawn into the line, the pump is again stopped. The dipper is moved back to the carrier position, the pump restarted and the carrier is pumped to the nebulizer carrying the sample with it. The whole operation is under computer control, is very elegant and can clearly be applied to i.c.p.a.e.s.

The large majority of workers in i.c.p.a.e.s. use the Fassel torch, and argon has remained the preferred gas in all streams therein. The small number of initiates who use the large Greenfield torch generally use nitrogen or air in the coolant flow. Barnes and Meyer [101] have operated all-nitrogen plasmas at as little as 1.3 kW. They reported better detection limits for Ca(II) emission in an argon plasma than in a nitrogen plasma and worse detection limits for Ca(I). However, in both cases the plasmas were not running under optimized conditions. Despite the obvious economical advantages of running with an all-nitrogen plasma, it has not been used extensively.

There is very little doubt that low-power argon plasmas in small torches are not in local thermodynamic equilibrium. The inequalities which were found in ionization and excitation temperatures led Mermet and Jarocz [102—104] to postulate excitation reactions with metastable levels of the argon atom and ion by Penning ionization to account for the high intensities of certain ion lines compared to atom lines. Boumans and de Boer [105] also suggested that the metastable species could be considered as easily ionizable constituents so as to explain the high sensitivity of ionic lines and the low ionization interferences. Although low-power argon plasmas may not be in local thermodynamic equilibrium, there is strong evidence to suggest that the nitrogen [101] and the nitrogen/argon [106, 107] plasmas are. The present position would appear to be that even after 25 years of experiment and debate, certainty of the exact nature of excitation mechanisms is not yet with us.

Most users of i.c.p.a.e.s. are agreed that chemical and ionization interferences are relatively small and spectral interference is the biggest problem [108], not only from the viewpoint of direct spectral overlap but because continuum and background shifts are also troublesome. This has led to careful design of spectrometers with emphasis on the use of baffles and light guides together with holographic gratings to reduce stray light. Vibrating quartz plates have been used to measure background radiation as have stepping motor-driven entrance slits. Because of the problem of spectral interference, especially when dealing with matrices which produce many lines, there is a persistence of interest in high-resolution spectrometers and more recently in Fourier transform spectroscopy [109]. Much change has taken place over the 25-year period in the data processing end of spectrometers; sophisticated graphic outputs are the order of the day and most functions of the whole instrument are under microprocessor control. Surprisingly, electronic spectrometers (the image dissector Echelle spectrometer (IDES) [110] which is an analogue of a scanning monochromator in which the scanning is done electronically and another variety, which is an analogue of the direct reader, where the whole spectrum is collected on a vidicon screen and as many wavelengths as are wanted are chosen to have their intensities measured sequentially) have not made the impact that was expected.

Plasmas were used as atomizers [111] and as line sources [112] in the 1960s. Since then they have found use in these roles in atomic fluorescence

spectrometry (a.f.s.) [113—122]. There is now a commercial [123] a.f.s. instrument incorporating an i.c.p. A combined atomic emission/fluorescence spectrometer incorporating a high-power plasma has also made an appearance [64, 124]. In the 1970s, i.c.p.'s also found use as ion-sources in mass spectrometry [125] and this attractive technique is now commercially available to those who have an interest in isotopic and isotope dilution techniques, as well as for more general elemental analysis.

It has always been possible, since its inception, to introduce liquids, gases and, to a limited extent, solids, into the i.c.p. for elemental analysis. This state of affairs has not changed over the last 25 years. Most elements in the periodic table can be determined in the i.c.p.

It is small wonder then, that i.c.p.a.e.s., which is firmly established, has been applied to almost every material under the sun. Given a successful instrument, the rest is chemistry and manipulation, two things analytical chemists are supposed to be good at!

A QUARTER CENTURY OF ATOMIC ABSORPTION SPECTROMETRY

Optical atomic spectroscopy has been used for elemental determination for more than 150 years. Of the chemical methods based on physics, atomic spectrometry provides some important advantages. After all, the Old Testament reports that early in the world's history it was commanded "Let there be light". It took until recent philosophical time for light to be harnessed effectively into the service of analytical chemistry.

By the middle of this century, atomic emission spectrometry (a.e.s.) had already accumulated a century of experience. Emission spectrographs were widely used in metals and mining and in many industrial applications. These instruments used a high current arc across graphite electrodes and photographic recording of the spectra. Harrison, at the Massachusetts Institute of Technology, and others had developed gratings to provide the high resolution that these emission spectrographs required to minimize interference between closely spaced lines. At about mid-century, simple photoelectric detectors were yielding to the newly developed photomultiplier. Lundegardh in Sweden had developed the premixed burner that provided an opportunity for flame emission photometers to determine the alkali and alkaline earth metals in biological materials.

Arnold Beckman and Howard Cary were successfully developing the elegant u.v.-visible spectrophotometers that converted colorimetric molecular photometry to photoelectric instrument-based measurements. Those who did not use arc or spark spectroscopy, flame photometry or colorimetry for metal determinations used the new science of polarography developed in Czechoslovakia in the first half of this century.

Flame atomic absorption spectrometry

It was in this environment that Alan Walsh resurrected the recommendation of Bunsen and Kirchhoff from a hundred years earlier that atomic absorption,

as well as atomic emission, be used for elemental determination. Walsh used the Lundegardh burner to atomize the sample and, essentially, the same spectrophotometer that had been developed for molecular absorption spectrometry. He made several key contributions to atomic absorption spectrometry (a.a.s.) including the use of lamps made of the desired element. The lamps were modulated so that emission from the flame could be ignored by tuning the detector to the source modulation frequency.

Walsh's first paper appeared in 1955 [126] and it would have been difficult to develop a useful atomic-absorption instrument before the decade in which he worked because the flame, spectrometer and photoelectric detectors used were all critical to the success of a.a.s. In addition, Walsh did not work in a vacuum. In the hundred years since Bunsen and Kirchhoff, astronomers had used atomic absorption widely to identify elements in the sun and stars and to understand the structure of these bodies. Woodson described a mercury photometer in 1939 [127] that was based on atomic absorption. Finally, Alkemade also published a description of an atomic absorption system in 1955 [128]. But Walsh and his colleagues at CSIRO in Australia persisted with the development of the technology and nursed it through infancy and adolescence.

The late 1950s were spent improving the technology and coaxing people to use the technique. The first applications papers appeared in 1958 [129, 130] and commercial instruments appeared in the early 1960s. The first half of the 1960s was devoted to applications and to understanding the limits of analytical performance that could be expected.

It is illuminating that the efforts to understand and apply the technique were hampered in this period by those who compromised important aspects of the system and, naturally, found the technique to be less attractive. The first a.a.s. book, in 1961 [131], found the technique to be useful only for a few elements because the instrument they used abandoned Walsh's requirement to use a modulated source and a tuned detector, thus elements determined at wavelengths in the near-u.v. and visible regions were hampered by emission from the flame. Other commercial instruments, opting against the Lundegardh burner recommended by Walsh, chose the total-consumption burner that was fashionable at that time in the U.S.A. for flame emission. A vivid memory of the mid-1960s, is a Pittsburgh Conference symposium on a.a.s. with an audience of over 1000. One paper from a prestigious American laboratory found that the first published application paper had been overenthusiastic in finding no a.a.s. interferences. It was difficult for that American laboratory to realize that the difference between their experience and the previous experience in Australia [130] and New Zealand [129] depended upon the choice of burners. This difficulty, resulting from the use of non-optimum systems, was repeated later in the graphite furnace technology pioneered by L'vov in the U.S.S.R. and generally misused for more than a decade.

By the mid-1960s the major limitation of flame a.a.s. was the inability to

determine the more refractory metals. Many of us turned our labs into pyrotechnic displays as we experimented with higher temperature flames. But it was Willis in Australia in 1965 [132] who found that the nitrous oxide/acetylene flame penetrated that last flame a.a.s. barrier. The last half of that decade saw the publication of several books, numerous reviews and, generally, the emergence of flame a.a.s. as a mature analytical technique.

In the last 15 years, the computer and electronics revolution has greatly improved the convenience and speed of flame a.a.s. but the spectroscopic and physical technology has not changed much. Automated systems can provide reliable determinations at a rate of over 500 h^{-1}, with operators of only limited experience. Burner safety has received considerable attention. In recent times, instruments have become available that automatically set up operating conditions from a "memory" within the on-board microprocessor. The microprocessor even senses the element to be determined by electrical coding of the lamp that has been moved to the source position.

The future of flame a.a.s. belongs to the ongoing computer and robotics revolutions. Computer-based programs will help the analyst to assure the quality of his results. Software routines will detect and identify malfunctions and perhaps switch automatically to redundant systems that will permit work to continue. Many papers are describing automated flame a.a.s. systems based upon the flow injection technique [133] and commercial systems can be expected soon that will combine sample preparation with automatic sample input. Robotics systems are an attractive alternative with perhaps more flexibility, although greater expense.

Furnace atomic absorption spectrometry

Early in the 1960s, L'vov [134] proposed the use of a furnace as an atomizer to replace the flame. The early commercial versions of his idea adapted the furnace to flame instruments, but it eventually became apparent that flame and furnace a.a.s. were different techniques with different instrumental requirements. The original L'vov paper was long inaccessible because it was published in Russian, but it was recently translated into English [135]. The elegance of L'vov's concept is captured in the translation:

> "In order to bypass the difficulty of calculating the atomic concentrations in a column of atoms, we completely evaporated the sample in a graphite furnace (cuvette) heated to a high temperature."

Both Walsh [126] and L'vov [135] anticipated that their a.a.s. techniques would become absolute, that is, the signals could be interpreted by physical data to indicate the amount of analyte element present in the sample without using standards. By now it has become clear that incomplete atomization of many metals in the flame and other factors will make it very unlikely that flame a.a.s. will be susceptible to absolute computation. Walsh's technique created a stable column of analyte atoms in the flame whose steady-state absorbance at a characteristic wavelength was proportional to the element's concentration in the sample. Thus Walsh's philosophy adapted the elemental

analysis to the concept of the molecular spectrophotometers. In contrast, L'vov retained the philosophy of the arc and spark elemental spectrographs by converting the entire sample to atomic vapor and correlating the integrated absorbance to the amount of analyte.

Spectrochimica Acta published L'vov's first paper in English in 1961 [136]. An atomic absorption spectroscopy book [137] published in 1968 devoted only a page and a half to the L'vov furnace, almost a decade after his first publication. The remarkable detection limits were so curious that they were reported as $\mu\mu$g instead of the pg we use today. I (W. S.) met L'vov at the First International Conference on Atomic Spectroscopy in Prague in 1967. I had come to the meeting prepared to discuss with him a commercial version of his furnace. He steered us towards Massmann who in 1968 published [138] an adaptation of L'vov's furnace for use with commercial flame a.a.s. instruments. In 1969 a commercial system was introduced, and in 1970 the first paper [139] was published involving this system. In the same year L'vov's book of a.a.s. was published in English [140] and, in a page devoted to evaluating Massmann's simplification, he concluded with the simple judgment "Definitely, therefore, this... simplification is not advisable."

Despite this good advice, many instrument manufacturers continued to supply Massmann systems for almost another decade. L'vov summarized the history of furnace a.a.s. [141] with Fig. 1. He plotted the number of published papers each year using flame a.a.s. and furnace a.a.s. since 1970. Flame a.a.s., a mature field in the 1970s, witnessed a slow decline in published papers. In contrast, the growth of furnace papers displayed the usual exponential growth that characterizes all successful new technologies. This con-

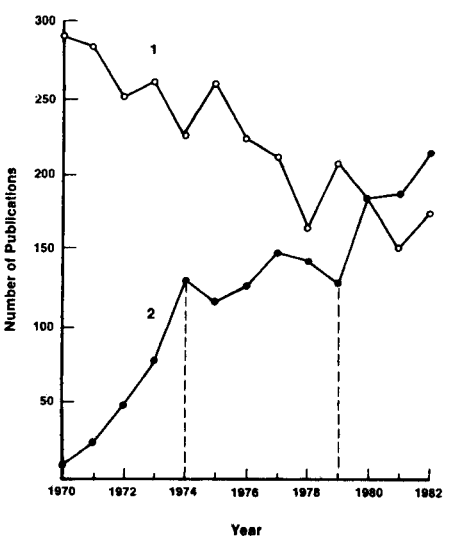


Fig. 1. Comparison of publications in analytical a.a.s.: (1) papers describing flame a.a.s.; (2) papers that use a furnace atomizer in a.a.s. Reprinted with permission [141].

tinued until about 1974 when the literature finally recognized that the Massmann furnace was plagued by interferences. The exponential growth resumed in 1979 when new furnace technologies began to emerge. What were the many compromises that cost a decade of graphite furnace a.a.s.? There were so many that the return to optimum performance was obscured for several years.

First of all, L'vov had shown that the absorbance signal must be integrated to avoid errors due to the rate of atomization, which is matrix dependent. But the flame a.a.s. instruments in which the furnaces were incorporated measured a steady state signal and had no means for integration. When, eventually, integrated absorbance was made available as an option, it became apparent [142] that the slow analog circuits of flame a.a. instruments could not follow the fast furnace signals. With the advent of modern digital circuitry this deficiency was remedied.

L'vov had recognized in his first paper that the integrated absorbance signal was proportional to the amount of analyte only if the gas phase temperature was constant during the full period when analyte vapor was present. While de Galan [143] and others have shown that the gas phase temperature follows the wall temperature quite closely, the Massmann simplification causes matrix components to alter the temperature at which vaporization occurs. Woodriff understood this problem and continued to use a furnace that stayed close to the L'vov principle, which Woodriff called a "constant temperature furnace" [144]. Woodriff's furnace was difficult to use but his work was particularly important because his system was free of the interferences observed in the Massmann system.

From 1977 to 1979 several alternatives were developed to bring the furnace to a steady, high temperature before vaporizing the analyte. Space does not permit these important contributions to be evaluated in this paper, but most of them stem from ideas of L'vov [145] expanded by Frech [146], Chakrabarti [147], Ottaway [148], Slavin [149] and many others. For some of these techniques to work, it was necessary to separate the function of achieving a particular final temperature from the function of the initial rapid heating. This was accomplished [150] by applying full power to the furnace until a diode detector sensitive in the infrared indicated that the desired final temperature was reached and a lower power was set.

With all of this, the technique still failed to achieve expected performance in most situations. L'vov had long ago pointed out that hot graphite was porous to atomic vapor. He suggested that pyrolytic graphite be deposited on the inside of the tubes to prevent diffusion of analyte through the tube walls. Until 1978—1979, commercial tubes were made either of a good quality graphite without coating or a poor quality graphite with a poor quality coating. Many workers supplemented this unsatisfactory situation by metal carbide coating of commercial tubes. By now, good pyrolytic coating on a good graphite tube prevents loss of atomic vapor through the tube.

The matrix of real samples provides a background absorption or scatter of

radiation used for the absorbance measurement that must be distinguished from atomic absorption. The use of a continuum corrector, invented by Koirtyohann and Pickett [151], was acceptable for flame a.a.s., but was inadequate for the much larger background signals found with furnaces. The Zeeman effect [152] and pulsed hollow-cathode lamps [153] have been used to provide the background correction that is routinely required for accurate analysis of most real samples.

There were other technological changes that were necessary to provide theoretically-anticipated furnace performance, but that performance is now available. In the past few years it has been realized that the sensitivity, i.e., the slope of the calibration graph, was largely independent of the matrix and had become quite stable from instrument to instrument, even those of different design [154]. The sensitivity is characterized by a value called characteristic mass, m_0, which is specific for each element and is expressed as the mass (in pg) that produces a 1% absorption-second signal, that is 0.0044 A·s. A striking breakthrough occurred in 1985 when L'vov was able to come within about 20% of the characteristic mass values for all but a few elements using physical parameters for the calculation. This raises again the prospect of absolute spectrochemical analyses using the graphite furnace, but there is still very much to be done.

Conclusions

The past 30 years of analytical a.a.s. provide a splendid example of the critical importance of the scientific literature in the development of technology. Walsh knew of the technology that would convert the Bunsen and Kirchhoff proposal to flame a.a.s. after it had lain dormant for a century. L'vov coupled Walsh's published idea with the prior literature of furnaces and arc emission spectroscopy. But L'vov's idea has been difficult to execute from the technology side. Many individual workers have pooled their skills using the medium of the scientific press to bring about the very successful products that are now becoming commercially available.

Another lesson to be drawn from this history is the futility of grafting one technique onto another. We must draw ideas from available technologies, but we should then proceed to solve the new specific problem with an optimized system. Some of the early failures in flame a.a.s. resulted from making flame a.a. an accessory for a u.v.-visible spectrophotometer. Fifteen years of competent furnace analyses have been postponed by trying to make the furnace an accessory to a flame a.a.s. instrument. While both flame and furnace a.a.s. rest upon the same physical principles, the technical requirements are very different. By separating the two instruments a furnace system can be made that is remarkably free of interference and a flame system can be produced that is not burdened by many furnace features of no use in flame a.a.s. Both systems work better and cost less.

ATOMIC AND IONIC FLUORESCENCE SPECTROSCOPY

General considerations

Fluorescence is based upon the absorption of photons of a given frequency followed by spontaneous isotropic emission of photons at the same frequency (resonance fluorescence) or at a different frequency (Stokes or antiStokes non-resonance fluorescence, according to whether the fluorescence frequency is lower or higher, respectively, than the absorbed frequency). In considering the historical development of atomic fluorescence spectrometry (a.f.s.), it is customary to date its analytical birth from a paper by Winefordner and Vickers in 1964 [155] after a suggestion, based on quenching studies of sodium atoms in flames, made by Alkemade two years before [156]. So, it can be said that the technique is 22 years old.

The mere fact that the title of this section, as suggested by its proponent [157], includes ionic fluorescence implies progress, i.e., that the fluorescence technique has been extended to ions as well (in this discussion, atomic fluorescence refers to that from neutral atoms and ionic fluorescence to that from atomic ions.) This is mainly due to the use of atomizers hotter than the conventional hydrogen- and acetylene-based flames of the pioneering experiments.

During its development, a.f.s. had, of course, to be confronted with a.a.s. during its phenomenal growth as an analytical tool. In the opinion of the author, while a.a.s. began its major expansion because of urgent analytical demands (from industry, geochemistry, metallurgy, etc.) a.f.s. was pursuing its own intellectual goals in a corner of atomic physics. Keen competition between manufacturers and the firm belief that a.a.s. could solve any elemental analytical problem better than other techniques were the major stimuli to the explosive development of a.a.s. Atomic fluorescence spectroscopy was confined to laboratory-constructed equipment, with its supporters competing with various excitation sources, none of which had all of the characteristics necessary to make the technique so superior to a.a.s. as to convince manufacturers to add the fluorescence facility to their absorption instruments. Meanwhile, however, the theoretical background of the technique (shape of curves of growth, effect of flame composition on quantum yields, excitation and de-excitation processes) was firmly established.

As a consequence of the similar atomizer requirements of a.a.s. and a.f.s., several improvements made in the absorption techniques had a direct impact on a.f.s. Typical examples include the "isolated droplet" technique used to study flame vaporization kinetics [158], electrothermal atomization ("out, out, brief candle" as quoted by Amos [159] in 1972) and hydride generation [160].

The use of lasers as excitation sources marked a turning point in the development of a.f.s., with no counterpart in analytical a.a.s., because it soon became evident that, in a.a.s., the advantages of the laser could not justify its replacement of the inexpensive hollow-cathode lamps. It was felt that

tunable dye lasers, because of their tunability, high peak power and narrow spectral bandwidth, were the ideal single-element excitation source for a.f.s. At the same time, saturation of the fluorescence signal was theoretically predicted and experimentally observed, opening new exciting possibilities of reaching extremely high sensitivities and of devising various approaches to flame and plasma diagnostics. As new optical and electronic equipment became available, they were rapidly incorporated, so that, nowadays, in its various fields of application, measurement of atomic and ionic fluorescence routinely involves the use of tools such as lasers, frequency-doubling crystals and Raman shifting techniques, electro-optical modulators, boxcar integrators and sampling oscilloscopes, time-resolved gated photon counting, correlation techniques, intensified image devices and streak cameras.

As a conclusion to these general considerations, it is interesting to compare early and more recent experimental systems for a.f.s. In a 1965 experiment, a turbulent air/hydrogen flame and a d.c. detection system were used. Thermal emission (if any) and especially light scattering had to be compensated. The excitation source was a vapor discharge lamp, available commercially for a few volatile elements (Zn, Cd, Hg, Tl, In, Ga). A 1985 system involves a double resonance fluorescence experiment in which two pulsed dye lasers, spatially and temporally coincident, are focussed into an argon i.c.p. to populate efficiently either excited argon levels, from which fluorescence is observed, or excited levels of alkaline earth metal ions nebulized into the plasma [161]. The argon-filled hollow-cathode lamp is used to tune the laser frequency to the near-i.r. argon transitions by monitoring the change in the discharge conductivity caused by the laser-induced ionization of the metastable argon atoms present in the glow discharge [162]. Thus, there have been major advances in instrumentation for a.f.s. during the past two decades.

In the following paragraphs, an attempt is made to evaluate the significant progress made in a.f.s. during this 22-year period. Specific reference to the literature is not always given and the reader should consult the many published review articles, books and monographs, where all the original studies are cited [163—174].

Evaluation of specific developments and studies

Excitation sources and atom/ion reservoirs. In a fluorescence spectrometer, the excitation source was immediately identified as its critical component. The replacement of a spectral vapor discharge lamp with a tunable dye laser was the outstanding change that took place over the last 22 years. Before the use of lasers (1971), many sources were tested, with varying degrees of success. The author agrees with Sullivan, who stated that "the lack of popularity of atomic fluorescence spectroscopy among analysts has been due in no small way to the problems encountered with the electrodeless discharge lamps" [175]. The problem of finding a universal excitation source has been associated with that of applying a.f.s. to either single-element or multi-element measurements. While the best single-element source has been found to be the

tunable dye laser, no multi-element source has yet been identified that would make atomic/ionic fluorescence spectrometry significantly superior to i.c.p. atomic/ionic emission spectrometry for simultaneous multi-element analysis. It is instructive to note that most multi-element fluorescence systems described in the literature use hollow-cathode lamps, often in the pulsed operation mode, which is particularly suited to multi-element work, as the pulse sequence can be synchronized with the detecting electronics. Also, the first commercial instrument, the Technicon AF-6, based on work by Mitchell and Johannson [176], introduced in early 1970 and since discontinued, operated six pulsed hollow-cathode lamps. One instrument currently available uses an argon i.c.p. as atomizer, and is also equipped with pulsed hollow-cathode lamps [123].

Apart from exploding wires [177], which have not been used for a.f.s., the use of most kinds of line and continuum sources for a.f.s. has been amply described and in some cases (e.g., the microwave-excited electrodeless discharge lamp and the Eimac arc continuum lamp) they have reached the point where no more development is expected. A very interesting source is the argon i.c.p. itself, in its almost unique dual role of excitation lamp and atomizer [117].

As mentioned above, the use of a laser provided new impetus to a.f.s. and considerable progress has since been made. Needless to say, if it were not for lasers, neither the concept of "single-atom detection" [178—181] nor the "multiphoton excitation" process, which allows the fluorescence detection of hydrogen [182], oxygen [183], nitrogen [183], chlorine [184] and carbon atoms [185], would have been brought to the attention of analytical chemists. Lasers may be frustrating, not only for their cost, but also for the skill and patience required to optimize their operation. This applies especially to pulsed dye lasers which are the most useful analytically because of their variable wavelength facility.

The choice of the excitation source in a.f.s. is sometimes closely related to that of the atomizer. It can be stated that, if an ideal atom or ion reservoir were available, many stringent demands put on the excitation source could be relaxed. Considerable effort has been devoted since 1964 to develop flames, electrothermal devices, plasmas, glow discharges and sputtering cells as atomizers. Among these, the argon i.c.p. again deserves to be mentioned because most of the peculiar attributes which make it the preferred source for emission spectroscopy are also retained in atomic [114, 186, 187] and ionic fluorescence [187]. Again, much benefit has resulted from the similar needs of the absorption and emission techniques.

The ideal atomizer/ionizer has still to emerge. Nevertheless, the combination of lasers, electrothermal atomizers and saturated fluorescence has pushed the limits of detection down to as few as 1000 atoms cm^{-3} [187, 188] or correspondingly to an absolute detection of sub-fg of analyte. Further developments with glow discharges and sputtering techniques are expected and single-atom detection might then approach reality [181].

TABLE 1

Useful combinations of excitation sources and atom reservoirs in atomic and ionic fluorescence spectrometry

Excitation source	Atomizer					
	Flames	E.t.a.[a]	I.c.p.	Modified i.c.p.[b]	G.d./S.c.[c]	C.v.t./H.g.t.[d]
Microwave excited e.d.l.[e]	*	*		*		*
Radiofrequency excited e.d.l.	*					*
C.w. and pulsed h.c.l.[f]	*	*		*		*
Boosted-output h.c.l.[f]	*	*				
Glow discharge					*	
C.t.g.l.[g]	*					
Discharge lamps continuum arcs	*	*		*		
Exploding wires	*	*				
I.c.p.	*	*	*	*		
Tunable lasers	*	*	*	*	*	

[a]Electrothermal atomizer. [b]Extended sleeve torch. [c]Glow discharge/sputtering cells. [d]Cold vapor techniques/hydride generation techniques. [e]Electrodeless discharge lamps. [f]Continuous wave; hollow-cathode lamps. [g]Controlled temperature-gradient lamps.

Table 1 collects many combinations of sources and atomizers, and the entries in the columns indicate which particular combination was experimentally tested.

Optics and detection systems; the multielement approach. The isotropic character of fluorescence emission is a disadvantage in single-element determinations because the solid angle of the radiation collected has to be small. However, it is one of the most-often quoted advantages for multi-element work because it permits a simple arrangement of collecting optics and detectors around the atomizer. Fluorescence spectra are also simpler than either absorption or emission spectra, especially with conventional excitation sources (i.e., not lasers) and therefore the use of non-dispersive detection systems [189] has been largely successful in several circumstances.

In order to increase the solid angle of collection, most optical arrangements have mirrors positioned around the atomizer. The value of such arrangements, which depends largely on the type of atomizer and detection system (dispersive or non-dispersive), has been exhaustively discussed in the literature (see, for example, [165, 166, 171]). The photomultiplier tube has been used in practically all experimental arrangements, varying from the solar blind type via that selected for photon counting applications to the fast tube wired for pulsed operation. Image devices such as the silicon target vidicon or its inten-

sified version have also been tested and abandoned for multi-element work, but probably warrant some further evaluation now that the state of the art of these detectors has been much improved.

Electronics and signal processing have developed according to the type of source and atomizer used. Simple d.c. amplifiers have, therefore, been replaced by synchronous (lock-in) detection when hydrogen was replaced by acetylene as a fuel, and boxcar integrators were the choice with pulsed excitation sources. Photon counting (d.c. and a.c.) was also characterized, and of course microprocessor technology has been a common denominator in several instrumental approaches.

With the experience acquired over the years with the above instrument components, it is now generally agreed that a flexible and reasonably simple atomic fluorescence spectrometer can be assembled for the simultaneous determination of a limited number of elements provided that ultratrace sensitivity is not required. In some cases, e.g., where either boosted-output hollow-cathode lamps or r.f.-excited electrodeless discharge lamps are available [175, 190], extremely good detection limits are indeed obtained.

A most welcome, non-dispersive, multielement fluorescence spectrometer that uses pulsed hollow-cathode lamps as excitation sources and a modified argon i.c.p. as atomizer was put on the market by Baird Corporation in 1982. Various examples of sensitive, rapid and accurate analysis have already been reported, and much development is expected to take place.

Scattering rejection. This problem in a.f.s. has received considerable attention from analytical spectroscopists; it tends to be either minimized by the supporters of a.f.s. or overemphasized by its detractors. In reality, it should always be a source of concern when making resonance fluorescence measurements. Understandably, therefore, much effort was directed, and progress made, since the very first applications of the technique, to overcome or correct this problem.

The turbulent flames used as atomizers in the pioneering fluorescence work were particularly prone to scattering because of the large droplets of the nebulized solution produced and consequently the inability of the flame efficiently to vaporize the solid residue left after desolvation. As a most striking example, the scattering signals obtained in an air/acetylene flame, for a solution of refractory elements (Al, Zr, Ti), were erroneously taken for fluorescence [191]. Once again, the methods experimentally tested for the correction of scattering were based on the same procedures already in use in a.a.s., i.e., on simultaneous or sequential two-line or continuum source compensation. Techniques such as wavelength modulation (and derivative spectroscopy) were not of general applicability. The advent of tunable lasers has enlarged the avenue to the correction procedures. First of all, full advantage could be taken of hotter flames and of non-resonance fluorescence transitions because no optical filters were needed to isolate the lines from the source. Secondly, other more exotic methods were shown to be effective. These included time resolution with pulsed excitation [192],

harmonic saturated [193] or intermodulated fluorescence spectroscopy [194], multiphoton or multistep excitation [195, 196] and polarization spectroscopy [197]. Finally, the Zeeman correction procedure was also applied to a.f.s. [198] and experiments were reported [199, 200] that were based on self-absorption and self-reversal of the exciting transitions.

Limits of detection. Limits of detection obtained for pure aqueous solutions should not be considered as the primary figure of merit for a technique such as a.f.s. Nevertheless, trends in such detection limits should reflect the progress made in the development of new excitation sources and of more efficient atom reservoirs. A review of detection limits for eleven elements published in the last 20 years shows an improvement of 2—3 orders of magnitude for most elements. Notable exceptions are copper and cadmium for which very intense excitation sources were available initially. Except for these elements, there have been major advances in the capabilities of a.f.s. since its inception. These advances have included applications of flames and plasmas as well as continuum and laser excitation sources.

Theoretical development and diagnostic studies. This is doubtlessly the field in which a.f.s. has witnessed constant progress and where it is difficult to foresee a slowing down of research. In the late 1960s, the basic theoretical needs of a.f.s. were the understanding of quenching effects in the atomizers, of the shapes of the analytical growth curves and the limiting noise of the overall apparatus. With the advent of tunable lasers, a significant effort was devoted to the study of the saturation of the fluorescence signal and related effects. These topics have largely been investigated and successfully understood [165]. A great deal of excitement began about ten years ago, and continues today, on the possibility of local sensing of physical parameters in flames and plasmas. Thus, profiling of the total number density of species and of the temperature was investigated, and soon the laser became the ideal tool in the study of the physics and chemistry of combustion [174].

The number of applications foreseeable for the combination of lasers and a.f.s. can hardly be overestimated. Dye lasers efficiently pumped by an excimer laser or better by a Nd/YAG laser, provide an excitation source with high peak power, narrow spectral bandwidth and continuous tunability from the u.v. to the i.r. These characteristics allow a variety of new experimental possibilities (e.g., multiphoton or multistep excitation) which will certainly help to elucidate complex excitation processes and excited-state dynamics in atmospheric-pressure flames and plasmas.

Concluding remarks

In attempting to present a bird's-eye view of the history of a.f.s. and its developments during the last 25 years, attention has purposely been focussed upon the analytical aspects of the technique rather than on its potential as a basic research tool in atomic physics. In addition, no other laser-based technique such as optogalvanic or resonance ionization spectroscopy, which can be used in flames, has been discussed.

Some conclusions can be summarized as follows. Conventional (non-laser) excitation sources, the i.c.p. and non-dispersive detection can join successfully to provide a fairly compact fluorescence instrument with adequate sensitivity, precision and accuracy for a large number of analytical problems. Similar developments would be instruments based on sputtering cells, glow discharges and resonance monochromator detection. With these facilities, a.f.s. has reached a stage of relative stability, similar to that of a.a.s. which was described by Koirtyohann as the period in which the technique had "put on its work clothes" [201].

The pulsed tunable dye laser and a.f.s. can be combined with any atomizer. The research potential and the overall flexibility of such a combination largely surpass the improved analytical results obtainable. With a properly devised atomizer, laser fluorescence is challenging as a single-atom detection method. The tunability of pulsed lasers can be extended down to 185 nm by stimulated Raman scattering and four-wave interaction processes occurring in hydrogen gas which is excited by a Nd/YAG pumped dye laser [202, 203]. The fluorescence technique can then reach elements such as As, Se, Te and Zn. Time-resolved fluorescence detection of laser microprobe plumes [204] and laser excitation in glow discharges [205] look very promising for trace element determinations in solid samples. "Fun Time" [201] with laser excited fluorescence is then expected to continue.

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ADVANCES IN CLINICAL CHEMISTRY OVER THE PAST 25 YEARS

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SUMMARY

A survey is presented of developments over the past 25 years of several important areas of clinical chemistry. These areas are instrumentation, reagents, electrochemical sensors, computing, statistics, quality control, enzymology, drug monitoring, trace elements and nucleic acid probes.

ADVANCES IN INSTRUMENTATION AND REAGENT SYSTEMS

The expansion over the past 25 years of clinical chemistry tests and the rapid development of new instrumentation and reagents for performing these analyses, has been phenomenonal. This growth equals or surpasses that seen in many other areas of analytical chemistry such as spectroscopy, electrochemistry, chromatography etc., which is not surprising when one considers that clinical chemistry draws on techniques from virtually all branches of analytical chemistry.

Clinical chemistry has been an expanding field for the entire 20th century. In the preface to the first edition of "Practical Clinical Biochemistry" [1] the author, H. Varley writes: "The rapid development of clinical biochemistry has been an outstanding feature of medicine during the past twenty to thirty years". This preface was written in 1953 and reflected the high level of sophistication in the methods available for the analysis of body fluids, and especially in the pathophysiological meaning of these analyses.

In order to appreciate the advances in clinical chemistry over the past quarter century, it is necessary to determine the state of technology around 1960. Several textbooks on clinical chemistry were published about this time [1—6] and provide information on the subject. The vast majority of organic analytes then, as is the case today, were determined using some type of spectrometric measurement. Spectrophotometers on the whole were crude. Visual comparison colorimeters, of which the Duboscq is best known, were rapidly

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being replaced by photoelectric instruments. Many instruments used cut-off filters but some used a diffraction grating or prism. Readings were taken off a scale which had a linear component for percent transmittance, or a log scale for absorbance. Often the technologist had a chart to convert percent transmittance to absorbance. Calculations were either made using a slide rule or by reading concentrations from a plotted standard curve. Almost all analyses were performed manually in test tubes with volumetric pipettes. Some crude automatic pipetter-dispensing units were available but were notoriously unreliable. Many assays were done at acid or alkaline pH which necessitated prior precipitation of proteins. The art of choosing the best method of protein precipitation was vitally important to providing accurate results. A classic chapter on protein precipitation is included in R. J. Henry's textbook "Clinical Chemistry Principles and Technics" [7]. Around 1960 only a few substrate measurements used enzymes as reagents, for example glucose oxidase for glucose, urease for urea and uricase for uric acid. These more specific methods, however, were used in only a few laboratories.

Other techniques used quite commonly in clinical chemistry laboratories 25 years ago were flame photometry, paper electrophoresis, and titrimetric and gasometric techniques. The introduction of flame emission methods for the determination for sodium and potassium was certainly a major breakthrough in providing convenient, accurate, and precise means for these vitally important clinical chemistry measurements. Electrochemical methods were available for blood pH, with a glass electrode, chloride using the Cotlove chloridometer, and the assessment of total base (Na^+, K^+, Ca^+ and Mg^{2+}) using the Sunderman Conductivity Bridge. Blood oxygen and carbon dioxide content were measured using gasometric techniques introduced by Barcroft and Haldane in 1902 [8] and refined by Van Slyke in 1917 [9a]. These elegant techniques unfortunately have been abandoned in all modern laboratories much to the dismay of the purists.

Titrimetric techniques which were used extensively for the measurement of serum calcium [10] and also for chloride [11] also have been abandoned. The elimination of gasometric and titrimetric methods occurred when automation was introduced. Neither of these techniques was adaptable to this new trend. Electrophoresis is still with us in a more sophisticated form, and is used at the cutting edge of our field in the application of DNA probes in laboratory diagnosis.

The first two decades following the introduction of automation into the clinical chemistry laboratory were dominated by the continuous flow-stream analysis system, the AutoAnalyzer (Technicon Instruments Co.) first described by Skeggs in 1957 [12]. The AutoAnalyzer was successful initially because of its mechanical simplicity and flexibility in being able to perform traditional analyses. Protein removal was accomplished by dialysis rather than precipitation. Incubation could be done in glass coils at ambient or elevated temperatures. Final measurements could be made using a flow-through colorimeter, fluorimeter, or a flame photometer. The success of the

Skeggs automated analyzer is now legendary. Multichannel instruments were developed and miniaturization allowing sample and reagent volumes to be reduced. Additionally, computers became an integral part of the system. Most of the readers of this article will be familiar with the new generation of Technicon AutoAnalyzers, the SMAC, which is based on analytical principles very similar to the old analyzer described by Skeggs. The other category of automation introduced twenty years ago was represented by robot systems combining the separate mechanical events of traditional chemical analysis into a sequence which was controlled by electronic, hydraulic or mechanical programs. The robot automated analyzer as originally designed by Research Specialties Co. (Richmond, CA) was much more complex mechanically than the continuous flow system, and the earlier instruments tended to be unreliable. Many clinical chemists of that era who thought along traditional lines were more attracted to the robot analyzer than the AutoAnalyzer because of the similarities in operation to most manual procedures. Fifteen years and many engineering innovations were needed before the "discrete" automated analyzer was to find a successful niche in the routine laboratory. At the present time there are several of these discrete-sample automated analyzers which are used successfully in most large laboratories. The Beckman Astra is the most widely used but there are several others which are mentioned later.

Along with the development of automated analytical instruments came the introduction of specific reagents using enzymes and antibodies. The development of immunoassays will be dealt with in a separate section. The use of enzymes as reagents for substrate determination or in coupled reactions for enzyme assays, certainly has made a great impact on improving accuracy as well as simplifying the technical aspects of the analysis. The introduction of serum enzyme assays as important diagnostic tests led to the need for "kinetic" analyzers. Enzymes are measured using pseudo zero-order reaction conditions, and continuous kinetic monitoring of the reaction is necessary to provide activity results which are related to enzyme concentration. Kinetic measurements can be made with a recording spectrophotometer but throughput is limited even with multiple cuvette chambers. A major advance in instrumentation occurred in 1969 with Anderson's development of the centrifugal analyzer [13]. This instrument which was originally conceived as a high throughput automated analyzer, made its major contribution in being a superb instrument for performing rate methods. This was one of the first instances in the development of clinical chemistry instrumentation in which a computer was an integral part of the analytical instrument. The centrifugal analyzer provided ultimate control of the time variable so that different kinds of kinetic analyses could be performed. Spectrophotometric performance was excellent because multiple absorbance readings could be taken within a short time period. Additionally, analytical performance was improved because standards and samples could be processed simultaneously. The centrifugal analyzer turned out to complement the multichannel, continuous flow analyzer, rather than become a replacement. At about the same

time other automated instruments for performing kinetic measurements were manufactured with a more conventional design. Features included automatic mixing of sample and substrate, and absorbance monitoring for a few s in either flow-through or disposable cuvettes. Among these instruments were the Abbott Bichromatic Analyzer 100 (ABA 100), Gilford 3400 Automatic Enzyme Analyzer, LKB-8600 Reaction Rate Analyzer and many others. The limitations of this type of instrument was that only one reaction could be monitored at a time. Thus, in order to achieve acceptable throughput, this monitoring time had to be quite short, being of the order of 10—60 s. The centrifugal analyzer could use much longer reaction times because all the cuvettes were monitored simultaneously.

One major disadvantage of the centrifugal analyzer was that analyses were performed in batch mode. Recently there has been considerable emphasis in the development of Random Access Analyzers. Here several tests can be performed on a single specimen. The Hitachi 705 and the Technicon RA-1000 have been among the most popular of these instruments. Samples and reagents are pipetted automatically into a cuvette which is indexed through a spectrophotometer at pre-selected times so that multipoint kinetic measurements can be made. The spectrophotometer has several filter positions so that several tests can be intermingled with each other.

The development of kinetic analyzers over the past 15 years has led to many changes in reagent systems. The use of enzymes as reagents has become commonplace and has led to improved accuracy for many tests. One useful development has been the introduction of the two-point pseudo first-order fixed-time kinetic analysis scheme for substrate analysis using enzymes as reagents. This approach, as described by Ingle and Crouch [14], has been a useful innovation for increasing throughput in clinical chemistry testing and also providing a means of reducing static and non-static interferences.

The most recent development which is changing clinical chemistry is the use of dry-reagent chemistry technology [15]. The potential value of this approach was demonstrated in the DuPont ACA analyzer which employed a heat-sealed transparent plastic envelope containing analytical reagents in separate compartments. The envelope became the reaction chamber and subsequently the cuvet for the spectrophotometric measurements. This instrument and reagent system simplified the operational aspects of the analysis because reagent pipetting was eliminated. The DuPont ACA was ideal for low volume and off-hours (emergency) testing, but was expensive to operate due to the complexity of the reagent envelope which contains both liquids and dry reagents. The use of dry reagents on a slide or a strip has been developed by Eastman Kodak (Ames Division of Miles Laboratories, Boehringher Mannheim) and others. The first development in this field was in the 1950's when the Ames Clinistix urine reagent strip was introduced for testing urinary glucose. Two approaches have been taken to produce the various layers comprising the basic components of dry-reagent chemistry carriers. One approach has been to use film casting techniques which was

that used by Kodak, obviously resulting from their extensive experience in photographic films. The second approach was to construct a reagent layer involving the use of a paper or a synthetic fiber matrix. These analytical systems provide a means of simplifying many assays and offer the opportunity to provide good quality testing in small laboratories in offices, clinics or on wards.

This brief introduction provides a superficial review of changes and advances over the past quarter century in the classical clinical chemistry tests which have formed the nucleus of most routine laboratories. The development of many other aspects of clinical chemistry such as ion selective electrodes, drug monitoring, immunoassays etc., form the basis of the remainder of this review.

ELECTROCHEMICAL SENSORS

There is an exciting revolution currently underway in academic and industrial laboratories around the world and it is focused on the development of novel chemical sensors for use in the clinical arena. This is evidenced by the ever increasing number of journal articles, new publications [16, 17] and national and international symposia [18, 19]. In the academic sector the excitement comes from the thrill and satisfaction of conceiving and demonstrating new sensor concepts. In the commercial sector the expectation of increased revenue and profits from new and expanded markets is luring many companies to invest in short- and long-term sensor research. Indeed, this is an exciting time to be involved in chemical sensor research and development. This brief review examines the role of electrochemical sensors in clinical chemistry in the last quarter century and presents some indication of their future role in the health care domain.

The major processes involved in any chemical sensor system are analyte recognition, signal transduction and read out. Table 1 lists in random order many of the key criteria or requirements for an ideal chemical sensor. New developments in fluorescence-based optical sensors, piezoelectric-based mass detectors, and other unique concepts offer great promise to satisfy many of these criteria and may lead to improved sensor performance and wider analyte scope. However, these approaches have yet to meet the demanding

TABLE 1

Important characteristics for chemical sensors

Selective	Reusable	Precise	Non-destructive
Inexpensive	High resolution	Multiple	Relevant data
Small	Adaptable	Fast	Durable
Manufacturable	Rugged	Simple	Accepted
Reliable	Non-toxic	Predictable	Easy to calibrate
Stable	Whole blood/turbid samples	Convenient	

requirements necessary for wide scale commercialization. In contrast, electrochemical-based sensors meet many of the ideal characteristics and have been used commercially for diagnostic purposes since the late 1950s. As such they serve as the foundation or reference point for much of the new work which is just beginning [20].

Each of the sensor processes for an electrochemical device varies depending on whether the sensor is a potentiometric or amperometric device. With potentiometric sensors (i.e., ion-selective electrodes), the recognition component is a selective binding event which transduces analyte ionic activity into a voltage developed across the sample/electrode interface. This voltage is measured (readout) under conditions of essentially zero current and presented to the user as the analytical signal. The system configuration can be as a classical glass pH-electrode or as the novel solid-state ion-selective field effect transistor (ISFET). With amperometric devices (redox electrodes), the recognition component is a selective electron transfer event that transduces analyte concentration into an electrical current passing through the cell. This current is measured under constant applied potential and becomes the analytical signal. The system configuration can be as conventional as the Clark PO_2 electrode or as the newer multicathode structure fabricated on a silicon substrate.

Commercialization of electrochemical sensors

Table 2 lists the major classes of clinical analytes and the current state of electrochemical sensor commercialization. It is fair to say that blood gas sensor technology is rather mature with no fundamental changes in the sensing concept since the original designs were first shown to be feasible. Reliable electrolyte sensors are now available for most of the key ions with no new breakthroughs anticipated in the short term. Metabolite sensors based on immobilized enzyme membranes have been shown to be feasible for over 15 years but commercialization is just now starting. Of the top ten blood components now determined in clinical laboratories, eight can be determined with electrochemical sensors. In descending order, these are: glucose, sodium, potassium, urea nitrogen, chloride, creatinine, total carbon dioxide and calcium. These constitute over one billion assays annually in U.S. hospital laboratories. Thus, it seems likely that electrochemical sensors for electrolytes

TABLE 2

Commercialization of electrochemical devices

Analyte	State of commercialization
Blood Gas	Mature
Electrolytes	Nearing maturity
Metabolites	Emerging
Biomolecules	Germinating

and metabolites will be a profitable growth area for at least the next 5—10 years.

The "final frontier" for electrochemical sensors is the class loosely labelled biomolecules, which includes all analytes not covered in the three classes above. This domain is the largest of all and includes analytes such as hormones, drugs, antibodies, antigens, proteins, enzymes, lipids, amino acids, vitamins and some toxic substances. While offering the most potential for new assays and new markets, this domain offers the biggest challenge to electrochemical sensors. The requirements for high selectivity with extremely low detection levels (10^{-12} M) will not be easy to satisfy. It is in this area that electrochemical sensors will face very strong competition from the alternative technologies just starting to be developed.

The next four sections explore the past, present and future of electrochemical sensors in each of these analyte domains.

Blood gas analysis

The traditional and major use of sensors in clinical chemistry has been for the measurement of the partial pressure of oxygen (PO_2), carbon dioxide (PCO_2) and pH of arterial blood. Currently, approximately 7×10^7 blood gas analyses are done annually in the U.S.A.

In the early twentieth century, Van Slyke and Neill [9a] described manometric and volumetric methods for the determination of gases in blood. These methods were tedious and difficult to perform. Over the years improvements were made; however, the methods remained impractical for routine use. During the 1950s several factors led to the commercialization of the first blood gas analyzer. These were the development of improved gas permeable membranes and more sophisticated electronics, increased awareness of the utility of blood gas measurements, and the development of reliable PO_2 and PCO_2 electrodes.

In 1956 Clark [9b] described a polarographic (amperometric) oxygen electrode. This electrode, still in use today, consists of a platinum cathode fused into a glass rod and a Ag/AgCl anode. The cathode is polarized at −0.6 V relative to the anode. The electrode is isolated from redox and protein interferences in the blood sample by an oxygen permeable membrane, typically polypropylene. Between the membrane and the electrode is a thin supporting electrolyte layer. Oxygen diffuses through the membrane and is reduced at the cathode. The current generated is linearly proportional to the PO_2 of the sample solution.

In 1958 Severinghaus and Bradley [9c] described an improved electrode system for the determination of PCO_2 in blood. This style of electrode is also still in use. A carbon dioxide-permeable membrane, usually silicone, is placed over a pH electrode with a dilute hydrogencarbonate solution entrapped between. Carbon dioxide from the sample diffuses through the membrane and shifts the equilibrium in the thin buffer layer. The resulting pH change is sensed by the pH electrode yielding near Nernstian type response to carbon dioxide over the physiological concentration range.

Current blood-gas analyzers measure the pH, PO_2, and PCO_2 on 60—150 µl blood samples in approximately 3 min. While the electrodes themselves are relatively simple, the overall system design is complex and requires very precise engineering and manufacturing procedures. Two of the main challenges are achieving and maintaining adequate sample temperature (37°C) and preserving sample integrity (loss of gases or contamination). Little has changed regarding these electrodes since their original invention; however, numerous advances have been made in blood gas systems. These include microprocessor-based electronics for improved data acquisition, signal processing and error checking, increased automation, smaller sample size, reduced maintenance, and the development of blood-like quality control samples.

One of the more recent trends in blood-gas analysis has been in the area of continuous or on-line monitoring of the critically ill patient. Transcutaneous or "through the skin" monitoring of oxygen ($tcPO_2$) and, to a much lesser extent, carbon dioxide ($tcPCO_2$) have become popular in the last five years. Modified Clark (O_2) and Severinghaus (CO_2) electrodes have been incorporated into small "button-like" sensors that are applied to well vascularized skin surfaces such as the forearm or stomach. Built-in heaters warm the skin to approximately 44° C to promote the diffusion of capillary blood gases to the surface where they are detected by the electrodes. To date, transcutaneous oxygen monitoring in neonates has been very useful and is the most widely accepted method. The use of $tcPO_2$ and $tcPCO_2$ data for adults is the subject of much controversy because of the poor correlation of transcutaneous data with data obtained by conventional blood gas analysis. Many factors affect the observed data, such as skin thickness, amount of fat, edema and the overall status of the circulatory system. For this reason, transcutaneous measurements in adults may be limited to use as simple trend indicators rather than for quantitative analyses. A related but non-electrode based technique is oximetry, which measures percent oxygen saturation through the skin using optical transmission or reflectance. Widespread clinical use has not yet been achieved; however, interesting advances such as the use of reflectance photoplethysmography may make oximetry more commonplace. In the long run, oximetry may be used to complement existing blood gas analysis for a better overall diagnostic evaluation.

Another type of continuous monitoring is the invasive or in-vivo type sensing. To date, relatively little commercial success has been achieved with electrochemical sensors. While it seems likely that electrochemical devices will play an important future role for in-vivo blood-gas sensing, it is clear that optically based sensors offer many advantages and will be competing for the same market.

Electrolytes

During the late 1950s and early 1960s clinical application of electrochemical sensors was limited to blood-gas measurements. In the mid-60s there was a

sudden increase in the development of new ion-selective electrodes. A major milestone occurred in 1969 with the development of the valinomycin based potassium electrode by Simon et al. [20a]. This electrode exhibited extremely high selectivity over sodium ions and was suitable for measuring potassium in blood. Within 3 years, sodium (glass membrane) and potassium electrodes were marketed as a new technology, replacing the conventional flame photometric method which had been in clinical use for over ten years. Since then the popularity of such Na^+/K^+ measurements has grown each year as evidenced by the plot shown in Fig. 1. Today, potentiometric sensors are available and commercially in use for the four key electrolyte species, Na^+, K^+, Cl^- and total carbon dioxide, and for ionized and total calcium.

All of the electrolyte sensors are potentiometric devices exhibiting a Nernstian (logarithmic) relationship between the potential developed and analyte ion activity. The three major types of potentiometric sensors are:

(a) glass membrane with silicate ion exchange sites (e.g., Na^+, pH);
(b) plasticized polyvinyl chloride-based membranes with neutral carrier binding sites (e.g., K^+, Na^+, Ca^{2+} and pH);
(c) solid state membranes based on insoluble metal salts (e.g., Cl^-).

In each category, the sensing mechanism is due to a selective binding or adsorption mechanism at the electrode surface. As the charged analyte ions associate with the surface sites, a charge separation is established, giving rise to the membrane potential. In the past decade there has been a great deal of research in developing new and improved ionophores or complexing agents for ion-selective electrode applications. However, now that most of the key analytes can be measured by ion-selective electrode, basic research interest in this area seems to have waned. Those ions for which selective ionophores are still needed, namely lithium and hydrogencarbonate (HCO_3^-), represent especially difficult challenges. This has caused many researchers to look to other analyte areas, such as metabolites and biomolecules, as discussed below.

Along with the implementation of sodium and potassium electrodes in the mid-70s came the realization that the ion-selective electrodes could be used

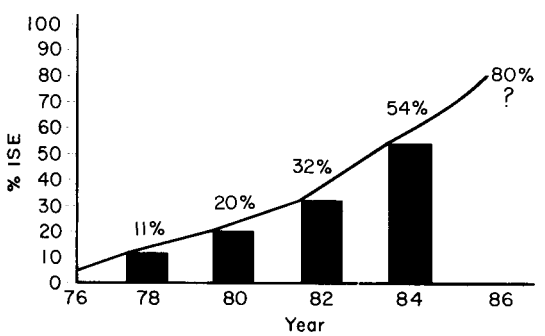


Fig. 1. Percentage of total sodium and potassium hospital assays performed with ion-selective electrodes during the last ten years.

in whole blood, plasma and serum (direct potentiometry; measurement in diluted samples is referred to as indirect potentiometry). Direct potentiometry is attractive because no sample preparation is required and because the assay values are not sensitive to the levels of solids (proteins and lipids) in clinical samples as is observed with diluted-sample techniques.

Indirect potentiometry is attractive because it is well suited for use on high throughput batch analyzers and because, being a dilution technique, it yields data equivalent to that obtained by flame photometry. This is still defined as the reference method for sodium and potassium. During the early years of ion-selective electrode commercialization little distinction was made between direct and indirect potentiometry and this was the source of much confusion on the part of clinicians and physicians. Furthermore, there was considerable debate among the manufacturers and academics as to the impact of activity coefficient and residual liquid-junction potential errors, bicarbonate binding, ion-pair formation, and water activity effects on the direct potentiometric data [21]. While complete consensus has not been achieved on the specific technical issues, it has been proposed that direct potentiometric sodium and potassium analyzers be designed to yield data on normal patient samples equal to data obtained by flame photometry. More importantly, a conscious effort is being made in the clinical laboratory to differentiate between assays obtained by direct and indirect potentiometry. It is now becoming understood that both methods yield data which are useful but not necessarily equivalent.

It is difficult to predict what the future will bring with regard to electrochemical ion sensing. Most of the short term improvements will be in the form of engineering improvements rather than changes in basic concepts. Improved ionophores are anticipated for sodium, lithium, ammonium, and possibly chloride and hydrogencarbonate ions. There continues to be an active interest in commercialization of ion-selective field effect transistors (ISFETs) due to the perceived opportunity for disposable devices for alternate site (non-hospital) testing. However, much work needs to be done before these devices are able to satisfy the many demanding performance standards required for routine use.

Metabolites

The demand for monitoring common metabolites of diagnostic use such as glucose and urea continues to provide the impetus for a major research effort directed towards improved metabolite sensors. Most methods for these two compounds rely on the specific enzymatic reactions

$$\text{glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2$$

$$\text{urea} + H_2O \xrightarrow{\text{urease}} 2NH_3 + CO_2$$

Modern enzyme immobilization techniques allow these reactions to be directly coupled to electrochemical sensors. These probes are typically

referred to as "enzyme electrodes" and consist of a membrane or membranes which possess certain selective permeabilities, an immobilized enzyme (physically entrapped, covalently attached, etc.), and an electrochemical detector.

An enzyme electrode for glucose was first conceived by Clark and Lyons in 1962 [21a]. Glucose oxidase was trapped between two cellulose acetate membranes held closely to the surface of a Clark oxygen electrode. Small molecules such as glucose diffuse into this layer. An inner hydrophobic membrane allows the selective permeation of oxygen to a platinum cathode. The consumption of oxygen in the enzyme layer is monitored amperometrically providing a measurement of glucose concentration. An alternative is to measure the hydrogen peroxide evolved in the enzyme reaction. In this latter case a thin cellulose acetate membrane is placed in front of a platinum anode. Hydrogen peroxide will permeate while interferents such as ascorbate and urate will not. Several membrane configurations have been disclosed; a laminated version was first commercialized by Yellow Springs Instruments. To achieve a rapid analysis time, the cellulose acetate must be extremely thin (1—2 μm). Currently, Japanese scientists are the most active in the pursuit of new membrane geometries and formulations.

To date, wide scale commercialization has not been achieved, probably due to the limited number of analytes for which enzyme electrodes have been fully implemented. These sensors do offer many advantages in terms of manufacturing costs, reagent usage and sample throughput, and it appears that commercialization will occur for a host of analytes in the near future. Other oxidase-based electrodes are listed in Table 3.

A second major class of enzyme electrodes was introduced by Guilbault et al. [21b] for urea assay based on an ammonium ions sensor. The ammonium ions generated in the enzyme reaction are detected at an ammonium ion electrode (nonactin ionophore in PVC membrane). Subsequent refinements included the addition of a gas permeable membrane to eliminate interferences. Similar systems have been studied for amino acids and creatinine.

Enzyme electrodes based on dehydrogenase systems have been reported for ethanol, lactate and malate [21c]. A dialysis membrane was used to constrain both the enzyme and cofactor (NAD^+) near the electrode surface. Electrochemical regeneration of NAD^+ generates a current that is linearly proportional to substrate concentration.

Simplification of the multilayer composite membranes now required would constitute a significant improvement in enzyme electrodes. The manufacture of these membranes is not trivial and the fairly slow diffusion processes associated with these membranes limits their response time and sensitivity. Recent developments in chemically modified electrodes may result in very elegant enzyme electrodes. An interesting example was reported by Hill and co-workers [22]. A ferrocenium derivative which serves as an alternate cofactor for oxygen is deposited onto a graphite electrode. Glucose oxidase

TABLE 3

Oxidase-based enzyme electrodes

Substrate	Enzyme/cofactor	Products[a]
Glucose	Glucose oxidase	Gluconic acid
Uric acid	Uricase	Allantoin
Lactic acid	Lactate oxidase	Pyruvate
Ethanol	Alcohol oxidase	Acetaldehyde
Galactose	Galactose oxidase	Galactose aldehyde
Cholesterol esters	Cholesterol esterase + Cholesterol oxidase	Cholestenone
Pyruvic acid	Pyruvate oxidase, Phosphoric acid, FAD, Thiamine pyrophosphate, Mg^{2+}	Acetylphosphate + CO_2
Amylase	Maltase, Glucose oxidase	Gluconic acid
Maltose	Maltase, Glucose oxidase	Gluconic acid
Creatinine	Creatininase, Creatinase, Sarcosine oxidase	Formaldehyde + Glycine

[a] H_2O_2 is also produced.

is also covalently attached to the electrode. The reaction scheme is:

$$\text{glucose} + 2FeCp_2R^+ \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + 2FeCp_2R$$

As the $FeCp_2R$ is formed due to the enzymatic reaction, it is immediately reoxidized to $FeCp_2R^+$ at the electrode surface. This occurs at low voltages where interference from electroactive sample constituents (e.g., ascorbate) is minimal. This is a very clever approach and is indicative of future trends in metabolite sensing.

Ultimately, schemes will be developed in which there is direct electron transfer between redox enzymes and the sensing electrode, eliminating the need for electron-transfer mediators. Overall, it is felt that the use of chemically modified electrodes for quantitations of metabolites is the most exciting arena for electrochemical sensor research and commercialization in the next 5—10 years. The analyte concentrations are within the sensitivity range for electrochemical detection and good selectivity will be provided by existing or newly isolated enzymes.

Biomolecules

There is little history to present in this section because to date there has been no significant commercialization of electrochemical sensors for biomolecules. Virtually all the approaches that have been published so far remain in the realm of basic research. One of the most popular areas is that of immunoassay. Immunoassays have been developed for many complex biomolecules and therapeutic drugs relying on the antibody-antigen recognition

for selectivity. Radioisotopic methods were first used because of their very low detection limits. However, in recent years alternative methods have gained popularity. Enzyme labeled assays have the necessary amplification and use electrochemical transduction with both potentiometric and amperometric sensors [22a].

Direct potentiometric sensing with an "antibody electrode" has been reported by Keating and Rechnitz [23]. Digoxin was covalently attached to a K^+ ionophore and incorporated into a PVC membrane. After equilibrating this modified K^+ electrode in a K^+ solution, the antibody to digoxin was added as the analyte species. In response, the electrode exhibited a substantial shift in output proportional to the amount of antibody added. Sensitivity down to 10^{-9} M was achieved. This interesting approach, while not yet fully understood or optimized, indicates that electrochemical based immunosensors are indeed possible.

Basic research with a bilayer lipid membrane (BLM) is another promising research area that could lead to an altogether new sensor concept based on conductance. The BLM is well known as a synthetic model for natural cell membranes. When biomolecules interact with specific receptors in cell membranes, the ionic conductance of the membrane changes. Thus, BLMs, carefully engineered to contain selective binding elements (ionophores, enzymes, antibodies, etc.), become capable of sensing biomolecules at low levels. This concept as studied by Krull and Thompson [24] is shown in Fig. 2. The BLM is a sandwich of lipids, typically 6—8 nm thick, and is held together by repulsive forces when immersed in an aqueous solution. An electrical potential is applied across the membrane resulting in a small transmembrane ion

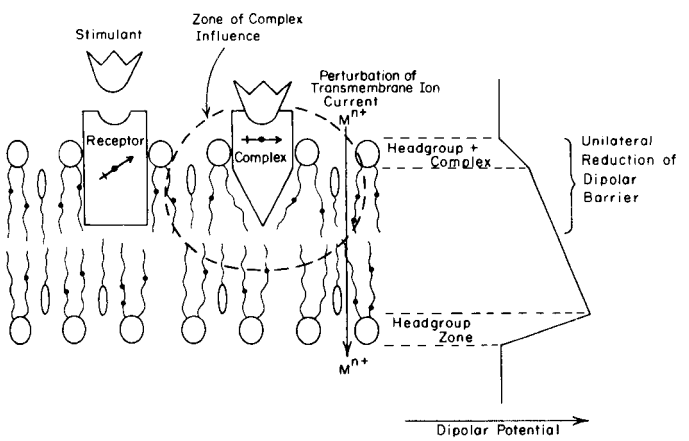


Fig. 2. Illustration of bilayer lipid membrane sensor. The binding of stimulant (analyte) to receptor molecule in the membrane causes large changes in ion permeability through the membrane. This may be the basis for extremely sensitive and highly selective biomolecule sensors. (Reproduced with permission, Trends in Analytical Chemistry, 4 (1985) 90.)

current. Interaction of the analyte with an embedded receptor site causes a perturbation in the BLM conductance resulting in a variation in the ion current. In this manner the biospecific binding event is directly transduced. Because one selective interaction may induce the flow of hundreds of ions, BLMs operate with detection limits of 10^{-8}—10^{-12} M, much below those of traditional sensors. The production of practical devices with clinical utility will rely on future developments to improve membrane stability, to optimize receptor technology, and to integrate the BLMs into an acceptable format. Indeed, this approach offers the hope of developing a "universal" biosensor capable of assaying a multitude of biomolecules by simply using a different BLM receptor for each analyte.

Based on the authors' experience with electrochemical sensors, it can be predicted that the evolution/revolution now underway in sensor development will lead to many new and exciting advances; however, it will take many years before wide-scale commercialization is a reality for all of the diagnostically important species.

ADVANCES AND TRENDS IN COMPUTING, STATISTICS AND QUALITY CONTROL

Computing

Of the developmental trends within clinical chemistry during the past 25 years, perhaps none have been so dramatic as those which have emerged with advances in computer technology [25]. With the dropping prices and increasing availability and sophistication of computer hardware, the field of clinical chemistry has begun to apply computers in everyday laboratory operations, utilizing approaches in statistics and quality control that were considered impractical before adequate computing facilities became so readily accessible.

Clinical laboratory information systems

Many of these revolutionary changes began to take shape with the pioneering applications of computers in early Clinical Laboratory Information Systems. Although these systems were primitive by modern-day standards, utilizing keypunched data and batch processing techniques, they, nevertheless, demonstrated some of the advantages of computerizing the clinical laboratory. Many of these systems were successful in automating the functions of printing blood collection lists and patient reports, the generation of laboratory billing, and preparation of worklists within the laboratory.

A number of groups were involved during the mid-1960s in the development of laboratory instrument computers which became precursors of the modern-day mini-computer. The thrust of these efforts was to develop small computers that could be programmed as dedicated on-line laboratory instrument controllers and data-acquisition devices. Once these functions were determined to be feasible, it was only a matter of time before full-fledged interactive laboratory computer systems, having all the features of the old batch processing systems plus the ability to acquire data directly from

on-line laboratory instruments, became a reality. Although these developmental efforts were initially concentrated in universities, once the commercial potential of these computer systems was realized, they resulted in the formation of several companies that manufactured minicomputer-based laboratory information systems. The report by J. Lloyd Johnson Associates [26] is an excellent compendium of early laboratory information systems.

Modern laboratory information systems have evolved in sophistication and size from these early, interactive, minicomputer-based laboratory systems. The modern system is capable of supporting dozens of on-line terminals, printers, and laboratory instruments and handling all of the admission-discharge-transfer information, request logging, the generation of specimen collection lists and worklists, the performance of automatic results checking, result reporting on a cumulative, interim or stat basis, billing, workload and other administrative reporting, quality control reporting and assistance in result interpretation. In addition, such systems may be interfaced with a wide variety of laboratory instruments and can provide assistance in monitoring the performance of these instruments.

Although laboratory information systems have become quite sophisticated, the further evolution of computer hardware and communications equipment promises to bring even greater changes to the practice of clinical chemistry. Impressive developments in the field of very large scale integration of electronic circuits have led to the availability of extremely powerful microprocessors that have been incorporated into virtually every laboratory instrument. In addition, they have been incorporated into microcomputer systems that have the power and features of small minicomputers but at a very modest price. The power and the low cost of these products has added impetus to the development of distributed processing systems.

Distributed processing. Fundamental to the concept of distributed processing is the sharing of computer workload among multiple processors that are tied directly with one another through a communications network. Several approaches have been taken in implementing a distributed processing system. One approach links together several minicomputer processors in a configuration designed to supply redundancy to the computer system, so that if one component of the system should fail, the other components will automatically detect that failure and take over the function of the defective component. Such systems have been labelled as "fault tolerant" and have been used in applications in which minimizing computer down-time is of critical importance. Several vendors have recently introduced "fault-tolerant" laboratory information systems.

Another approach for implementing a distributed processing system uses groups of microprocessors and other devices that are interlinked by a local area network. This approach has gained considerable popularity with the emergence of personal computers because they can communicate with centralized facilities and databases, as well as other personal computers that are linked into the network. Stand-alone programs that run on the more

popular personal computers have been developed for many applications in the clinical laboratory, and many groups now are working to develop distributed processing laboratory computer systems that consist of several personal computers (microprocessors) interconnected by a local area network. Each microprocessor is dedicated to servicing a particular function and/or laboratory area. Data is accessible for storage and retrieval by any of the microprocessor stations in a central database managed by one of the microprocessors. The appeal of this approach to distributed processing is that it allows the intercommunication of many varieties of computers, ranging from the microcomputer to the mainframe computer, each of which then can be applied to the functions for which it is best suited. It also provides the potential of connecting devices from multiple vendors, thus giving the customer greater flexibility and bargaining power.

Database management systems. The use of a generalized database management system to manage laboratory information is appealing since the operation of a clinical laboratory entails the generation and cataloging of many laboratory results. Numerous such systems have been marketed over the past five years and may be applied to very small research databases or to the problem of managing all of the information produced daily in a large clinical laboratory. There are several advantages to using a generalized database management system including a large reduction in the programming effort required to implement a specific database, flexibility in how the database is structured (or restructured), and the availability of generalized query languages that allow the user to quickly access selected portions of the database for use in solving specific problems. The widespread use of database management systems in the clinical laboratory will overcome many of the problems of inflexibility and inability to easily retrieve information that are experienced by many of the current-generation laboratory information systems.

Advances in telecommunications

The general availability of modems and other telecommunications equipment has led to the evolution of many dial-up services that can be of immense benefit to the laboratory professional in the area of searching and keeping up with the biochemical and clinical laboratory literature. Access to various abstracting services and literature searching services is available through a simple local telephone call in many areas. In addition, telecommunications has allowed laboratory services in widely separated geographic locations to become more efficient through the sharing of common data processing support at a centralized location. Many computer and instrument vendors are now troubleshooting their equipment in the field from their corporate field service headquarters by use of telecommunications gear. Laboratory results are frequently transmitted to a remote location by use of telecommunications equipment. Letters and correspondence can similarly be transmitted to and from any location that has the appropriate telecommunications equipment.

Other trends and future directions

It is outside of the scope of this paper to review in detail the explosive growth in the use of computers and computer applications in clinical chemistry, but there are several areas that merit special mention. The first is the increasing use of robotics and other programmable automation in new laboratory instrumentation. The programmability of the automation allows a given laboratory to exercise a great deal of flexibility in designing various laboratory procedures that are uniquely suited to that laboratory. Once the price of programmable automation equipment becomes more reasonable, there is every expectation that a similar revolution to that which accompanied the personal computer will occur in the application of these devices in the clinical laboratory.

Digital speech input and output devices will be available in the near future that will have a profound effect upon how laboratory professionals interact with computers. It is easy, for example, to visualize a scenario of the laboratory professional dictating a laboratory result to the computer, the computer responding back regarding whether that result fell within acceptable limits and asking for a verification password, followed by the verbal reporting of the result to health care personnel via telephone.

Computer aided instruction will also play a more important role in the training of laboratory personnel. The ability to simulate real bench-level problems and to intelligently interact with the student following branching logic according to the student's choices, distinguishes computer aided instructional methods from other audio-visual approaches to teaching. The computer can be used as an adaptable tutor in teaching basic concepts to students.

Future development of artificial intelligence applications for the interpretation of clinical chemical data appears to be likely. Expert programs for the interpretation of a variety of medical data are already existent [27, 28] and many investigators are working to extend these approaches to a wide variety of problems [29].

Statistics

The application of statistical methods to the analysis of clinical chemical data has grown in parallel with advances in computer technology. The average clinical chemist of 25 years ago needed little more knowledge of statistics than how to calculate a mean and standard deviation. Today, much more sophistication and knowledge of statistics is required. Statistical methods have evolved in two major areas: in the clinical use and interpretation of test results, and in the laboratory performance of tests. Knowledge of the statistical methods used in each area is of vital importance to the clinical chemist.

Clinical use and interpretation of test results

Among the earliest applications of statistical methods in clinical laboratory testing was the development of normal ranges (now called reference values). Although the mean, plus or minus two standard deviations, was the usual

calculation used in early reference value studies, subsequent studies have shown that other statistical methods will in general give superior reference-value estimates because of the non-Gaussian nature of laboratory data [30]. Even with statistically optimal reference values, however, there have been interpretational problems. Harris [31] has reviewed the statistical derivation and the limitations of reference values. Recent studies [32, 33] have explored the use of multivariate reference values as a possible means to overcome some of the limitations of univariate (single-test) reference values.

Because physicians often use laboratory tests to confirm or exclude diagnoses that they are presently considering, it has been necessary to evolve statistical methods for determining optimal decision limits and for evaluating the relative diagnostic values of laboratory tests. The Department of Clinical Epidemiology and Biostatistics at McMaster University [34] has reviewed the methods of receiver operating characteristic (ROC) curve analysis, Bayesian analysis as applied in predictive-value theory, and decision analysis and how these methods may be used as aids for test interpretation. Albert [35] has proposed the use of likelihood ratios in differential diagnosis. This latter approach may have advantages over decision limits because is supplies a relative probability for having a certain disorder rather than allocating a patient into one of two classes (i.e., disease or no disease) based on the value for a single test.

The development of automated classification rules that assign patients to specific diagnostic groups based on their laboratory (and/or clinical) data is frequently an objective in various clinical studies. Decision limits can be applied to single test results, but the correct classification of a patient into the appropriate diagnostic category often requires the results of more than one test. For this reason, several multivariate mathematical approaches have been developed to aid the analysis of classification problems. Most important in this category is the approach of discriminant analysis which has recently been reviewed by Solberg [36]. Other approaches have been reviewed by Boyd [37].

Some statistical approaches have been introduced into the practice of laboratory medicine as a result of the need to reliably detect a medically significant change in a patient's laboratory values for the purposes of therapeutic drug monitoring or tumor marker monitoring. Harris and Yasaka [38] have reviewed recent work in this area.

Laboratory performance of tests. Various statistical approaches have become standard tools in the development, performance and monitoring of routine laboratory procedures. Method development, with the attending problems of method validation and comparison with other approaches for measurement of the same analyte, relies heavily on statistical analysis of experimental data. Commonly used statistical procedures include regression analysis, detection of mean differences by t-tests or by non-parametric rank sum tests, detection of differences in variances by F-tests, response surface optimization techniques for determination of optimal assay conditions, and

curve fitting methods for fitting the dose/response curves of immunoassays and other non-linear methods.

Quality control

The establishment of a comprehensive scheme to monitor assays in both clinical and research applications has been an objective in the evolution of quality control programs for clinical chemistry laboratories. Important components of such programs are the derivation of analytical goals and development of methods for monitoring assay accuracy and precision in order to meet the analytical goals.

Analytical goals

Several methods have been used in assessing the analytical goals for an assay. Some workers have approached the problem of allowable analytic variability by deriving a consensus opinion of medical specialists [39, 40]. Others have specified that the analytic variation should not exceed a certain proportion of the biological variation among individuals [41, 42]. Cotlove et al. [43] felt that the assessment of analytical-precision needs should be based on variation within individuals. Turcotte et al. [44] have suggested that the median cumulative coefficient of variation, obtained in a multiple laboratory comparison study, could be used as a realistic guideline for the minimum precision required of an assay. Regardless of the approach used in deriving an analytical goal, careful monitoring of assay accuracy and precision is the only method for achieving that goal.

Assessment of assay accuracy

It was not until the early 1970s that the problem of achieving analytical accuracy was addressed systematically by workers concerned with the development of a highly accurate method for determining total calcium in serum [45, 46]. This early work, coupled with the joint efforts of numerous national and international agencies has spawned a model for a comprehensive measurement system in clinical chemistry [47] that can be applied in assessing the accuracy of analytical methods.

The model has a hierarchical structure consisting of definitive, reference and field (working) methods, as well as the appropriate reference materials (primary, secondary and control materials). A definitive method reflects the optimal state of the analytical art; it requires sophisticated instrumentation, superior and well defined reagents, and highly skilled personnel; it is performed only in the most reputable of laboratories. The analysis is traceable to base units of measurement and either has negligible systematic error or can be corrected to eliminate any systematic error. The high cost of analysis by a definitive method generally limits its use for any purpose except the development and evaluation of primary reference materials and reference methods.

A reference method is somewhat less demanding technically; it is an analytical method that has thoroughly documented accuracy and precision,

low susceptibility to interfering substances, and has been validated by direct comparison with a definitive method. Reference methods are not intended for general, day-to-day use, but for the evaluation and validation of field methods and secondary reference materials.

A field method is any routinely used analytical method that has been found to have adequate accuracy, precision and specificity for its intended use. Field methods are practical and economical, suited to everyday use in routine service laboratories for measuring analytes in biological fluids. They are also used to assign values to control materials.

A few assays now have completely developed measurement systems with definitive, reference and field methods and primary and secondary standards. Use of definitive and reference methods has yielded useful information about the performance of field methods [48]. Much work remains to be done in the development of accuracy standards for clinical laboratory tests.

An additional method that has been utilized in the assessment of assay accuracy is for laboratories to analyze specimens provided by external agencies. The resources offered by such programs allow laboratories to regularly compare the results of their assays with other laboratories, including reference laboratories.

Assessment of assay precision

Most laboratories routinely analyze serum pools at each analytical run as a means for assessing the quality of analytical results from assay to assay. Interpretation of these quality control data is aided by plotting the data on graphs of the type first proposed by Shewhart [49] and later introduced into clinical chemistry by Levey and Jennings [50].

The frequency of assay of the control materials and the limits or rules to be followed to ensure that the analytical variability of an assay is adequately controlled has been approached by the formulation of statistical control procedures. Recently, Westgard and co-workers [51—53] have done a series of studies that provide a formal statistical basis for the design and implementation of systems of quality control. Many different control rules have been proposed for quality control of chemical measurements. Westgard et al. [51] have assessed the performance characteristics of the more commonly used control rules in terms of their tendency to generate "false alarms" and their ability to detect errors. Based on this analysis, they advised the selection of at least two control rules to be used in combination; one rule that detects random error, and one rule that detects systematic error. In a subsequent study, Westgard et al. [52] evaluated the power of several control-rule combinations for detecting varying amounts of random and systematic error and provided graphs that allow determination of the minimum number of control observations required per analytical run to maintain a desired level of analytical variability.

Although the approach of Westgard and co-workers to designing systems of quality control requires several assumptions and is not perfect, their

studies have shown that, with careful attention to the medical needs of an assay and characterization of the inherent performance of the assay method, rational, statistically-based quality control procedures can be devised that are capable of detecting predetermined levels of inaccuracy or imprecision in assay results.

CLINICAL ENZYMOLOGY

The period 1960—1985 has seen rapid growth in the analytical abilities of clinical enzymologists and in the diagnostic power of enzyme tests. In this section the developments that affected the most commonly measured serum enzymes, beginning in 1960—1961, are considered.

Clinical enzymology in 1960

By 1960, much of the biochemical and clinical foundation was in place to support the ensuing period of rapid growth. A large number of enzymes had been observed in blood, including all of the enzymes that are most commonly determined today. For example, the presence of amylase in blood had been described as early as 1846 and its presence in urine had been reported in 1863 [54], while the presence of alkaline phosphatase in serum had been reported by 1926 [55]. Key reports had described associations of increased enzyme activities with specific diseases. In 1929, Elman et al. [56] described increased amylase in acute pancreatitis and in 1930 Roberts [57] described increased serum alkaline phosphatase in biliary obstruction. In 1936 Gutman et al. [58] described increased acid phosphatase in bone that contained metastatic prostatic cancer. The seminal finding of transiently increased serum transaminase, after acute myocardial infarction, had been reported just before 1960 (in 1954) by LaDue et al. [59]. This was followed by rapid growth in clinical enzymology including the demonstration in 1956 of increased lactate dehydrogenase following infarction [60] and, finally, increased serum creatine kinase following infarction [61]. This latter report appeared in 1960.

In retrospect, the development of clinical enzymology prior to 1960 was limited by several key factors. First among these was instrumentation. It is a tribute to earlier investigators and clinical chemists that progress was made without widespread availability of the analytical instrumentation that today is taken for granted. As Wilkinson wrote [62] in December, 1975; "Perhaps the most important single factor leading to the rapid development of clinical enzymology has been the introduction of the ultra-violet spectrophotometer". The throughput of today's instrumentation exceeds even Wilkinson's 1975 description [62] of instruments "which enable a single technician to make hundreds of determinations in a few hours". The topic of this instrumentation is addressed elsewhere in this review and will not be further discussed here.

Clinical enzymology in 1960 was also limited by problems related to optimization and standardization of assays. Younger analysts today may find it difficult to appreciate that many of the early clinical enzyme assays did

not allow multi-point or continuous monitoring of the enzyme-catalyzed reaction. Important factors that made true kinetic assays possible were the use of modern instrumentation and the development by a host of investigators of coupled-enzyme assays and certain chromogenic substrate assays. The performance of enzyme assays has been further improved by much work and effort devoted to optimization of reaction conditions. The quality of clinical analyses has been improved further through the efforts of numerous laboratory organizations including the College of American Pathologists and the International Federation of Clinical Chemistry. Nonetheless, the clinical enzymologist of 1960 was only slightly worse off than his present day counterpart in the area of standardization. It can be said, however, that most laboratory workers have accepted some definition of an enzyme unit that can be easily related to micromoles of substrate consumed per minute. This acceptance occurred following a time of (well-founded) concern, expressed by Henry [7] in 1964 when he wrote: "Although it is possible that such units will eventually supplant the older arbitrary units, the latter have become so firmly entrenched... that it is unlikely that this will occur easily or soon". Those involved in current efforts at standardization may find some encouragement in reflecting upon progress made in this area during the last 25 years.

Isoenzymes in clinical enzymology

Perhaps the single most noticed development in enzymology has been the introduction of isoenzyme determinations in hospital laboratories. In this writer's view, the past 25 years might be considered a golden age for this field. Biochemical and analytical developments in the 1960s paved the way for these measurements; their clinical acceptance was assured by physicians who were eager to obtain tools that had greater diagnostic specificity than did measurements of total enzyme activities.

The clinical study of isoenzyme measurements, with the exception of occasional reports in the 1940s and 1950s of somewhat selective inhibitors of isoenzymes, seems to have begun in earnest in about 1960. Notable reports in 1961 and 1962 alone included the electrophoretic separation of isoenzymes of lactate dehydrogenase [63], the distinction of mitochondrial and cytoplasmic forms of aspartate aminotransferase [64], clinical studies of isoenzymes of alkaline phosphatase [65], the detection of multiple forms of creatine kinase [66], and electrophoretic separation of acid phosphatase [67]. By 1964, creatine kinase isoenzymes (from heart, brain and skeletal muscle) had been separated on agar gel [68] and the first published demonstration appeared of true separation of pancreatic and salivary isoenzymes of amylase [54]. Thus, separations had been described of all the isoenzymes most commonly measured in serum today.

Many methods have been introduced, by a host of creative investigators, for the specific measurement of isoenzymes in biological samples. Heat stability tests and a variety of inhibitors and activators have been introduced for measurement of various isoenzymes. Although these methods, in general,

have met with only limited success, several new ones have been recently introduced, including a commercially available inhibitor method for the study of pancreatic amylase in serum. Different substrates and various substrate concentrations have been used in an attempt to achieve some degree of specificity for isoenzymes of clinical interest. In general, these have been replaced by other approaches.

Separations of isoenzymes based upon physical properties have been used extensively. Electrophoretic separations have enjoyed wide-spread popularity in clinical laboratories. In these methods, isoenzymes are most commonly visualized in situ following incubation with reagent containing substrate. Limitations have included difficulty in achieving optimal substrate concentrations in the gel and problems with densitometric quantification. Electrophoretic methods that involve gelslicing generally are too cumbersome for routine clinical use. Ion exchange chromatography and related techniques have been used for virtually all of the commonly-measured isoenzymes, but these methods have achieved only limited popularity. The reasons for this are not entirely clear, because throughput and turn-around times can be quite acceptable. The limited acceptance of the methods may reflect the complexity of some techniques and the critical importance of pH, temperature and ionic strength in some other methods. Separations of isoenzymes based upon molecular size have been useful in identifying macroenzymes of various types, including complexes of enzymes with immunoglobulins, lipoproteins or carbohydrates and complexes formed by enzyme self-association.

Immunochemical approaches to the measurement of isoenzymes have achieved remarkable successes. Radioimmunoassays were among the first approaches taken, followed by immunoradiometric assays and related non-isotopic immunoassays. Immunoassays of these types are discussed elsewhere in this review. These approaches allow estimations of concentrations of isoenzymes without reliance on detection of enzymatic activities. This avoids potential problems of inhibitors of enzyme activity, although few examples exist at this time of superiority of immunoassays over activity assays in this regard. Rapid advances can be expected in the ease and precision of these assays, and some immunochemical assays of creatine kinase isoenzymes appear to offer important advantages already.

A second type of immunochemical assay employs antisera (or monoclonal antibodies) to inhibit the catalytic activities of selected isoenzymes or to precipitate them prior to measurement of enzyme activity. For example, creatine kinase isoenzyme-1 activity can be inhibited by antisera prior to measurement of creatine kinase activity in the sample [69]. A variant of this method is specific for isoenzyme-2 [70]. Lactate dehydrogenase isoenzyme-1 can be measured following removal of isoenzymes 2—5 by precipitation using antiserum against a subunit common to those isoenzymes [71]. A similar approach has been described for pancreatic amylase in which other isoenzymes of amylase are precipitated by a monoclonal antibody [72]. Each of these approaches allows the isoenzyme of interest to be measured

under optimal conditions (cf., electrophoresis) with minimal sample dilution (compared to most chromatographic procedures).

The future of clinical enzymology

The accuracy of long-term predictions in clinical chemistry has been singularly unimpressive. The foundation for the advances made during the past 25 years was laid before 1960, but that foundation did not guarantee accurate prediction of what would happen between 1960 and 1985. It may be best to assume simply that the foundation probably exists now for the advances that lie ahead in the next 25 years. It is even possible that advances in fields such as molecular biology and nuclear magnetic resonance imaging will make many of the current clinical enzyme assays irrelevant to patient care. If so, this would be consistent with one time-tested principle: chemical measurements become unimportant to physicians just at the time when adequate analytical tools seem finally to be (almost?) available.

THERAPEUTIC DRUG MONITORING

The past 25 years has seen major advances in drug therapy. One unfortunate consequence of this vital part of medical practice is that drug-induced disease has become a major health problem. The field of therapeutic drug monitoring (TDM) has evolved as one approach toward a more rational drug therapy and the avoidance of adverse side affects.

Only when one considers the current level of analytical sophistication and pharmacokinetic knowledge does the rapidity of these advances become apparent. In the 1960s the realization was just beginning that the plasma concentration of certain drugs was often a better predictor of therapeutic effect than the dose given. Data providing therapeutic and or toxic blood concentrations were available for only a few drugs [73]. The gaps in knowledge resulted from the lack of the widespread availability of suitably sensitive and specific analytical techniques. In the 1970s, TDM was just beginning to make the transition from the research laboratory to the routine clinical chemistry laboratory. Many of these early techniques were modifications of extraction methods developed originally by the forensic toxicologists. Usually massive drug ingestions and the availability of a relatively large sample size allowed the toxicologist to make quantitative measurements with fairly insensitive instrumentation. Unlike the post mortem investigation, clinical TDM had been available for only a limited number of drugs which were given in large doses and attained high plasma concentrations. While it is now of historical interest only, the sulfonamide drugs are a good example of this early type of drug analysis. They were important currative drugs prior to the availability of penicillin and were among the first drugs routinely determined in the clinical chemistry laboratory. Data provided by this analysis revealed that there were well defined therapeutic and toxic blood concentrations which could not be predicted on the basis of dose administered. Perhaps

more importantly, these analyses provided for increased awareness among clinicians of the value of individual drug dosage regimens.

In the late 1960s the foundation of TDM in the clinical laboratories was made possible by the widespread use of gas-liquid chromatography (g.l.c.) which provided superior methods for the separation and quantification of several important classes of drugs. During the next ten years, the technique and instrumentation were greatly improved and g.l.c. was routinely used in many clinical laboratories for TDM. However, by 1979 only one quarter of the laboratories participating in a nationwide TDM quality control program used g.l.c. for their anticonvulsant drug analysis. Even with the advances in sensitive and specific detectors such as the nitrogen-phosphorus detector, photoionization and even mass spectrometers, there was a continued shift toward newer, less complex technology.

The rapid growth of high pressure liquid chromatography in the 1970s undoubtedly accounted for part of this shift in methodology. High performance liquid chromatography currently referred to as simply liquid chromatography (l.c.), is an old chromatographic technique whose rapid rise in popularity coincided with the development of the bonded reversed-phase column packing material. The reversed-phase column technology represented a major breakthrough because of its versatility and stability over a wide pH range and mobile phase composition. The power of the technique for quantitation of drugs in microsamples is still largely unexploited. Microbore columns and more specific detectors will be important factors in the continued role of l.c. in the clinical laboratory. Liquid chromatography will have a secure future for the analysis of newly introduced drugs for which there are no commercial kits available. Despite the continuing large number of publications describing the applications of l.c. techniques [74], in the past two years there has actually been a decline in these applications. In the field of TDM, this recent decline has been the result of satisfying clinical needs with totally new technologies.

Since its introduction over 25 years ago, radioimmunoassay (r.i.a.) has provided the clinical laboratory with sensitive and specific analyses for a wide variety of compounds as is discussed later in more detail. In the 1970s, r.i.a.s for the TDM of digoxin and the aminoglycosides, as well as kits for the screening for drugs of abuse were widely used in the clinical laboratory. Except for a few drugs such as cyclosporine, where few good alternatives exist, r.i.a. has now been largely abandoned for routine TDM because of the inconvenience of the radioactive label, and the availability of non-isotopic immunoassays.

Not all of these non-isotopic immunoassays are new techniques. The introduction in 1977 of the homogeneous enzyme multiplied immunoassay technique (EMIT) for the semi-quantitative analysis of urine for drugs of abuse was soon followed by similar quantitative EMIT techniques for a number of anticonvulsant drugs. The major advantages of this system are the small sample requirements and simplicity. Precision has improved while

reagent cost and analysis time have decreased as these reagents have been adapted in recent years to automated and semi-automated clinical analyzers. In 1979, by some estimates, more than half of laboratories involved in anticonvulsant TDM used some form of enzyme immunoassay.

About this same time immunoassay procedures for drug analysis using fluorescent labels became commercially available and in the past five years have captured a considerable portion of the TDM reagent and instrumentation market. Fluorescent immunoassays for TDM are all based on similar binding principles but differ in instrument requirements and the need for separation of "bound-" from "free-" labeled drug [75]. In the latter heterogeneous assays, the antibody is immobilized on polyacrylamide beads or on glass fiber mats. The homogeneous fluorescence immunoassays do not require such a separation step but they do differ in their fluorescent instrumentation requirements. Quenching and substrate-labelled fluorescence immunoassays can be measured with most fluorometers while fluorescence polarization requires a polarization analyzer dedicated to TDM.

There are several additional variations of fluorescence immunoassays that have been described that may be useful for TDM in the future. As the immunoassay portion of these techniques increases in selectivity and new, more sensitive labels (e.g., chemiluminescent) are developed, these techniques will continue to be important for TDM for several years to come.

In this brief discussion it is only possible to highlight some of the technological advances seen in the field of TDM. This sub-specialty of clinical chemistry is an area where the rapid evolution of both new instrumentation and technology continues. The trend today is certainly toward automation and rapid test turnaround. How the recent health-care cost containment changes will affect the future of TDM is uncertain. If the analytical advances of the next decade continue at the rate of the past decade, the potential for increasing the quality of the clinical practice of therapeutic drug monitoring is enormous.

THE APPLICATION OF IMMUNOASSAYS

An entirely new era in clinical chemistry has emerged since the discovery by Yalow and Berson in 1960 [76] that antibodies could be used for saturation isotherm analysis. In this pioneering work the first r.i.a. was developed to measure serum insulin by allowing labeled and unlabeled insulin to compete for antibody binding sites. Prior to the first r.i.a., analytes present in small quantities in biological fluids could only be measured by bioassay. Bioassays were at best only semi-quantitative because they relied on the physiological response to an injection of the analyte into laboratory animals. Since the development of r.i.a.s, it has been possible to measure a wide variety of analytes in virtually any biological fluid. Many of the analytes measured by r.i.a. have characteristics such as a high degree of molecular complexity or they exist in such small quantities that their measurement by conventional

chemical isolation becomes impractical. Radioimmunoassays have such a high degree of sensitivity that they remain unrivalled by any other technique to date, with the exception of some modern variants of a r.i.a. This section traces the evolution of r.i.a.s into the realm of clinical immunodiagnostics.

The evolution of the immunoassay can conveniently be illustrated by tracing the technological progress that has been made with the determination of human chorionic gonadotropin (hCG). The determination of hCG has presented some of the most challenging complexities to researchers developing immunodiagnostics. The earliest r.i.a. methodologies to isolate and measure hCG, which followed standard immunization protocols, were inadequate. The antisera for glycoprotein hormones displayed cross reactivity and nonspecificity; these were primarily attributable to the lack of purity of the immunogens used to elicit antibody production [77]. Other specificity problems were attributable to structural homologies between different antigens. For example, hCG is a placental glycoprotein hormone which exits as a dimer of dissimilar subunits. The alpha subunit has a high degree of structural homology with the pituitary glycoprotein hormones, LH, FSH, and TSH. The beta subunit, on the other hand, has only structural homology with the amino terminus of LH [78].

Other approaches to improve the specificities of r.i.a.s which were developed during the late 1970s were attempted for the analysis of hCG. First, because the antisera for hCG consisted of a mixed or polyvalent population of antibodies directed at a variety of epitopes including LH, the interfering LH antibody was absorbed using an hLH-Sepharose affinity column [79]. This method of specific absorption of interfering antibodies was effective in improving assay specificity but had the additional cumbersome purification step and markedly reduced the yield of available antibodies. For routine clinical use, long term batch-to-batch consistency of antibody production was difficult to achieve when different animals were used for immunization.

The advent of more sophisticated purification and isolation techniques for antigens in the early 1970s improved markedly the specificity of the antisera produced during this time. In 1972 when a highly purified preparation of the beta subunit was used for immunization by Vaitukaitis [80], the specificity for measuring hCG improved, but the cross reactivity with LH was not completely eliminated. In 1979, the carboxy-terminal region of the beta chain was isolated which did not demonstrate any homology with the interfering pituitary hormones. This peptide was used as the immunogen to produce antisera with a high degree of specificity for hCG [81]. Coincident with the development of this highly specific r.i.a. for hCG were the development of variations of the r.i.a. technique which would make even greater improvements in specificity and convenience of assay, but first large amounts of homogeneous antibodies had to be available.

In the last decade, a major improvement in the immunodiagnostic field occurred with the pioneering work of Milstein and Kohler [82] and the

development of hybridoma techniques of monoclonal antibody production. Monoclonal antibody production is based on the careful selection of a lymphocyte that secretes antibodies which possess the highest specificity and affinity for the analyte being determined. Antibodies are selected for a variety of useful characteristics such as affinity, lack of cross reactivity, ability to be labelled, and yield. This particular lymphocyte is then effectively immortalized by fusion with culturable plasmacytoma cells. The selected hybridoma can be cultured and produce essentially pure, homogeneous antibody indefinitely.

The use of monoclonal antibody technology in the measurement of hCG falls into two main categories of analytical techniques, analyte-observed or reagent-observed. In the analyte-observed method, the residual labelled hCG is measured after steady state has been achieved and antibody bound hCG is removed. This approach constitutes the classic r.i.a. type procedure where unknown analyte is incubated in the presence of known amounts of labelled analyte and specific antibodies. A second but closely related technique measures the distribution of antibody after the reaction with the analyte. Immunoassays of this type are referred to as immunometric assays because the antibody carries the label. Modern assays for hCG have been developed which have employed both types of analytical techniques. The immunometric configuration yields an assay which has its best performance characteristics in the lower analyte region where a r.i.a. performs less satisfactorily, hence the immunometric assay is generally the method of choice.

Modern immunometric assays for hCG have been developed which rely on the use of several monoclonal antibodies directed at a variety of epitopes on the hCG molecule. The two-site immunoradiometric assay developed by Schimizu et al. [83] relied on the binding of the alpha subunit of hCG to monoclonal antibodies immobilized to a plastic bead. The solid phase was designed to have essentially infinite capacity for hCG in the range of clinical concentrations. A second radiolabelled monoclonal antibody directed against the beta subunit of hCG then was added forming a sandwich with the hCG in between the two antibodies. The relative merits of immunoradiometric assays are many. Labelled antibody techniques can achieve sensitivities several orders of magnitude greater than r.i.a. Factors which alter the thermodynamics of steady-state reactions do not affect immunoradiometric assays. The dynamic range of the assay is often several fold greater than by r.i.a. Finally, reaction times are faster at lower analyte concentrations in the immunoradiometric assay.

Other changes in the field of immunodiagnostics have been aimed at convenience factors instead of improving overall assay performance. For example, hCG assays are offered with a variety of non-isotopic labels in an effort to reduce the cost of reagents, instrumentation, and to reduce inconveniences in handling radioisotopes. Some institutions are already adding radioactive waste disposal costs to analytical procedures employing isotopic labeling. A significant improvement in reagent shelf life has been realized with the use of non-isotopic reagents. Enzymatically labelled immunoradiometric assays

have been commercially available for hCG during the last several years with assay performances which are equal to their radioactive counterparts.

The future of immunodiagnostics will bring techniques which will concentrate on improving sensitivity in non-isotopic assays. As indicated in the section on drug monitoring, two methods, fluorescence and luminescence, hold some promise in achieving sensitivities at least as good as radioisotopes. Fluorescence quenching with proximal linkage or simply the proximal linkage of enzyme substrate pairs should open up new areas for immunodiagnostics [84, 85]. Homogeneous assays will be possible through these techniques in which separation steps are no longer required. With careful monoclonal antibody selection and production techniques, combined with modern detection methods, an increased availability of immunoassays for many clinically relevant antigens is expected.

TRACE ELEMENTS

Eleven elements (carbon, hydrogen, oxygen, nitrogen, sodium, calcium, potassium, magnesium, chloride, phosphorus and sulfur) comprise more than 99.9% of the human body. The remaining 0.1% of body weight is made up by the so-called "trace" elements; at present these include the essential elements iron, copper, zinc, manganese, cobalt, chromium, selenium, silicon, nickel, molybdenum, iodine and fluorine. Also included are aluminum, scandium and titanium, which have no known essential biological function but are nevertheless ubiquitous in living tissue. A complete review of advances in the analytical chemistry of these metals over the past 25 years could fill several volumes. This short review focuses on those innovations that have had the greatest impact on trace metal analysis in the clinical chemistry and toxicology laboratory.

Prior to 1960, and indeed well into the 1960s, clinical analyses of trace metals were limited by matrix complexity and availability of instrumentation. Spark- and arc-source spectrographic methods were available and substantially free from interferences but instruments were quite expensive and precise quantitation, an absolute requirement for clinical assays, was very difficult [86]. Complexiometric methods, combined with u.v./visible spectrometry were widely available and economical, but quite susceptible to matrix interferences [86]. Despite these difficulties, the essential nature of iron and cobalt to animals was demonstrated in the early 1900s, and they were soon joined by zinc, copper and manganese as essential trace elements [87]. The 1950s saw the addition of molybdenum, selenium, and chromium to this list [87]. Since 1960, tin, silicon, fluorine, nickel, vanadium, and arsenic have each, at times, been considered essential although at present only silicon, fluorine, and nickel are considered essential [87] to human life. It was not until the late 1960s, however, that instrumentation was available to make the clinical assay of many of these elements feasible.

In 1955, Walsh [88] first described the method of flame atomic absorption spectrometry, and this has since become the most widely used method

for determination of metallic elements. Approximately ten years later, the nitrous oxide/acetylene flame was introduced for atomic absorption applications [89], and electrodeless discharge lamps were introduced some years later [90]. Flame atomic absorption spectrometry has thus become an integral part of clinical chemistry and toxicology laboratories, having sufficient sensitivity for determination of most essential elements at concentrations of 0.1 $\mu g\ ml^{-1}$ or greater [91]. Lower detection limits have been obtained for certain trace elements by generation of the volatile metal hydride prior to atomic absorption measurement [92]. This method has been applied to the measurement of antimony, arsenic, bismuth, mercury and selenium.

The most widely applicable innovation in atomic absorption spectrometry has been electrothermal atomization, first described in 1961 by L'vov [93]. Electrothermal atomization lowered the limit of detection for many metals to less than 10 $\mu g\ l^{-1}$ [91], making possible the direct quantitation of such metals as aluminum and nickel in serum. Microprocessor control of furnace-temperature programming and automatic sampling systems have also simplified this method, thereby increasing its range of application in the clinical chemistry laboratory [94].

Flame atomic emission methods, while suitable for the alkali metals, are not sensitive enough for most metal analyses. The recent development of inductively coupled plasma emission spectrometry has, however, rejuvinated interest in atomic emission techniques. Sensitivities approaching those obtained with electrothermal methods are available for many clinically relevant trace elements [91]. As instrument costs are lowered, inductively coupled plasma instruments are likely to have a major impact on multi-element trace analysis in biological samples.

Neutron activation analysis is a versatile method that, while not available to most clincial laboratories, is widely applicable to quantitation of trace metals in biological specimens and its applications are certain to widen the understanding of the metabolism and distribution of trace elements in humans in the future, as it has over the past two decades. Similarly, x-ray and laser microprobe methods have made determinations of localized trace elements within cells possible, and contributed to an understanding of metabolism and toxicity of trace metals in the microenvironment of living tissue. These analytical tools, when coupled with the growing availability and applicability of atomic absorption and emission methodologies, provide a sound basis on which research directed toward an understanding of the unique roles trace elements play in human health will be based.

THE ADVENT OF NUCLEIC ACID PROBES

Since the description of the DNA double helix in 1953 by Watson and Crick [95], a phenomenal amount has been learned about the genetic organization of life and the molecular biology of disease. Introductory texts

[96, 97] and reviews [98] are available for a comprehensive overview of the field of recombinant DNA. In surveying the past 25 years of progress in this field, the focus of this brief review will be nucleic acid probes and their clinical applications.

Theoretical principles

Nucleic acid probes (or simply probes) are segments of labelled nucleotides arranged in a specific sequence. This sequence is complementary to that of the probe's target (another strand of DNA or RNA) in the preparation under study. Complementarity is the specific base pairing which normally occurs between the two strands of the DNA double helix or between DNA and RNA. Adenine pairs with cytosine (in DNA) or uracil (in RNA) through two hydrogen bonds, while guanine pairs with cytosine through three hydrogen bonds. When the DNA double helix is heated in solution, these hydrogen bonds break forming two single strands of DNA (a process called melting). These strands reanneal due to their complementarity when the solution is cooled. By providing an excess of probe, the formation of native DNA-probe hybrids is favored. This hybridization is detected by developing the label appropriately.

While the specificity of this hybridization can be exacting, it may be modulated by the stringency of the hybridization conditions. The variables here include temperature, salt concentration and formamide concentration (an agent which lowers the melting temperature of the DNA double helix). Probe length also affects specificity; long probes may cross-hybridize to imperfectly complementary partners because the favorable free energy of the matching pairs overcomes the unfavorable mismatches. All other factors being equal, the most complementary probe will continue to form a stable hybrid at the higher temperature.

Probe synthesis

The approach to preparation of probes is dependent on what is known about the DNA (gene) or protein (gene product) under study. If the sequence of the protein is known, an oligonucleotide (e.g., 14—20 bases long) probe can be synthesized (see [99] for a review). The sequence of nucleotides is selected for synthesis based on an amino acid sequence which is unique to the protein and which has minimal degeneracy (i.e., is coded for by only one or two codons). These short probes can be used to search for more complete gene probes (see below) or can be used in their own right. An oligonucleotide probe allows a 5—10°C [100] difference in dissociation temperature, T_d, which is dependent on probe length, total G—C content, and most importantly, the site of the mismatch (ideally near the center of the probe).

Another approach to probe synthesis is to isolate the mRNA from a preparation producing the protein of interest in large amounts. This isolation can be achieved using a poly-T chromatography column which binds the poly-A tail of mRNA. The isolated mRNA can be used to make full length cDNA (complementary DNA) copies of the mRNA [101].

If the location of a gene is known (through linkage studies) to be near a gene for which a probe already exists, chromosome walking can be used to find an insert which contains the known gene and the desired product, using either oligonucleotide or cDNA probes in a colony hybridization [102]. This method is used to obtain a probe or for other recombinant work. Genomic DNA is isolated, cut into fragments using restriction enzymes, combined with a vector (a plasmid, phage or cosmid), and bacteria are infected. Using appropriate selection techniques, the vectors which contain the appropriate insert of genomic DNA can be detected, will multiply and can be isolated for use.

Probe labeling. Since its beginnings, radioactive nucleotides have been used in modern molecular biology. The use of ^{32}P remains the most common and sensitive isotope, although its short 14.3 day half-life is a disadvantage. Other radioisotopes used include ^{3}H, ^{14}C, ^{35}S, and ^{129}I. The radiolabel is incorporated by nick translation [103] using DNA polymerase I, and its presence is detected by autoradiography.

Within the last four years, biotinylated probes have been developed [104]. Biotinylated probes can be detected using streptavidin bound to an enzyme such as horseradish peroxidase, alkaline phosphatase or acid phosphatase, which allows development of a colored reaction product. Alternatively, an antibiotin antibody can be used [105]. At present, biotinylated probes are two or three orders of magnitude less sensitive than ^{32}P labeled probes [106] but compounds such as photobiotin may improve their sensitivity [107].

Rather than label the probe itself, it is possible to use either type of label on a probe directed against the vector used to replicate the first probe and to allow some portion of that vector to remain with the complementary probe. This sandwich method [108] has the advantage that several different primary probes can be detected, while only the secondary (vector) probe needs to be labelled.

Signal to noise ratio

A number of manoeuvers are routinely used to decrease the signal to noise ratio. Prehybridization of the nitrocellulose filter (see below), using a solution containing carrier DNA, decreases non-specific binding to the filter. Stringent washing also decreases non-specific binding. Intensifying filters help increase the signal generation from a given decay in radiolabeled preparations.

The best signal for blots with biotinylated probes is obtained using alkaline phosphatase linked to a poly-A probe which is directed against a poly-T tail added to the probe for the sequence of interest [109]. Another method of increasing the signal is the formation of probe complexes which are possible when a portion of the vector is left attached to the actual probe [110]. The use of single-stranded RNA probes followed by post hybridization treatment with RNase to remove non-hybridized RNA [111] is also a useful method for reducing non-specific background.

Blotting techniques using probes

Several blotting techniques have evolved since 1963 when Nygaard and Hall [112] first noted the ability of nitrocellulose to immobilize DNA. An excellent review of many aspects of blotting is available [113] so most types will be mentioned only briefly. The most exacting and widely used technique is known as Southern blotting [114]. Briefly, DNA extracted from cells is digested using one or more restriction enzymes (which cleave the double-stranded DNA at known sites) and electrophoresis separates these fragments based on molecular weight. The actual Southern blot is an exact replica of the electrophoretic gel which is transferred to a nitrocellulose (or nylon) filter. This filter is dried and subsequently used for hybridization to detect the sequence of interest. A similar technique for RNA [115] is commonly referred to as a Northern blot. Simple detection of the presence or absence of a particular viral DNA can be performed using techniques known as dot blot, cytoblot or quickblot.

In situ hybridization

Detection of specific DNA or RNA sequences is possible in situ using probes, and has been recently reviewed by Haase et al. [116]. Applying the probe to the deparaffinized proteinase treated tissue sections or to tissue culture cells allows histologic detection and localization of viral genomes or mRNA. Localization of a gene to a particular chromosome is also possible using a similar technique [117].

Clinical applications

While the techniques for producing and utilizing nucleic acid probes are relatively new, many examples of their clinical utility have been described. Prenatal diagnosis of various genetic disorders is possible with a high degree of accuracy, especially for the various haemoglobinopathies [118] and single base pair mutations [119]. The presence of virus in antigen-negative [120] or culture-negative [121] patients is also possible, often within 48 h. Using probes to detect the presence of immunoglobulin gene rearrangements [122] allows detection of a monoclonal population of lymphoma cells before malignancy is histologically evident. The use of oncogene probes to diagnose and understand cancer also holds promise [123].

The future

Advances made in the past 25 years have been impressive in adding to the understanding of the molecular biology of disease. While the techniques are currently labour-intensive, sometimes slow and time consuming, continued advances, especially in non-radioactive probes and automation [124], should aid in making these powerful techniques more widespread. The characterization, standardization and application of nucleic acid probes and the techniques to use them will require application of the principles of analytical chemistry that have been used with great success in clinical chemistry during the past 25 years.

CONCLUSION

Clinical chemistry has seen dramatic changes over the past 25 years. Several techniques not ever imagined in the early 1960s are now commonplace in routine laboratories. The need for automation, speed and reliability means that the major emphasis has been on relatively simple detection techniques with more complex reagent systems.

The authors look forward to even more exciting developments as we enter the twenty-first century. Better diagnostic tests are needed in the areas of hypotension, cancer, neurological diseases, nutrition, prenatal diagnosis, infectious diseases, etc. New developments will require the skills and innovations characteristic of analytical chemistry.

This article comprises several reviews covering important areas of development in clinical chemistry. The contributors were:

Advances in instrumentation and reagent systems: J. Savory, M. R. Wills
Electrochemical sensors: J. D. Czaban, K. F. Coffey, K. M. O'Connell
Advances and trends in computing, statistics and quality control: J. C. Boyd
Clinical enzymology: D. E. Bruns
Therapeutic drug monitoring: J. R. Shipe, Jr.
The application of immunoassays: R. A. Felder
Trace elements: R. L. Bertholf
The advent of nucleic acid probes: M. Lovell

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LIQUID-LIQUID EXTRACTION IN INORGANIC ANALYSIS: CURRENT STATUS AND PROSPECTS

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SUMMARY

This review surveys the progress in inorganic liquid-liquid extraction in the last 25 years. Achievements are emphasized in the theoretical foundations of the method, and in the synthesis, characterization and application of new extractants. Special attention is paid to the implementation of extraction methods and to the automation of extractive separation. The combination of liquid-liquid extraction as a method of preconcentration with the methods of subsequent determination of trace concentrations of elements is described in detail.

Liquid-liquid extraction constitutes one of the most important methods of analytical separation and preconcentration. Separation and preconcentration as such are almost never objectives in chemical analysis. These are essentially auxiliary processes employed when direct methods cannot, for some reason, achieve the objectives of the analysis: identification of components and determination of their amounts. The spectrum of direct methods constantly widens and their analytical characteristics are constantly improved, so that it would be logical to predict a decrease in the importance of separation for analysis. Actually, the opposite trend seems to prevail, being caused by at least two factors.

The first is well known. A number of multicomponent samples cannot be analyzed as such by direct methods: either the methods have insufficient sensitivity, or interference from some components is too great, or the distribution of components in the sample is inhomogeneous, or calibration is difficult because of the absence of reference materials. Separation and preconcentration are techniques that overcome these difficulties.

The second factor needs to be discussed in more detail. It lies in a certain change within the methodology of analysis. For decades, if not for centuries, it was a tradition to separate a mixture into its components and detect or determine each component by using more or less the individual properties of this component, e.g., its ability to form a typical residue or coloured solution, or to give the flame a certain colour. This methodology governed, for example, all programs of qualitative analysis, as well as most schemes of quantitative inorganic analysis such as the analysis of geological samples.

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Gradually, however, a new ideology was growing among analysts, holding that it was possible to find universal methods of identification of the separated components. In other words, it is possible to find such properties that are typical of all components but have a specific magnitude for each. The use of these properties dramatically simplifies detection, by eliminating the necessity of looking for some characteristic manifestation of each component. An example of such a universal property is the retention time in chromatography.

A similar approach also proved to be effective in quantitative analysis. After a clear-cut separation, all components can be determined on the basis of the same property, not of different properties of different components. The essential characteristic is that the extent to which a component exhibits this property, i.e. the magnitude of the analytical signal, is a certain function of the concentration of this component. Among such universal, non-specific properties are the electrical conductivity of a hydrogen flame after the introduction of almost any organic substance, the thermal conductivity of gases, and the electrical conductivity of solutions. This gave birth to analytical instruments (detectors) that are seldom used independently but typically function in a single unit with a separating device.

While formerly analytical chemistry turned its back on non-selective determination techniques, the new situation resulted in renewed interest in them. The less a technique is selective, naturally the more universal it is found to be. Separation grew to form an integral part of the methods of the new type, in which the separation of mixtures, the identification of components and the determination of their amounts ceased to exist in isolated stages (which they were before, and still are in other methods) but evolved to form the parts of a whole. Gas and high-performance liquid chromatography are among such "hybrid" methods. The appearance and development of such methods constitutes the second factor that attracts attention to the separation of mixtures and to preconcentration. This does not mean, of course, that the selectivity problem is a thing of the past. In fact, it remains of paramount importance for many methods of analysis.

Extraction has not yet reached the degree of universality and automation achieved in chromatography, but, nevertheless, it is widely used in direct combination with determination techniques. One example is the widespread combination of metal extraction with subsequent flame atomic absorption spectrometric (a.a.s.) determination of the metal directly in the extract.

Extraction has a long history, which need not be detailed here. However, the summarizing papers by Irving [1] and Kuznetsov [2], which attempt to systematize the available data on extraction, appeared as late as the 1950s. In 1960, Diamond and Tuck [3] published a classification of liquid-liquid extraction systems which served as a basis for subsequent, more general and rigorous classifications [4, 5].

Extraction proved its usefulness especially well in the years after the Second World War, mostly after the middle 1950s. Nuclear technology

provided a powerful impetus to the development of liquid-liquid extraction techniques. Among other things, it led to the development and wide utilization of new, phosphorus-containing extractants, especially tributyl phosphate, as well as high molecular weight amines. Later these extractants proved to be useful for analytical chemistry. Other extractants travelled in the opposite direction, from analytical chemistry to industry, e.g., chelate-forming reagents (hydroxyoximes, 8-quinolinol and its analogues) and to some extent, salts of quaternary ammonium bases. Many of the principal aspects of the chemistry of liquid-liquid extraction were not understood at this time when its applications were already fairly widespread; it is clear that some of the processes were misinterpreted. The period of intensive research that followed in 1960—1985 clarified the chemistry of most of the important extraction processes. The pace of development in extraction techniques is still high.

The scope of applications of liquid-liquid extraction has greatly widened in the last 25 years and hundreds of new systems have been introduced. As a result, a fairly consistent theory of extraction has evolved. The current classification principles make it possible to "compartmentalize" vast amounts of data. Nevertheless, a new problem has taken shape: that the behaviour of metals in utilized extraction systems is so diverse that much further research is needed to learn how to choose a priori the optimum extraction systems for the recovery of a specific metal. Another problem to be faced is the constant proliferation of combinations of extractive separation with various determination techniques. General principles are thus required in order that liquid-liquid extraction as a method of sample preparation can be matched to determination methods, and that the adequacy of the separation and determination techniques can be assessed. Consequently, it is necessary to assess the behaviour of extracts under the determination conditions in order to be able to optimize the methods and to implement automatic procedures.

The problems of extraction methods in analytical chemistry have often been discussed [6—15]. The present review aims both at evaluating the current state-of-the-art in liquid-liquid extraction as a method of separation and preconcentration, and at discussing the promising directions of development.

THE THEORY OF LIQUID-LIQUID EXTRACTION

Liquid-liquid extraction is a complicated physicochemical process, in spite of the simplicity of its implementation. The method cannot be used efficiently unless the chemistry of the processes inherent in the extraction is profoundly understood.

What is the ultimate objective of the theory of extraction? Simply, it is to instruct how to select systems and conditions that ensure complete and selective extraction of a component. The objective, formulated so plainly, is achieved, if it is achieved at all, only with difficulty. The theory of liquid-liquid extraction involves chemical thermodynamics, theory of solutions and

chemical kinetics, and makes use of the achievements of organic and, especially, coordination chemistry.

The theory thrives on the understanding of the most important and practicable extraction processes, on the ability to discern order in their diversity, and on the above-mentioned possibility of classifying them under certain attributes. A classification is a cognitive tool, but it is also, and to a greater extent, a result of cognition. A list of extractants is given below which helps to organize the experimental data. The list contains two large groups, viz. neutral compounds and ion association compounds; 6–7 groups of compounds can be distinguished within these groups.

Neutral compounds

(1) Coordinatively unsolvated neutral compounds, such as $HgCl_2$, AsI_3 and OsO_4.

(2) Uncharged metal chelates.

(3) Coordinatively solvated neutral compounds, such as $AuCl_3L$ and $UO_2(NO_3)_2L_2$ where L is a molecule of an uncharged extractant bound within the inner coordination sphere of the complex.

Ion association compounds

(4) Coordinatively unsolvated ion association compounds of the type Kat^+An^- where Kat^+ and An^- are the large, hydrophobic cation and anion, respectively, with predominantly electrostatic bonding between them.

(5) Mineral (strong) acids.

(6) Complex (metal-containing) strong acids, such as $HGaCl_4$, $HAuBr_4$, $HNbF_6$; heteropoly acids can also be included.

(7) Various compounds not included in groups 1–6.

Three aspects of the theory deserve special emphasis.

Coordination chemistry of liquid-liquid extraction

The above list of groups of compounds demonstrates that inorganic compounds employed for extractive separation and preconcentration of metals are, with rare exceptions, complex compounds. Complex formation in aqueous and non-aqueous solutions is indeed a decisive factor for the efficiency of extraction. Correspondingly, one of the most fruitful developments in the theory of extraction has been the incorporation of the achievements of coordination chemistry. This is especially important for resolving selectivity problems, which have grown to be the main problem in this field.

Complexation in extraction systems has been the subject of extensive research. The results obtained have helped to establish the mechanisms of many extraction processes, and in some cases even to predict the type of compound that must form in the system and to evaluate the conditions for selective extraction. Let us consider several examples.

Extractable halide and related complexes are coordinatively unsolvated species MX_m (M^{m+} is the metal ion, X^- is a halide or similar anion), coordinatively solvated compounds MX_mL_n (L is the extractant), or complex

anions MX_{m+n}^{n-} incorporated as ion association compounds. It is possible to explain why and which complexes of these types are formed by using the principle of soft and hard acids and bases [16—19].

A simple estimate of the possible coordination numbers of the central metal atom and of their involvement in extraction is often useful. For instance, coordination aquation of inorganic complexes has been studied; the results indicated the possibility of extraction of intermediate compounds, hydrated in aqueous solutions, such as lanthanides and trivalent actinides [20, 21]. Thus, lanthanides are extracted from chloride solutions by amines as singly and doubly charged tetra- and pentachloride complexes, respectively. As the coordination numbers of the lanthanides are high (i.e., not less than six) it is logical to expect that the complexes will be aquated. If this is true, the degree of extraction can be increased by adding to the system a second extractant capable of replacing water in the inner coordination sphere. An example of such a compound is tributyl phosphate. This prediction was tested and it was found that a mixture of a cationic and a neutral extractant does give a considerable synergic effect, and the distribution coefficients are increased. This new type of synergic effect was employed to increase the efficiency of lanthanide and actinide extraction from chloride and nitrate solutions.

Knowledge of coordination chemistry is especially important for the extraction of chelates. The composition, charge and coordination saturation or unsaturation of a chelate can often be evaluated a priori from four parameters, viz. the charge on the central metal ion, the coordination number of the metal ion, the number of ionogenic groups in the reagent molecule and the identity of this molecule [22]. These parameters can be used to formulate the conditions of formation of charged chelates. A number of separation procedures for the extraction of metals using cationic and anionic complexes of the types outlined have been developed [23]. These complexes can be extracted by adding hydrophobic counterions to the system [24].

Mutual effect of metals

Some time ago the absence of conjugated processes, similar to coprecipitation or to azeotrope formation in distillation, was regarded as an advantage of liquid-liquid extraction. It has been proved, however, that such conjugated processes (coextraction or suppression of extraction) are widespread and are observed in practically all important extraction systems. It can be stated that the mutual effect of metals in liquid-liquid extraction is one of the attributes of the process and as such can be controlled and used. This constitutes a significant success for the theory of liquid-liquid extraction. The most progress was achieved in studying the interaction between ion association species, as for complex metal-containing acids extracted by oxygen-containing or amine extractants.

The reason for coextraction in this and similar cases is the formation of mixed ion aggregates in the low-polarity organic phase. The suppression of

the extraction of a trace element by an extracted major element is caused by the dissociation of the extracted ion association compound in the extract (provided the polarity of the solvent is sufficiently high) and by the manifestations of the common-ion effect. The cases described above involve the extraction of complex acids, so that the common ion is the hydrogen ion. If the system contains a second element, and if its amount predominates and its compound dissociates in the extract, this compound acts as the source of hydrogen ions in the organic phase; correspondingly, the distribution coefficient of the trace element is decreased [25—27].

Extraction kinetics

In recent years the study of extraction kinetics has gained in importance. This is caused by wider use of extraction equipment with a short residence time of the phases in the mass transfer zone and with the possibility of separating components by differences in their extraction rates. Furthermore, such kinetic studies yield information about the mechanisms of reactions.

Substantial improvement took place recently in the experimental equipment used for studying extraction kinetics. Experimental procedures play a decisive role in this field. For example, instruments are already available that enable the diffusion and kinetic stages, and the bulk and surface reactions to be distinguished, thus allowing them to be studied separately [28].

Among the important results of the latest research is the finding that many chemical reactions producing extracted compounds take place not in the bulk of the aqueous phase but at the interface [29]. Fast surface reactions were found and studied in a number of systems and the minimum admissible contact time was measured for the phases in centrifugal extractors in a number of practically important systems, e.g., in uranium back-extraction from the tributyl phosphate phase. It was established that surfactants can either catalyze or inhibit the reactions at interfaces. Surfactants thus become a means of effective control over the rate of such reactions. For example, a method was suggested for accelerating the rate of equilibration by a factor of 5 to 10 in copper extraction by LIX-type industrial extractants. Anionic surfactants were used, but the mechanism involved is not yet clear.

A series of very interesting papers on the kinetics of chelate extraction was published by Freiser et al. [30—32].

Current problems of theory

Apparently, the main problems of liquid-liquid extraction are the creation of high selectivity and a priori evaluation of the conditions required to extract a particular metal. Obviously, these problems are interrelated. They can be resolved if integrated research is undertaken.

In addition to the progress of theoretical work described above, it is necessary to develop the theoretical foundations of the synthesis of extractants for particular purposes, and to use on a wider scale the physical and physicochemical techniques for evaluating the real state of metals in aqueous and organic phases.

EXTRACTANTS

This section outlines progress that has been made in discovering and studying new extractants. The main problem is to look for selective extractants. The use of such compounds greatly decreases the number of extraction stages and cycles, and simplifies and decreases the cost of extracting the required components. The problem of achieving high distribution coefficients is not as acute now as it was before, and is resolved rather easily.

The general principle that can be used as the basis for designing a selective extractant is that of maximum compatibility of the properties of the central metal ion and of the extractant molecule [33—35]. The most important factors for compatibility are: the nature of the metal and of those atoms in the extractant that directly interact with the central atom; the size of the metal ion, its coordination number, the type of coordination polyhedron in the given compound; the number and arrangement of active atoms in the extractant molecule; the properties of the rest of the molecule and its geometry. Of course, all this is true for compounds with direct extractant—metal bonding. Effects connected with the dissociation or aggregation of the extracted compounds in the aqueous and especially organic phases become significant for ion association compounds with the extractant in the outer coordination sphere.

The strength of the metal—extractant bonding, primarily determined by the nature of the metal and of the active atoms of the extractant, is a factor of paramount importance. The principle of soft and hard acids and bases was successfully used to evaluate this factor, explain the available data and make predictions. In many cases this was enough for a theory-supported choice of selective extractants. Selective extraction of "soft" bismuth or mercury will be better with "soft" sulphur-containing extractants than with "hard" tributyl phosphate. Likewise, "soft" triphenyl phosphine is more effective for "soft" silver than is "hard" triphenyl phosphate etc. A consistent and purposeful application of this principle (which may seem obvious now) is an important tool for finding selective extractants.

A search for a relationship between the structure of organic compounds and their extraction efficiency still constitutes an important problem in the theory of extraction and in its applications. A significant fraction of papers on extraction chemistry are devoted, to some degree, to solving this problem. The following conclusions are drawn by approaching the problem from the standpoint of predicting the optimal structure of extraction reagents as ligands of extracted complexes [36, 37].

In all likelihood, group reagents need a sufficiently flexible electronic and geometric structure. For relatively simple structures, the possibility of complex formation with a large number of metal ions results from the mobility of reagent electrons. Furthermore, the ligands in the complexes of various metals must exist as various tautomers. Such properties are found, for example, in cupferron and alkyldithiocarbamic acids, which are typically

group reagents. In contrast, a sufficiently rigid electron and stereochemical structure is preferable for selective extractants. In selecting and synthesizing such reagents, it is necessary to take into account simultaneously both the electron and the stereochemical requirements imposed by the metal. It must also be borne in mind that the properties of the extracted metal complex (its stability and solubility) are interrelated. A classical example of this correspondence is the nickel(II)/dimethylglyoxime complex.

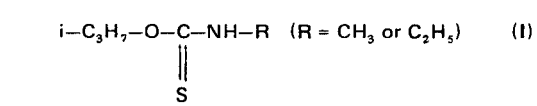
The selectivity of extractants is very important for such analytical techniques as spectrophotometry and spectrofluorimetry, and for preparing active components of membranes of ion-selective electrodes. The selectivity of extraction reagents and systems can also be of significance when working with spectrochemical methods of analysis, e.g., indirect determination of elements by a.a.s., or direct and indirect determination by molecular emission cavity analysis [38, 39].

Types of extractants

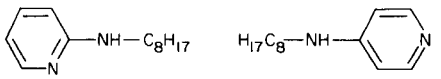
Both neutral, cation-exchange and anion-exchange extractants are widely used. Among them we find neutral organophosphorus and oxygen-containing reagents, amines (especially tertiary and aromatic), salts of quaternary ammonium bases and numerous cation-exchange (especially chelate-forming) extractants, including organophosphorus and carboxylic acids. New extractants are being suggested even among these fairly familiar compounds.

Various types of new extractants have been suggested and tested: neutral, cation- and anion-exchange extractants with various active atoms, including sulphur-containing reagents; those with linear and cyclic molecules. It would be impossible to give here an exhaustive survey of all the achievements in this field, and so only a few examples are included.

Neutral extractants. Among neutral extractants, sulphoxides deserve special attention [40, 41]. New extractants are especially numerous among neutral sulphur-containing compounds (in fact, sulphoxide can hardly be subsumed under this group). These are organic sulphides which extract Ag, Au, Hg and Pd from nitric acid. Isopropylalkylthiocarbamines (I) are used for silver extraction [42].



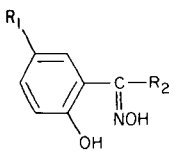
Among nitrogen-containing compounds acting as neutral extractants, aromatic amines are worthy of mention. n-Octyl aniline was proposed for the extraction of platinum metals [43, 44]. 2-Octylaminopyridine (II) [45] extracts iridium and rhodium, forming complexes of unusual composition. Unusually, iridium and rhodium are extracted better than the other platinum metals. 4-Octylaminopyridine (III) readily extracts iridium [46].



(II) (III)

Among the oxygen-containing neutral extractants reagents with two or more phosphoryl groups are worthy of mention. Much attention has been paid to them recently.

Cation-exchange extractants. These are mostly extractants that form chelates. Attention has mostly been paid (but not by analytical chemists) to hydroxyoximes of general formula (IV):



(IV)

and other similar extractants [47—49]. They were proposed for industrial-scale extraction of copper and other non-ferrous metals. Their prototypes were the familiar analytical reagents α-benzoin oxime and salicylaldoxime. Other chelate-forming extractants proposed for industrial application are also mostly analytical reagents with substituents which decrease solubility in water and increase it in organic solvents, or make the reagent less expensive and easier to obtain.

Anion-exchange extractants. These include tin compounds such as $Sn(NO_3)_2R_2$ [50, 51]. Their extraction capability with respect to phosphate, arsenate and other oxo-anions is unique. Complete extraction takes place in the interval from pH 6 to 5 M nitric or perchloric acid. Metal cations, such as Na, Cd, Zn, V, Cu, Co and Ni, have little effect on the extraction. Separation factors for phosphorus(V) and arsenic(V) with respect to cations exceed 10^4—10^5. The extractants were used for extractive spectrophotometric determination of phosphorus and arsenic, and for the neutron activation determination of arsenic with substoichiometric extraction. Phosphorus(V) and arsenic(V) can be extracted as HPO_4^{2-}, $H_2PO_4^-$, $HAsO_4^{2-}$ or $H_2AsO_4^-$. The extracted anion is incorporated into the inner sphere of tin. A formal description of the extraction process is similar to that used for the standard anion-exchange mechanism. It can be assumed that the special feature of the chemistry of extraction of oxo-anions by organotin extractants is precisely the capability of the extracted anion to enter the inner coordination sphere of tin and form a strong Sn—O bond. This leads to highly selective extraction: the only anions that are extracted are those that can do this. This behaviour may be common to all organometallic compounds of this class, containing Sn, Pb, Sb or Hg.

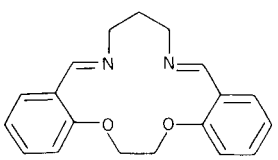
In addition to the widely-known ammonium bases, phosphonium, and sulphonium bases have also been studied; nothing of exceptional interest was found.

Macrocyclic extractants. Cation-exchange and neutral macrocyclic extractants merit special discussion. Macrocyclic species are of general and substantial interest in analytical chemistry. The most important property of these compounds, so attractive to specialists in chemical analysis, is their unique selectivity with respect to metal ions. Indeed, it is this property on which all known applications of macrocycles in analytical chemistry are based. The most important field of such application is the extraction of metals, especially because in some cases liquid-liquid extraction enhances the selectivity of the reactions between the ions of these elements and the macroheterocyclic compounds. A number of metal-macrocycle complexes, as well as the reagents, are poorly soluble in water, so that the method of extraction becomes additionally advantageous. This is also one of the reasons why liquid-liquid extraction developed into a significant technique for studies of complexation between macrocycles and ions in solutions. Nevertheless, the analytical utilization of the extraction of these compounds is a considerably more important application.

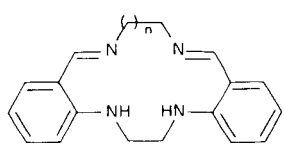
The largest number of papers on extraction by macrocyclic compounds is devoted to oxygen-containing macrocycles, i.e., crown ethers. The selective extractants for potassium, 18-crown-6 and dibenzo-18-crown-6, are of special interest. Methods have been proposed for the extraction of lithium and sodium, and for separating alkaline earth elements.

The first results on the use of nitrogen-containing macrocycles were reported at the beginning of the 1980s; it was possible to develop methods for selective extraction of transition and post-transition metals (copper, silver and mercury). The same objective is achieved with sulphur-containing macrocycles. The possibility of complexation and the selectivity of a macrocycle are always governed by the nature and location of the donor atoms in the molecule, the size of the cavity in the macrocycle, the general structure of the molecule (e.g., the presence of flexible chains capable of hampering or stimulating the formation of a complex, the rigidity of the molecule) and possibly by other factors.

Some examples of selective macrocycles can be given. The compound 5, 6; 14,15-dibenzo-1,4-dihydroxy-8,12-diazacyclopenta-deca-7,12-dien (V) [52] selectively extracts silver (in the presence of picrate or some other counter-ion). Silver can be extracted in the presence of predominant amounts of a number of metal ions, including Cd, Co, Fe(III), Cu(II), Hg(II), Ni, Pb and Zn. Only copper(II) among the investigated 13 elements is extracted by macrocyclic Schiff bases (VI) [53].



(V)



(VI) n=1,3,4,5

Many other extractants were suggested (both with selective and with group action; there is a need for the latter). A question arose (it was discussed in 1980 at the International Solvent Extraction Conference at Liege) as to whether it is preferable to search for new extractants or to obtain further and more reliable information on those already available. Although the discussion involved only industrial extractants, it obviously touched on analytical chemistry. As could be expected, it was impossible to give an unambiguous answer; or rather, the general opinion was that both types of work are essential. Indeed, it is very important to look for new extractants, but good knowledge and versatile application of the older extractants is also essential. Quite a few of the achievements of liquid-liquid extraction have arisen by choice and control of the conditions under which separation and preconcentration are carried out.

Binary extractants. In addition to the three currently known classes of liquid-liquid extraction processes, based on using cation or anion exchange, or neutral extractants, the class of binary extraction is also worthy of discussion [54]. This process consists of the extraction of various inorganic compounds by salts of organic acids and organic bases (binary extractants) producing two extracted compounds in the organic phase. Binary extraction differs from cation- and anion-exchange extraction by the type of mass transfer between the phases (whether it is molecular transfer or the transfer of cations and anions in the stoichiometric ratio) and from extraction by neutral extractants by the type of chemical reaction. In some cases systems with binary extractants successfully increase the efficiency and selectivity of extraction of metal salts, acids and hydroxides.

As for the problems in searching for new extractants, the following are general objectives at which the research must be aimed: the development of selectivity theory as a tool for extractant selection; the synthesis of new compounds as potentially highly efficient extractants; the extensive testing of substances produced on a wide scale for other uses (flotation agents, pesticides, additives to oils and polymers, accelerators for rubber curing etc.) as potential extractants; the search for new techniques that make extractants out of compounds which were formerly regarded as hardly suitable for the purpose (e.g., organometallic compounds); a detailed study of the physicochemical interaction between metals and extractants; a broadening of the range of applicability of extractants to fields beyond extraction per se (e.g., extractants may form the chemically active part of selective complex-forming sorbents).

EXTRACTION TECHNIQUES

Efficient application of liquid-liquid extraction is greatly promoted by the appearance and judicious use of new techniques. Those listed below are now conventional tools in the field.

Exchange extraction. This is based on using extractants that are complexes or salts of metals. When the compound is used, one metal substitutes for

another in accordance with their extraction constants. The advantages of such extraction lie in high selectivity and in the possibility of using stable complexes instead of unstable reagents, as in the case of, say, cupferron.

Synergic extraction. This consists of using a mixture of non-additively functioning extractants (with positive deviation from additivity). This technique is especially effective in the extraction of chelates and related compounds.

Substoichiometric extraction. This is an important technique with applications in radioanalytical chemistry [55, 56]. Its main feature is that the amounts of reagent used are lower than those dictated by stoichiometry. If the constants of formation and extraction of the extracted complexes are high, the amount of extracted metal is dictated by the amount of extractant introduced; this has proved to be very useful, e.g., for isotope dilution.

Application of low-melting extractants [57—59]. The extraction is performed while the extractant is liquid, after which the system is cooled; as a result, the extractant (now the extract) solidifies and separates from the aqueous phase. This technique has certain advantages, especially if the extract will subsequently be used for analysis by methods requiring solid samples.

Application of three-phase extraction systems [60]. As a rule, the separation of the organic phase into two phases is an undesirable effect; however, in some cases the formation of a third phase is specially stimulated, because it serves to concentrate trace elements.

Utilization of elastic polyurethane foams [61—63]. Cross-linked porous foamy polyurethane, whose open pores are filled with an extractant, was found to be useful for the group preconcentration and selective separation of trace elements. The main characteristics of the methods using such foams is high reproducibility, high speed, versatility of the concentration and separation processes, and relative ease of automation.

When applied to multistage separation methods, extraction systems may prove to be as effective, and in some cases more effective for achieving selective extraction of elements from complex mixtures than the use of selective reagents. One example is extraction chromatography which is capable of separating even lanthanides [64]. Among the imperfections of extraction chromatography using solid supports are a relatively low rate of separation and only moderate reproducibility, especially when changing from one column to another and when changing the stationary phase; this is why extraction chromatography, unlike other chromatographic methods, still resists automation.

When considering techniques introduced recently, the coating of various solid supports, especially porous supports, with extractants can be mentioned. Presumably polyurethane foams are the most promising from this standpoint. It is now possible to manufacture foams in such a way that their pores are open; such supports reliably retain considerable amounts of extractants.

Various versions of membrane extraction have also been studied. One defect of membrane extraction is its long duration. Several approaches are

known to overcome this obstacle. Thus, attempts were made to intensify the process of extraction of ionic compounds by applying an electric field (so-called electroextraction); this line of work is pursued at the Institute of Inorganic Chemistry of the Academy of Sciences of the Latvian S.S.R. (Riga, U.S.S.R.). Another interesting suggestion was membrane extraction using specially prepared droplets. However, this method seems to be of interest primarily for technological applications. Extraction was also suggested in two-phase aqueous systems based on water-soluble polymers [65, 66].

Each of these techniques has its peculiarities and advantages, and extends the potentials of liquid-liquid extraction as a whole from the standpoint of analyzing a broader spectrum of systems, achieving higher preconcentration coefficients and speeding up the process of separation. For example, extraction in two-phase aqueous systems makes it possible to extract inorganic and organic compounds in the absence of normal organic solvents, making the process safer, and to use as extractants certain water-soluble organic reagents many of which are highly selective. This is achieved by using systems that separate into layers, one phase being the aqueous solution of an inorganic salt (e.g., sulphate or carbonate in water) and the other an aqueous solution of a polymer (e.g., polyethylene glycol) [65, 66].

So far extraction methods have not been sufficiently automated. Some suggested equipment did not find wide application in inorganic analysis, e.g., Craig's apparatus, and equipment for discontinuous and continuous countercurrent extraction [11]. This state-of-the-art in laboratory equipment for extractive separation does not seem justifiable, for at least two reasons. First, industrial-scale extraction is a completely automated process. Second, a combination of a separating funnel with a modern analytical instrument, such as an inductively-coupled plasma emission spectrometer, is certainly far from an effective combination, and not because of the imperfections of the spectrometer. Consequently, the development of modernized automatic methods of extractive separation appears to be a pressing problem. It is important to bring automation not only into multistage methods but also into single-stage separation techniques, such as flow injection analysis.

COMBINATION OF EXTRACTIVE SEPARATION WITH SUBSEQUENT DETERMINATION OF ELEMENTS

Individual and group extraction

Two factors dictate needs in individual and group separation and preconcentration of elements: the objectives of the analysis and the capabilities of the subsequent determination. Group extraction reagents are advisable in the case of a multielement analysis of a sample and a multielement determination technique (e.g., atomic emission or x-ray fluorescence spectrometry) or in the case of a high-selectivity method (e.g., atomic absorption spectrometry). In a single-element analysis or with non-selective detection techniques, it is mandatory to use selective extractants or multistage separation schemes which are capable of separating single element fractions of the determined elements.

In what follows the specific features of extraction are discussed when it is used in combination with various determination methods. Here only one example is given which demonstrates how selective extraction can, in principle, extend the potentials of some analytical techniques.

Molecular absorption spectrometry is a method which determines, with high sensitivity, halide ions (X^-), e.g., from the absorption spectra of AlX and InX molecules. However, a practical application of this technique is hampered by non-selectivity caused by strong interference from other atoms and especially metal cations. It has been shown recently, however, that chloride and bromide can be determined by molecular absorption spectrometry, after a practically complete separation from all interfering cations by selective extraction with triphenyltin hydroxide [67, 68].

Spectrophotometry and fluorimetry

Historically, photometry was the first method of analysis with which liquid-liquid extraction was coupled. The history of extractive spectrophotometry covers several decades; a considerable number of reviews and monographs were devoted to it. Some of its modifications that occurred in recent years or are being currently developed, are discussed below.

Spectrophotometry generally has a lower selectivity than many other modern analytical techniques, so that a search for more selective reagents is still an important task. One of the more promising approaches to extractive spectrophotometric analysis is to use macrocyclic reagents. At least four examples of the use of macrocycles in spectrophotometric analysis are known. The most widespread method is to form an ion association compound between a cationic metal-macrocycle complex and a coloured anion. A well-known example is the determination of potassium in extracts by reaction with dibenzo-18-crown-6 and the yellow picrate anion. A second version is to use macrocycles whose molecules include a chromophoric group in the ring but which do not participate directly in complex formation. The chromophoric group would also be involved in metal complexation, which could lead to the highest colour contrast. Presumably, Calcichrome was historically the first reagent of the third type [69]. Porphyrins and formazans also belong to this group. The fourth version uses ion association compounds containing two metals, one in the cation, the other in the anion. The cationic part is a complex of a macrocycle and an alkali metal. The element to be determined is used as a coloured anionic complex with inorganic or organic ligands.

The selectivity and other analytical properties of extractive spectrophotometric and spectrofluorimetric analyses can be improved by using two reagents consecutively. In the first stage, trace components are separated by the more selective reagent, and then a reagent (not necessarily selective but best suited to the final determination) is added to the extract. Thus, phosphorus(V) and arsenic(V) can be selectively extracted by dialkyltin dinitrates and then determined by formation of coloured heteropoly compounds after adding molybdate [70]. A highly selective method of determination of

mercury(II) is based on the extractive separation of mercury as $HgBr_2$ from all other elements and subsequent reaction of the extract with an aqueous solution of a basic dye [71].

Extractive preconcentration that uses amplification reactions may decrease not only the relative but also the absolute detection limit of spectrophotometric determinations [72, 73].

Spectrophotometry and spectrofluorimetry are, with rare exceptions, single-element analytical techniques. However, several elements can be determined if the absorption or luminescence spectra of their compounds do not overlap or if overlapping does not hamper a numerical interpretation of results. The latter approach seems to be especially attractive owing to an intensive use of digital electronics, derivative spectroscopy and computers. Another promising approach, as mentioned above, involves the use of automated analyzers, as in the case of the flow injection technique with photometric detection.

Atomic absorption spectrometry

In the early days of a.a.s. it was believed that the high selectivity of this technique eliminated the need for preconcentration and separation. However, it soon became clear that the separation of the matrix and the preconcentration of the elements to be determined are often necessary for a.a.s.

Liquid-liquid extraction proved to be the most widespread method of separation and preconcentration of elements prior to their determination by a.a.s., especially in its flame version [5, 74]. Extraction combined with flame a.a.s. considerably decreases not only the relative but also the absolute detection limit. This is caused by the lower viscosity and lower surface tension of the common organic solvents in comparison with aqueous solutions so that the rate of solution introduction into the flame increases. The efficiency of nebulization is improved by increasing the degree of dispersion of aerosols. Flame temperature and composition produce smaller effects on determination sensitivity [75, 76].

The properties of solvents best suited to the requirements of flame a.a.s. were discussed earlier [76]. Apparently, the best solvents are methylisobutyl ketone and butyl acetate, but other solvents are also admissible. Considerable difficulties arise in working with chloroform, carbon tetrachloride and other non-combustible chlorine-containing solvents. If their use is unavoidable, preliminary drying of aerosols, dilution of extracts by a combustible solvent, stripping, evaporation of the extract and dissolution of the residue in an acid or in another solvent are recommended [75].

An injection method of aerosol introduction which puts into the flame small volumes (<200 μl) of solution [76] appears to be very promising. Its advantages (the possibility of analyzing very small samples, the determination of a large number of elements after the preconcentration stage, analysis of solutions with a high content of salts and acids) are essential for extractive a.a.s. The pulsed injection of samples into the flame makes it possible to

analyze extracts based on chloroform and other solvents "unsuitable" for flame a.a.s. [77, 78]. Another important direction of development in extractive flame a.a.s. is connected with designing automatic systems for sample extraction and analysis [79, 80], including flow injection analysis.

The number of papers reporting the combination of extractive separation and preconcentration of elements with their determination in an electrothermal atomizer (e.t.a.) has increased dramatically in recent years [81, 82]. To a large extent this is a result of a growing interest in the analysis of environmental samples, such as biological, geological and other multicomponent specimens containing the elements to be determined at a level of 10^{-6}–10^{-5}% or less.

Pioneer papers on e.t.a./a.a.s. hypothesize that, in addition to a better sensitivity, this version of the technique has other advantages over the flame a.a.s. of organic extracts. It was assumed that the elimination of the nebulizer would allow viscous extracts to be analyzed; that complete removal of organic solvents from the e.t.a. during drying and ashing should eliminate non-selective light absorption during atomization; and that it will be possible to analyze any type of extract, including extracts based on non-combustible solvents. Indeed, in most cases the modern e.t.a. enables extracts to be analyzed with the same sensitivity and precision as that achieved for aqueous solutions. However, effective analytical techniques can only be developed by taking into account the nature of the extract and by learning to suppress the interference of certain components.

Low sensitivity may be observed in e.t.a./a.a.s. for elements which form stable chloride complexes (Ga, In, Sn, Fe, Pb, etc.) when chlorine-containing organic solvents are used. The effects of chlorine-containing substances are eliminated by adding ascorbic acid to the sample. It must also be taken into account that the use of extracts based on aromatic solvents may result in substantial non-specific absorption during atomization [83].

The nature of the extractable complex may affect the loss of the element to be determined at the ashing stage and the mechanism of atomization. For example, many chelates, halides and some other metal compounds sublime without decomposition. In e.t.a./a.a.s. this phenomenon is most often observed, as it seems, in the analysis of extracts containing iodides of the determined elements; the high volatility of the iodides obstructs the direct analysis of the extracts. To solve the problem, it is recommended that ashing conditions are optimized, matrix modifiers are applied, or back-extraction is used. The nature of the compounds produced by thermal decomposition of extracts is to a large extent dependent on the nature of the donor atom. Consequently, the mechanisms of atomization of an element incorporated in a diethyldithiocarbamate, cupferronate or chloride complex may be different. It is recommended that reagents should be used whose donor atoms do not form very stable compounds with the metals to be determined [81, 82].

It can be concluded that the difficulties encountered in using e.t.a./a.a.s. for the analysis of extracts are mostly related to insufficient knowledge about

this hybrid method, not to any fundamental constraints. A choice of extraction system, taking into account the details of its behaviour in e.t.a., and the utilization of modifiers, graphite platform atomization, and varying atomizer heating rates result in accurate analyses of extracts, with high sensitivity and precision.

The achievements in the theory and techniques of the method described above make it possible to employ in flame and electrothermal a.a.s. most of the known types of extractants and solvents. The most widespread are chelates, especially pyrrolidine dithiocarbamates, with methylisobutyl ketone as the solvent [83]. More than 50 elements are determinable by direct a.a.s. after extractive separation. The range of determinable elements is widened by using indirect methods based on a reaction between the element of interest and some other species, and subsequent determination of a reaction product or excess reagent. It is possible to determine some elements for which a direct analysis is very difficult or impossible: halides, phosphorus, sulphur, boron, rhenium, silicon etc. [5]. It must be noted, however, that the latest achievements in a.a.s. and in the methods of preconcentration of non-metals has led to the direct determination of some of the elements which were determined earlier by indirect techniques. Thus, phosphorus in metals and alloys is determined with fairly high sensitivity by e.t.a./a.a.s. after its extractive separation with dialkyltin dinitrate [84].

Atomic emission spectrometry (a.e.s.)

Gorbach and Pohl proposed a scheme of extractive preconcentration of trace elements as chelates at the beginning of the 1950s [85–89], and applied it to the analysis of pure acids, plant ash, natural water, soil and rock. After a sample was dissolved, the interfering iron was precipitated by the ammonium benzoate method, and Ag, Al, Au, Bi, Cd, Co, Cu, Ga, Hg, In, Mo, Mn, Ni, Pb, Pd, Pt, Sb, Sc, Sn, Ta, Th, Ti, V, Y, Zn, Zr and lanthanides were extracted, as the pH values were varied stepwise (3, 5, 7, 9), into chloroform as chelates with 8-quinolinol, dithizone and sodium diethyldithiocarbamate. The organic solvent was distilled off, and the dry residue was then calcinated and analyzed.

This scheme rapidly progressed after the publication of results on the effect of organic solvent on chelate extraction [90]; this is an example of undelayed spin-off from fundamental research to applications oriented work. Polar organic solvents, oxygen-containing solvents among them, are convenient for simultaneous extraction of coordinatively saturated and unsaturated chelates, and for increasing the number of determinable elements, provided the elements of the matrix are not extracted. However, a better solvent is a mixture of a polar solvent and carbon tetrachloride or chloroform because it increases the extract density and simplifies its separation from the aqueous phase in a separating funnel. This modernized scheme improved the methodological characteristics and proved to be useful in the extractive analysis of high purity materials by a.e.s. [11].

It is also important to decrease the effect of organic substances on the results of determinations by a.e.s. Accordingly, a mixture of two chelate-forming reagents, viz. cupferron and diethyldithiocarbamate, was used, and a donor-active admixture, trioctylphosphine oxide, was added to the chloroform [91]. The result was a simultaneous determination of Ag, Al, As, Bi, Ca, Cd, Cr, Cu, Fe, Hg, Mg, Mo, Ni, Pb, Sb, Sn, Ti and Zn.

A group preconcentration of trace elements is achieved if the matrix is extracted. The capacity of the organic phase then must be sufficiently large, and the extraction per se must be selective with respect to macroelements, otherwise the most significant advantage of a.e.s., the possibility of multi-element determination, is lost. Matrix extraction is convenient in analyzing metals, alloys, simple salts, oxides and hydroxides, i.e., relatively simple substances. The matrix elements are extracted from halide, thiocyanate or nitrate media mostly by oxygen-containing organic solvents, amines, tri-n-butyl phosphate or di-2-ethylhexyl phosphoric acid [5, 11, 92]. Extraction of ion association and coordinatively solvated compounds is also effective in this situation; chelates are more difficult to use.

Developments directed towards the extractive preconcentration of trace elements with arc and spark sources can also be utilized in the inductively-coupled plasma version.

X-ray fluorescence spectrometry

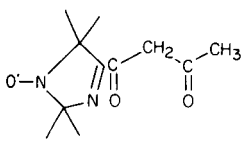
This method is more suitable for solid samples, so that liquid-liquid extraction is not the optimal approach to preconcentration. Melt extraction has been successfully used [93]. A solidified low-melting solvent or extractant is separated after the extraction is completed and the solution cooled. The solid concentrate obtained is suitable for analysis. Nevertheless, conventional liquid-liquid extraction can also be used. Thus, a methylisobutyl ketone or diisobutyl ketone extract with traces of Co, Cu, Fe, Mn, Pb, Ni and Zn as their diethyldithiocarbamates was placed, in droplets, at the centre of a circular filter paper and was analyzed after drying [94].

Electron spin resonance (e.s.r.) spectroscopy

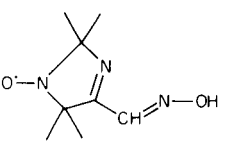
Systematic work on e.s.r. applications to inorganic analysis was carried out by Guilbault who measured the paramagnetism of the central atoms in complexes. A considerable number of analytical extractive spectroscopic techniques were developed, employing the paramagnetism of metal complexes in common oxidation states (Cu(II), Mo(VI), V(IV), Co(II), Cr(III)) and also in unusual oxidation states (e.g., complexes of palladium(III) and platinum(III)) [95]. This is another case where liquid-liquid extraction improves the conditions of determination by allowing the use of solvents with dielectric constants lower than that of water. The sensitivity of e.s.r. spectroscopy is comparable to that of u.v./visible spectrophotometric methods. Its advantage is its selectivity; the method makes it possible to determine, e.g., gadolinium against the background of all other lanthanides.

The field of analytical application of e.s.r. was greatly expanded with the advent of organic reagents which contain a functional complex-forming group and a group with an unpaired electron. Such complex-forming stable radicals represent a new class of analytical reagents that serve to determine the concentration of practically any metal from the intensity of the e.s.r. signal of the radical component of the reagent. Furthermore, there is an additional gain in sensitivity, since the lines in the e.s.r. spectrum of organic radicals are very narrow.

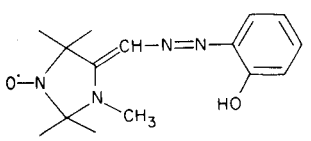
Reagents were synthesized which are related to β-diketones (VII), oximes (VIII), azo dyes (IX) and xanthates (X) [96]:



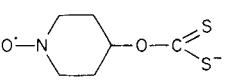
(VII)



(VIII)



(IX)



(X)

A study of complex formation between such reagents and metal ions demonstrated that if the radical group does not participate in complexation its effect can be estimated by regarding it as a relatively strong electron acceptor group comparable in acceptor properties with a CF_3 group.

A metal complex MA_n, where A is the anion of a chelate-forming reagent, is a polyradical system which contains, at least in the case of diamagnetic central atoms, n unpaired electrons. Electron spin resonance spectra of polyradical chelates are fairly diverse. In the simplest case there is no exchange between unpaired electrons within the MA_n complex, and the e.s.r. spectrum of the complex is practically identical with that of the original reagent, e.g., the spectrum of potassium xanthate coincides with that of zinc xanthate ZnA_2 [97]. The e.s.r. spectrum is a triplet due to the splitting at the ^{14}N nucleus. In this case the measurement of the intensity of the signal from the complex requires that the complex be separated from the excess of reagent. But more often, owing to a direct or indirect exchange interaction between the paramagnetic centres, the spectrum of the complex is more or less complicated, and deviates from that of the original reagent. It is then possible to measure the intensity of the e.s.r. signal from the complex against the background of the reagent. The situation is the most uncertain for complexes of paramagnetic metals [98].

Radioanalytical methods

For a number of years, liquid-liquid extraction was successfully used in activation analysis, which is predominant among radioanalytical methods.

Two versions are possible for the combination of extractive preconcentration and activation analysis: in the first the components to be determined are separated from the matrix prior to irradiation, in the second separation follows irradiation. The latter, radiochemical version, is of greater interest because it makes use of one of the main advantages of the activation method: it eliminates the blank correction. In its turn, radiochemical separation allows for two variants; one to remove the interfering high-activity components of a sample and measure the activity of the determined elements by using high-resolution γ-spectroscopy with computer processing of the spectra, the other to separate the elements individually or as a group [99]. In both these types, progress depends on the development of selective extractants.

The separation of the determined elements into groups becomes a more efficient technique owing to the application of modern automatic γ-spectrometers which simultaneously determine several elements in a sample. Gilbert et al. [100] employed group extraction of elements with dibutyl sulphide for analyzing metallic silver. The group separation making use of pyrrolidine dithiocarbamate [101] and dinonylnaphthalene sulphonic acid [102] also proved to be a very efficient technique. Alimarin et al. [99] suggested a scheme based on the group extraction of the required elements and subsequent substoichiometric extraction of all or any one selected element individually. The scheme has been applied to more than 20 elements in various samples.

Liquid-liquid extraction is also actively employed in radioanalytical methods based on the use of radioactive labels [103]. In addition to such familiar techniques as isotopic dilution, relatively new methods have also been perfected, such as those of radioactive reagents and concentration-dependent distribution. An important advantage of these methods is the possibility of determining not only inorganic substances but organic substances, including extractants, as well.

Mass spectrometry

Spark source mass spectrometry is used in combination with liquid-liquid extraction or other methods of separation and preconcentration less frequently than most of the available instrumental methods of analysis. This is because of the high sensitivity of mass spectrometry and by the possibility of determining simultaneously more than 60 elements, with little sample consumption. However, very small samples may yield non-representative results. Moreover, the analysis of liquid samples is not always an easy task for spark source mass spectrometry. And finally, mass spectrometry is not free from the effects of the matrix on the results of analysis. Consequently, it would seem promising to develop chemical mass-spectrometric analysis using a solid collector which is analyzed after the concentrate of the elements to be determined is placed on it; this can be the extract or back extract. Such collectors could be made, for example, of powdered graphite or aluminium oxide, because of their good sorptive properties and because they provide a constant rate of sample feed into the discharge gap.

A technique based on aluminium oxide was developed for determining eight elements in steels and concentrated sulphuric acid [104]. The required elements were extracted into methylisobutyl ketone from an iodide/sulphate solution and back extracted into a hydrogen peroxide solution, after which the back extract was evaporated on the collector and the concentrate analyzed mass spectroscopically. A low detection limit (10^{-6}–10^{-7}% for sulphuric acid) and high precision were achieved, owing to the stabilization of the relative sensitivity coefficients because of the single collector. Another method was suggested for determining traces of platinum metals in natural and industrial samples with non-uniform distribution of the elements to be determined [105]. Platinum metals are extracted as complexes with hexamethylenephenylthiourea from dissolved copper-containing samples; the concentrate is obtained by distilling off the solvent and by subsequent calcination of the dry residue (the collector being copper oxide or sulphide).

Voltammetry

The main problem of liquid-liquid extraction applied to voltammetry is how to increase selectivity. The trend apparent since the middle 1960s, towards the direct polarographic analysis of extracts, is still prevalent. No new approaches were found for selecting the conducting electrolyte. Nevertheless, some papers reported the use of new extractants. Thus, "petroleum sulphides" were applied to the extraction of gold and silver from solutions obtained by dissolving standard samples of copper/zinc ores [106]. Unfortunately, progress in extraction stripping voltammetry, which combines extractive preconcentration with the subsequent electrochemical preconcentration of elements on the electrode from the extract mixed with electrolyte, was insignificant [107]. Nevertheless, interesting results were reported on using "amplification" reactions in classical polarography. One example is the determination of phosphorus [108], involving the formation of 12-molybdophosphoric acid, extraction of this acid, back-extraction of molybdate into an alkaline solution, and its determination from the catalytic wave in the presence of nitrate ions.

Ion-selective electrodes

One of the postulates of liquid-liquid extraction is the assumption that the compounds distributed between the phases are electrically neutral. In the 1960s intensive research was launched into ion-selective electrodes with liquid membranes. The build-up of potential at the organic membrane/aqueous solution interface in an electrochemical membrane is governed by the distribution of cations or anions. In its turn, the distribution of charged particles maintains the equality of electrochemical potentials. At the same time it was found that the detection limit and selectivity are determined by the extraction properties of the appropriate two-phase liquid system.

The electroactive compound of the ion-selective electrode must contain the potential-determining ion whose amount is to be measured. Consequently,

the equilibrium value of the potential of the electrode reflects the total concentration of the test ion. For this reason the detection limit is governed by the distribution constant of the electroactive compound: the higher this constant, the lower the detection limit. In bi-ionic systems, an exchange reaction between the test ion in the electroactive compound and the interfering ion of the analyzed aqueous solution is realized at the interface. Therefore, in systems with not very different mobilities of the test and interfering ions the selectivity is governed by the ratio of extraction constants of the corresponding ions, and in systems with similar distribution constants by the ratio of stability constants. However, an analysis of a large amount of data indicates that these conclusions are true only in a relatively narrow interval of stability constant values [109, 110].

There is, therefore, a close relationship between liquid-liquid extraction and liquid-membrane ion-selective electrodes, and the information amassed in extraction can be used both for selecting electrochemical systems suitable for preparing ion-selective electrodes and for choosing optimal conditions for the analysis.

CONCLUSIONS

The material surveyed in this review demonstrates that there could hardly be another method for separation and preconcentration of elements, which would couple as successfully to as many determination methods as liquid-liquid extraction does. For this reason further progress in extraction used as a method of separation and in various analytical techniques utilizing extraction, and a search for new combinations of this type would appear to be extremely promising for chemical analysis.

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OPTICAL, ELECTRON AND X-RAY SPECTROMETRY IN SOIL ANALYSIS

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SUMMARY

The development of the applications of optical, electron and x-ray spectrometry to the analysis of soils over the last quarter century is reviewed.

The top 15–20 cm of the soil constitute our most valuable natural resource; without it life could not have developed nor could it survive. Spectrometry in all its forms has played the major role in unravelling the mysteries of the constitution and nature of soils. Today it is still the major source of new information and the means of monitoring the release or fixation of essential major plant nutrients, and of bioessential trace elements such as Co, Cu, Fe, Mn and Zn for the conduct of the normal operation of modern intensive, high yield, agriculture. Spectrometry also provides watchdog monitoring of biotoxic elements released from, or in various ways added to, soil, e.g., Cd, Hg and Pb.

Structural techniques such as x-ray diffraction, not reviewed in this article, have done much to establish the nature of the clay minerals which play such a large part in soil fertility whilst x-ray fluorescence spectrometry (x.r.f.), which is discussed, has yielded an enormous amount of elemental analytical information which is particularly useful for elements such as sulphur that are difficult for optical atomic spectrometry because of the presence of most of their diagnostic lines in the far ultraviolet part of the spectrum. Flame emission spectrometry and molecular absorption spectrometry which played a big role in the earlier days of trace element work at a level lower than that usually possible by x.r.f. spectrometry, are now less used having been overtaken by emission spectrography, atomic absorption and fluorescence spectrometry and more recently, radio-frequency plasma emission spectrometry. The first two techniques are not discussed here, except in passing, whereas the others are reviewed in some detail because of their present significance. Infrared absorption spectrometry has proved to be invaluable in the characterization of soil organic matter, the resolution of details of clay minerals and recently even the discovery of new aluminosilicate structures in soils that explain their weathering behaviour.

For some time, it has been recognised that the chemistry of the surfaces of soil particles is perhaps the most important feature in relation to their ability to support plant growth and nutrition. In this respect, electron microprobe, (i.e., electron beam microanalysis) analysis has been one of the most useful tools at our disposal. Basically it provides average analytical data over a number of layers below the 'surface' in as much as the latter concept can apply to soil particles. Recently, however, considerable improvement to the techniques of x-ray photoelectron spectrometry (x.p.s., or e.s.c.a.) has provided much more surface-specific information.

In the following pages the above-mentioned techniques are featured to provide a state of the art view of the spectrometric investigation of soils in 1985—6.

X-RAY FLUORESCENCE SPECTROMETRY

Origins and development

The basis for chemical analysis by x.r.f. was established over seventy years ago with Moseley's study of the x-ray spectra of the elements [1] and the first text book on the subject appeared in 1932 [2]. The first commercial x-ray spectrometer was produced about 1948 and it was some ten years before x.r.f. spectrometry became commonly used as a reliable method of chemical analysis. Technological advances since then have been mainly concerned with improved detectors, higher-powered x-ray tubes with a range of anode materials, and a wider range of crystals, all resulting in increased sensitivity and versatility. The increasing use of computers to control the instrument and continually improving software for data handling, matrix corrections etc., has led to a high degree of automation and increased speed of analysis. The single most significant advance has been the development of the energy-dispersive detector which made possible the simultaneous detection of all elements, with atomic number >10, at major concentrations.

Capabilities

Wavelength-dispersive x.r.f. spectrometry is capable of detecting the spectra of at least 80 elements (atomic number >8) and analyses can be performed on samples of wide-ranging compositions over a concentration range from 100% to less than 1 $\mu g\ g^{-1}$. The method is essentially non-destructive (although fusion is often advantageous for major element determination), is very precise, analysis times are short, and, with correct procedures for calibration, accurate quantitative analyses can be obtained on a routine basis. Energy-dispersive x.r.f. spectrometry has the disadvantages of lower counting rates (and consequent loss of sensitivity) and poorer energy resolution resulting in serious line overlaps for elements of low atomic number. Consequently, energy-dispersive x.r.f. spectrometry is not suitable for the quantitative analysis of minor and trace elements, but with spectrum-stripping programs to handle line-overlap problems, it is becoming more commonly

used for determining major elements in soils [3]. Energy dispersive detectors are particularly useful for quick qualitative analysis and are commonly used on scanning electron microscopes to provide chemical information for phase identification and for element distribution studies. As regards analysis of soils by x.r.f. spectrometry, wavelength-dispersive x.r.f. spectrometry is still the most commonly used x-ray method and the rest of this section is restricted to this.

Analysing soils by x.r.f. spectrometry, whether it be whole soils or particular size fractions such as the clay fraction (<2 μm), is very similar to analysing rocks, i.e., the matrix is essentially siliceous, the main difference from rocks being that soils contain much more variable amounts of moisture and organic matter. For qualitative analysis, very little sample preparation is required. The dried soil sample can simply be placed in a sample cup with a Mylar film covering the base, it can be pressed hydraulically into a pellet, or the sample can be dried from suspension on to a suitable substrate.

To obtain an accurate quantitative analysis of the major elements, it is necessary to overcome problems related to particle size, mineralogy and interelement effects, and the most satisfactory method is to fuse the sample with a borate flux. A number of fusion methods with various fluxes and various dilutions have been described, but one which is particularly appropriate for a wide range of compositions is that of Norrish and Hutton [4], a more recent variation [5] of which employs a lower dilution and so is more suitable for trace element determinations. The sample is best pre-ignited to remove moisture and organic matter before fusion, although in this laboratory soils with up to 25% loss-on-ignition have been successfully analysed without pre-ignition. To maximise sensitivity for the determination of trace elements at lower levels the soil must be finely ground (preferably to <15 μm) and pressed into an aluminium cup or on to a cellulose or boric acid backing.

X-ray fluorescence spectrometry also has the capability to analyse liquids, and this can be useful in the study of soil extracts. The elemental coverage in solution is more restricted because of path limitations; the elements of low atomic number have x-rays of relatively long wavelength which are absorbed by air and the liquids would have to be dried under vacuum on to cellulose or filter paper for analysis. Direct analysis of liquids in air is restricted to shorter wavelengths (<0.15 nm, equivalent to the K spectra of elements with atomic number >29). Elemental coverage can be extended to lower atomic numbers by flushing the system with helium which does not absorb long wavelength x-rays to the same extent as air.

Some applications to soils

The multielement capability combined with simple, non-destructive sample preparation make x.r.f. spectrometry a useful tool in soil studies. Many of the early applications were concerned with major element analyses in soils and clays [6, 7] but Beavers [8] recognised that determination of

minor elements such as Ti, Cu and Zn could be used to evaluate uniformity and distribution of parent material. Zirconium and titanium in soils tend to occur in resistate minerals and x.r.f. spectrometry has been widely used to determine these elements in order to obtain information on the pedogenic history of a soil [e.g., 9, 10]. Unusually high contents of elements have been detected as a result of the ease and rapidity of the method, e.g., U and Mo in soils [11] and Ti in soil clays [12]. Thin-film techniques can be used for clays enabling a few milligrams of material to be analysed [13]. Accurate analysis of soil clays can be used to calculate structural formulae of constituent minerals [14]. Cation-exchange capacity of clays can also be calculated after measurement by x.r.f. spectrometry of the amounts of Ba, Cs or Sr and Cl retained after saturation with chloride solutions [15—17]. The slight shift in wavelength of the characteristic x-radiation for the lighter elements according to valence state and coordination number has been utilised in soil studies to determine the coordination number of Al in clay minerals [18] and poorly-ordered materials such as allophane [19], but has only had mixed success as the shift for AlK_α has been shown to be related primarily to the mean Al—O distance and only indirectly to the coordination number [20]. The sensitivity and speed of x.r.f. spectrometry for the analysis of S has led to the technique being applied to soil problems such as locating acid sulphate soils [21] and S-deficient sites [22] and determination of S in soil extracts [23]. A more detailed account of soil analysis by x.r.f. spectrometry has been given by Wilkins [24].

ARC EMISSION SPECTROMETRY

The foundations of practical spectrochemical analysis by optical emission methods were laid in about 1860 by Kirchhoff and Bunsen [25] who by its use established that sodium existed in the atmosphere of the sun. At that time it was clearly established that the spectrum emitted by an atom is characteristic of that atom and that the spectroscope could be used as a new means of qualitative chemical analysis. After Kirchhoff and Bunsen, the use of the spectroscope declined probably because the very low temperature flames then available as excitation sources limited its use to very few elements. Developments in other fields such as photography, production of prisms and gratings, improvements in wavelength measurement and the compilation of charts of spectra during the latter part of the 19th century assisted the progress of emission spectrochemical analysis.

During the first quarter of the 20th century spectrochemical analysis was referred to as the forgotten method and the spectroscope was used mainly by physicists and astronomers. With the extension of electrical facilities the use of arc and spark sources of excitation became possible. The work of Meggers et al. [26] in the USA during the 1920s gave impetus to analytical spectroscopy and, at about the same time, the introduction by Gerlach and Schweitzer [27] of the internal standard principle made possible the development of quantitative emission spectrochemical analysis.

Optical emission methods were first developed extensively for metallurgical analysis using mainly spark excitation. During and after the second world war, however, techniques and equipment were developed to broaden their application into many other fields including agriculture. The problems in the analysis of non-conducting powders of variable composition such as soils or plant ash were, however, considerably greater than those encountered in the analysis of metals and alloys.

In Britain the development of d.c. arc spectrographic methods for the analysis of biological materials was pioneered by Mitchell [28] at the Macaulay Institute. Inspired by the geochemical studies of Goldschmidt and of Mankopff, who used the cathode-layer arc source for the analysis of rocks and minerals, he developed the potential of the d.c. arc and devised methods for both semi-quantitative and quantitative analysis of non-conducting powders including soils [28]. The method is ideally suited to investigations involving large numbers of samples and for long-term projects and he brought its multielement capabilities to bear on many of the trace element problems prevalent in Scotland and elsewhere.

The widespread adoption of spectrographic methods of analysis at this time was due to the development of the photoelectric cell and other devices facilitating the measurement of spectral lines on a photographic plate. For some time, the opinion had been held that the d.c. arc source was not suitable for quantitative analysis and that its use should be restricted to semi-quantitative applications. Provided, however, that attention is paid to operating details and to choice of internal standard, relative standard deviations of 2—3% are obtainable. In a review of spectrochemical methods in soil investigations Mitchell [29] stated that "the d.c. arc is the most convenient means of obtaining an overall picture of the inorganic composition of a soil". His review was part of a complete issue of the journal Soil Science devoted to spectrochemistry in soil-plant research in which the editor F.E. Bear expressed the view that the emission spectrograph merited much more consideration than it had yet received from biological scientists.

At the same time, d.c. arc methods of soil analysis were being developed and used by Oertel [30] in Australia, by Rogers [31] and by Specht et al. [32] in the USA, by Pinta [33] in France, by Ivanov [34] in the U.S.S.R. and subsequently by many others working on trace element problems in agriculture. Others such as Ahrens and Taylor [35] had also effectively applied the d.c. arc to the analysis of rocks, minerals and soils. The latter workers and others have favoured anode rather than cathode excitation, but there is little difference in relative sensitivity between the two, although the former generally employs a larger sample.

The advantages of d.c. arc spectrographic analysis include speed, low cost, high sensitivity, the provision of a permanent photographic record, both of the elements sought and of others which may later turn out to be of interest, and a very wide range of elements which can be detected and estimated. At least seventy of the elements can be detected using the d.c. arc excitation

method. The main weakness is the instability of the arc and its tendency to wander about the electrode in a random manner. Numerous ideas such as the use of magnetic fields or tangential gas flows have been used to stabilize it. Sensitivity is known to vary with the matrix, electrode material, volatility, excitation potential of the element, the excitation source, the controlled atmosphere and other means of background control, and the characteristics of the spectrograph. Matrix variations can be a problem, but these can be overcome by use of a spectroscopic buffer, usually with some loss in sensitivity. It is important that the material for analysis should be in the same physical and chemical condition as the synthetic mixture used as the powder standard.

Although the d.c. arc source enabled the total contents of many elements in soil to be determined directly, it was not possible to determine the much smaller amounts of extractable trace elements, particularly cobalt and molybdenum. Concentration procedures to improve sensitivity were, therefore, devised to collect and concentrate groups of biologically important trace elements simultaneously prior to d.c. arc analysis [36]. Other selective preconcentration techniques that separate the analytes from the matrix in order to enhance sensitivity, such as solvent extraction and ion exchange, prior to spectrographic analysis, have also been developed.

The development of the photomultiplier tube for the detection and electronic measurement of spectral line intensities, in place of the photographic plate was a major advance. Direct reading spectrometers such as the Polychromator and the Quantometer became available and these greatly increased the speed and precision of analysis by eliminating the steps involving photographic processing and microphotometry. Their introduction has been accompanied by increased use of grating dispersion, not only for direct-reading spectrometers, but also for spectrographic work, since their linear dispersion gives them an advantage over prism instruments.

In recent years the inductively coupled plasma has proved a more popular source than the d.c. arc. Its use, however, normally entails dissolution of the soil, which can itself present problems, and entails a dilution of the sample. For solid geochemical samples, including soil, the sensitivity of the two sources is similar when this dilution effect is taken into account. The d.c. arc is a simple, inexpensive and easily operated source of excitation and, with the possibility of computerised evaluation of photographic spectra, will remain a very versatile and powerful analytical tool.

ELECTRON BEAM MICRO-ANALYSIS

Although Moseley [37] was the first to realise the possibility of chemical analysis through the examination of the x-ray spectrum generated by the electron bombardment of a specimen, it was not until 1949 that Castaing and Guinier [38] described an instrument called the "microsonde electron-

ique", or electron microprobe. In 1955 at a meeting of the Societe Francaise de Physique, Castaing displayed an instrument which served as a prototype for the first commercial instrument. An electron beam scanning capability was later developed by Coslett and Duncumb in 1956 and Birks [39] presents a detailed history of instrumental development in the mid-1950s. In Britain the Geoscan electron probe x-ray microanalyser was produced by the Cambridge Instrument Co. in the mid-1960s, which was specifically designed for geological and mineralogical applications. With such an instrument it was possible to carry out quantitative analysis on μm-sized features selected by optical microscope observation on, say, a petrological thin section. Since then the development has been on computer control, the introduction of energy dispersive detectors and the interfacing of x-ray spectrometers of one type or another to both scanning and transmission electron microscopes.

Two types of x-ray detector are available for microanalysis. In the crystal spectrometer a divergent beam of x-rays emanating from the specimen strikes a curved crystal and is diffracted according to Bragg's law. The curvature of the crystal converges the diffracted x-ray beam on to the detector, a gas flow proportional counter. The crystal and detector are mounted on motorised goniometer movements calibrated in terms of the Bragg angle so that as the angle of the crystal changes the counter sweeps through an arc to catch diffracted convergent x-rays.

In the non-dispersive or energy-dispersive spectrometer a liquid nitrogen-cooled, solid-state detector is used in which voltage pulses proportional in magnitude to the energy of the incident x-ray photons are produced. These pulses are sorted, measured and counted by a multichannel analyser, the output of which is displayed as a complete x-ray spectrum on a visual display unit.

The wavelength spectrometer is superior for the determination of light elements. Using special crystals and thin-windowed counters, elements down to atomic number 4 (beryllium) can be detected. It is also superior in accuracy and resolution for most other elements, e.g., detecting a small amount of Si in the presence of a large amount of Al or vice versa. However, wavelength spectrometers are mechanically complex, cumbersome, slow for qualitative analysis and require a high level of operator skill. Their size means that the detector is at some distance from the specimen, necessitating lengthy analysis times and high beam currents. Each crystal in the spectrometer is only useful for a small range of wavelengths so that there must be an arrangement whereby a selection can be made from several crystals. Although the Cambridge Instrument Co. were successful in interfacing a semi-focussing type of wavelength spectrometer to their early scanning electron microscopes, their use in this context has largely been eclipsed by the introduction of energy dispersive detectors. Nevertheless wavelength-dispersive spectrometry is still the definitive method of microanalysis for accuracy and is used to calibrate standards. Indeed the introduction of computer control, which relieves the operator of the tedium of manual manipulation,

has led to a revival of interest in wavelength spectrometry and fully focussing wavelength-dispersive systems are now available as options for most top-of-the-range scanning electron microscopes.

The spread of interest in microprobe analysis, particularly in conjunction with scanning electron microscopy, has undoubtedly been due to the introduction of the energy-dispersive detector. This small semi-conductor device can be fitted to most types of electron microscope and can be positioned very close to the specimen, collecting x-rays over a large solid angle which allows the use of short analysis times and small beam currents, the latter being desirable if a reasonably high resolution imaging system is required simultaneously with the microanalysis. The presence of a thin beryllium window through which x-rays must pass to reach the detector excludes low energy x-rays, but any element of atomic number >11 can be detected. Some recent detectors have been fitted with a removable window which extends the range down to atomic number 5 (boron).

The applicability of electron microprobe analysis, allied to scanning electron microscopy, to studies in soil mineralogy is best illustrated with examples. Figure 1a is a scanning electron micrograph of a cleaved biotite particle isolated from the C horizon of a soil profile at Rehiran in Nairnshire. A distinctive feature of these biotite flakes was a remarkably distinct white core when viewed optically. This white core can just be discerned in the electron micrograph by a slightly rougher texture as compared to the edge, but a potassium x-ray distribution image (Fig. 1b), produced by using x-rays characteristic of potassium to modulate the display, shows clearly that the central part of the flake is markedly depleted in potassium. Such a conveniently flat specimen lends itself to quantitative microprobe analysis where peak areas in an x-ray spectrum from a point of interest are compared with those in x-ray spectra from appropriate standards recorded under identical electron-optical conditions. To a first approximation the concentration of element A is given by [peak area (sample)/peak area (standard)] X (concentration of A in standard). Where several elements are present, inter-element interferences, which arise because of the physical processes involved in the emission of x-rays from a solid bombarded by electrons, necessitate the application of three correction factors. These are known as the atomic number (Z), absorption (A) and fluorescence (F) coefficients. Sophisticated and comprehensive software packages are now readily available for the mathematical treatment of spectra and computing the ZAF correction factors to produce quantitative data for a variety of scanning electron microscope (s.e.m.), microprobe and transmission electron microscope (t.e.m.) applications. For example, quantitative microprobe analysis of the two regions of the biotite flake depicted in Fig. 1a shows that the outer rim contains 5.5% K_2O while the inner core contains only 0.9% K_2O.

Another way chemical information can be displayed is illustrated in Fig. 1c. The micrograph is of iron oxide inclusions in a serpentinite as demonstrated by the accompanying Fe distribution image in Fig. 1d. This serpen-

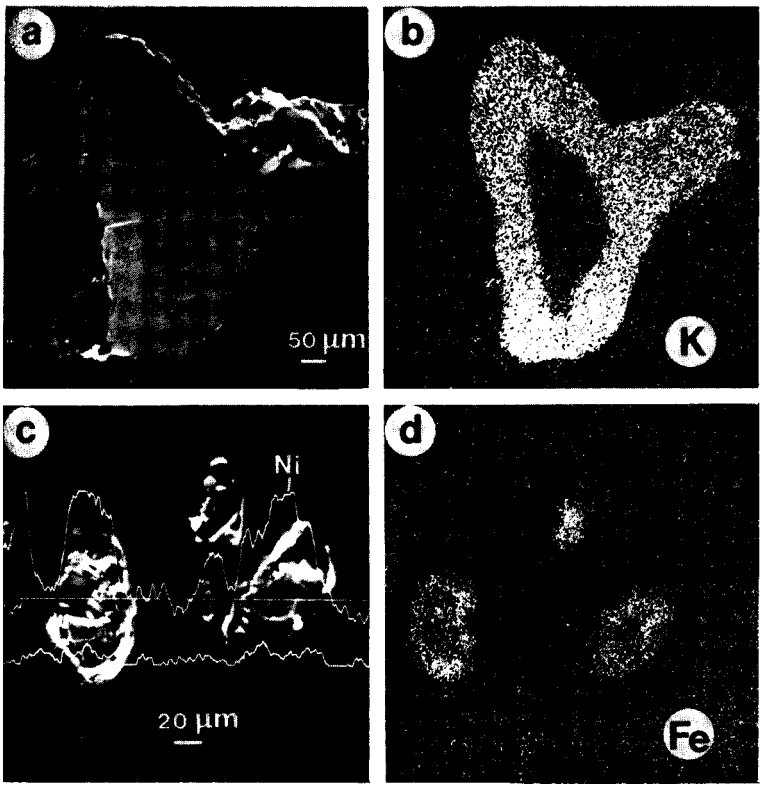


Fig. 1. (a) Scanning electron micrograph of a cleaved biotite flake separated from the C horizon of a soil profile; (b) a K x-ray distribution image of the same field of view as (a); (c) scanning electron micrograph of a serpentinite section containing inclusions, with a Ni concentration line profile across the field of view. The upper line is the profile, the straight line the line of analysis and the lower line a "background" profile; (d) an Fe x-ray distribution image of the same field of view as (c).

tinite contains Ni, and the amount of Ni in the iron-rich inclusions relative to the serpentinite is demonstrated by the Ni line profiles. The straight line along the middle of the micrograph is the line of analysis; the lower line, the background and the upper profile are the output from a ratemeter tuned to the Ni x-ray signal. It shows that although there is a little Ni in the serpentinite, there is about three times as much associated with the iron oxide inclusions.

An excellent account of the fundamentals and applications of electron-beam microanalysis with particular emphasis on the quantitative capabilities is given by Beaman and Isasi [40]. Microanalysis is also dealt with in a book on the microscopy of materials by Bowen and Hall [41]. Analytical electron microscopy, i.e., x-ray microanalysis of electron transparent films, is described in books by Chandler [42] and Hren et al. [43].

X-RAY PHOTOELECTRON SPECTROMETRY

It has long been recognised that surfaces play an important role in many processes that occur in soil, particularly in sorption and exchange reactions that are crucial in the retention and release of nutrient ions. Thus, it is clear that the explosion in the range and availability of surface analytical techniques which has taken place over the past decade [44] will have a considerable impact on the ability to describe and characterise the surfaces of soil components. However the application of these techniques to soils is still in its infancy. In view of this, the main features of one of the principal surface analytical techniques, x-ray photoelectron spectrometry (x.p.s.), previously known as electron spectrometry for chemical analysis (e.s.c.a.), will be discussed here in the context of its potential or actual application to soils and soil components.

In x.p.s., irradiation with x-rays causes photoelectron emission from both core and valence electron levels within the constituent atoms of the samples. The electrons have kinetic energies of 0—1500 eV and thus have a relatively short escape depth in solids, <100 Å, giving the technique a high degree of surface specificity. All elements of atomic number >2 may be detected which is a distinct advantage in studying a heterogeneous material such as soil. Indeed, it is interesting to note how few analytical techniques are capable of even detecting the presence of the most common element, oxygen, in the lithosphere. The spatial resolution of x.p.s. is very low and, although efforts are being made to extend it into the microscopic region, the areas currently being analysed are of the order of mm^2 [45]. In contrast, the energy resolution is high and this enables x.p.s. to be used very effectively in the characterisation of the electronic structure of solids through variation in peak positions, the presence of satellite structure and the splitting of particular photopeaks [46].

In principle, quantitative analysis using x.p.s. is straightforward for a homogeneous solid, with a simple proportionality between peak area and element concentration, the proportionality constant being given by the so-called cross section of the transition. In practice the relationship is much more complex with contributions from instrumental factors, surface contamination and considerations of the electron mean free-path in the solid phase [47]. For most of the elements encountered in clay minerals, interferences are uncommon and can normally be overcome by using another core line for the required element [48]. When a defined methodology and available data bases are used, accuracies of ca. 10% are attainable [49] although, using a series of aluminosilicates, Adams et al. [49] obtained an accuracy of 5% for Main Group elements. However, these quantitative schemes can only be rigorously applied to homogeneous solids and, in these cases, give the atomic composition of a surface layer of a thickness dependent on the nature of the material. In the case of heterogeneous powders many other factors may come into play but relatively little work has been

carried out. Sample preparation will clearly be of importance but, even with very good technique giving reproducible surface compositions, the physical nature of the analysed volume must always be borne in mind.

X-ray photoelectron spectroscopy has been widely used in adsorption studies where the high degree of surface specificity enables direct study of the adsorbed phase. An interesting example of a quantitative application of x.p.s. in the study of cation exchange by a beidellitic clay is provided by the work of Adams and Evans [50] who showed that the clay sorbed excess Ba^{2+}, K^{+} or Pb^{2+} during cation exchange compared to the amount of Ca^{2+} exchanged. By comparison with bulk chemical analysis, this excess sorption appeared to be associated only with the external surfaces of the clay and led to the postulate that these large cations can undergo cation exchange with edge sites normally inactive with respect to Ca. Potentially one of the most significant aspects of the use of x.p.s. in sorption studies is the multi-element capability of the technique with the consequent opportunity to study sorption from multi-component solutions. In this way co-operative effects, such as the effect of Ca on the sorption of silicate by gibbsite as observed by Alvarez et al. [51], may be fully explored enabling studies of nutrient sorption and release in solutions close in composition to the soil solution.

In addition to qualitative and quantitative analysis, x.p.s. can also provide structural information both from the binding energy of specific core-lines and spectral fine-structure associated with multiplet splitting effects and shake-up phenomena. For soil components, binding energy measurements are complicated by charge build-up on these electrical insulators. Charge referencing to some standard, either an element in the sample or an introduced species such as Au or more commonly C contamination can be used to normalise spectra, but some authors [52] have argued that charge neutralisation using an electron flood gun is a necessary prerequisite in the examination of silicates. Indeed, it has been suggested that the flood gun response can be used further to characterise the system. In the authors' experience peak broadening due to heterogeneous charging has proved to be a troublesome feature in some silicates and in these instances the use of an electron-flood gun can improve the situation without necessarily reducing the static charge to zero. Chemical speciation of adsorbed components has been widely studied and the early work of Counts et al. [53] showed that no redox processes were involved in the sorption of Pb by montmorillonite from Pb(II) solutions. On the other hand, more recent work by Murray and Dillard [54] has shown that, in the sorption of Co by manganese oxides, oxidation of the supplied Co(II) to Co(III) occurs during sorption thus accounting for the observed geochemical partitioning of Co and Ni.

In conclusion, it can be seen that x.p.s. is playing a valuable role in the study of processes and reactions of significance in soils and, indeed, in the natural environment as a whole.

INFRARED ABSORPTION

In the mid-1950s, the major application of infrared absorption (i.r.) spectrometry was to characterize organic compounds for which it was mostly used empirically as a fingerprinting technique. Applications to inorganic and mineral systems were relatively few. Even then, however, its potential to differentiate between complex anions and between molecular water and structural hydroxyl groups was already recognized. Gradually, as an understanding of the effects of composition and structure on i.r. spectra grew, and as better instruments and sample-handling techniques evolved, the application of i.r. spectrometry to mineral studies became a viable, and indeed vital, adjunct to the more widely used x-ray diffraction and thermal methods of analysis. Infrared spectrometry was seen to have a unique role to play in soil analysis.

Soil is a complex, heterogeneous assemblage involving both organic and inorganic components that range from relatively simple compounds to highly condensed polymeric structures. In addition, these components may be crystalline or amorphous, and have either fixed or variable composition, and all of them will make some contribution to the i.r. spectrum. The i.r. spectrum of whole soil is, therefore, too complex to provide anything but the most elementary information. Fortunately, soil can be readily separated into organic and inorganic fractions, and separate study of these by i.r. spectrometry with reference to pure analysed standard reference material has provided much information.

Inorganic soil clay minerals

Among the most important of the clay minerals are the layer silicates, and it was the pioneering work of Farmer [55], Tuddenham and Lyon [56], Stubican and Roy [57—59], Farmer and Russell [60] and Vedder [61] which laid the groundwork for an understanding of the relationship between the i.r. spectrum of a mineral and its chemical composition and structure. From the early deuterium exchange work of Stubican and Roy and their studies of synthetic minerals of known composition, combined with a knowledge of crystal symmetry and lattice vibrations, it has become possible to make very full, if not complete, assignments of the i.r. spectra of layer silicates.

Similar advances in interpretation of i.r. spectra of the other types of silicate minerals and also the carbonates, phosphates, sulphates etc., have meant that the structures of the major mineral types to be found in soil are now understood in some detail, and have provided possible explanations of soil properties. The monograph by Farmer [62] gives excellent coverage of these topics. In soil-mineral analysis, i.r. spectrometry is almost unique in providing direct structural information on the inorganic components amorphous to x-rays, particularly the aluminosilicate allophanes. The early work by Leonard et al. [63], again on synthetic model compounds, clearly showed

that their i.r. spectra gave a direct indication of the degree of substitution of Si by Al, a structural feature responsible for the cation exchange properties of these compounds.

Infrared spectrometry has been of paramount importance in recognizing the presence of imogolite and its related allophane in many acidic soils [64]. Their presence in such soils, including podzols, provides strong evidence for the translocation of Al, Fe and Si as a soluble inorganic complex during horizon development.

Perhaps the most important contribution that i.r. spectrometry has made in layer silicate research is in the characterization of the expansible interlayer region in montmorillonites and vermiculites. These minerals in the clay fraction of soil, play a key role in the adsorption of most soil additives (fertilizers, herbicides, pesticides, conditioners) and i.r. spectrometry gives direct information about the sites and mechanisms of adsorption, and the molecular orientation of the adsorbed species: Fripiat et al. [65], Mortland et al. [66] and Russell [67] showed that basic molecules, organic and inorganic, were protonated at the clay surfaces and adsorbed as cations; Serratosa [68] and Farmer and Mortland [69] showed that, depending on the layer charges on the silicate and its hydration state, the aromatic ring of the pyridine molecule may lie either parallel to the layer silicate sheets or perpendicular to them.

Of the interlayer species in layer silicates, water is probably the most significant agriculturally. Infrared spectrometry has again provided fundamental information concerning its site of adsorption and its function. One proton of each water molecule is weakly hydrogen bonded to silicon-oxygen surfaces, the other being directed towards exchangeable interlayer cations [70]. In this arrangement the water molecules in the interlayer redistribute positive charge on the interlayer cations to the sites of negative charge on surface oxygens arising from Al-for-Si or Mg-for-Al substitution in the silicate lattice [7].

A unique contribution has been made by i.r. spectrometry to the study of the surfaces and surface reactions of minerals. Much of the early work was concerned with the field of catalysis and involved treatment at high temperatures before the surface OH groups were detectable by i.r. spectrometry. While many of the catalysts have some relevance to soil, for example montmorillonite clays and natural zeolites, the pretreatments did not. The observation that the surface OH groups of synthetic goethite and gibbsite (oxyhydroxide, hydroxide of iron and aluminium, respectively) that occur widely in soil, could readily be detected by merely removing adsorbed water by evacuation at room temperature [72], is of great significance for soil in providing a model for subsequent sorption studies involving fertilizer anions such as phosphate, sulphate and nitrate. The i.r. results allowed the sorption mechanism and the structure of the adsorbed species to be clearly defined.

Organic soil components

The strength of i.r. spectrometry in this application to soil analysis lies in its empirical recognition of the absorption patterns of various types of organic compound, e.g., protein, polysaccharide, in acidic and neutral fractions of organic matter separated by physical methods. In this context, the identification of specific functional groups, OH, NH, COOH and C=C, is also of great value in characterizing what is, after all, a random, heterogeneous and amorphous polymeric material. Stevenson and Goh [73] provided evidence for the existence of three spectral types of humic and fulvic acid. One of the fulvic fractions contains predominantly neutral polysaccharide and protein, while the other contains little or no polysaccharide but is very rich in carboxylic acid groups. This second fulvic acid has been shown by i.r. spectrometry to be modelled very closely by a synthetic polymer of maleic anhydride [74], an observation which has stimulated renewed interest in structural studies of soil organic matter using i.r. spectrometry.

Future development

Research on soil components by means of i.r. spectrometry to date has used conventional spectrometric methods involving transmission or reflectance measurements on pure minerals or separated soil fractions. With the rapid development of Fourier-transform i.r. instruments capable of utilizing, in a reflectance mode, the microprobe facility of a microscope, direct study in whole soil of individual mineral grains or surfaces of <1 mm diameter has become possible. With such instrumentation, the ability to identify organic and inorganic components directly in soil pores, at root surfaces and in coatings on mineral grains would open up the whole field of soil research, keeping i.r. spectrometry at the forefront as an investigative technique.

INDUCTIVELY-COUPLED PLASMA OPTICAL EMISSION SPECTROMETRY

Origins and capabilities

The first analytical applications of inductively-coupled plasma optical emission spectrometry (i.c.p.o.e.s.) were reported in 1964 by Greenfield et al. [75], followed a year later by Wendt and Fassel [76]. Today i.c.p.o.e.s. is an established technique for elemental analysis comparing favourably with, and offering complementary capabilities to, atomic absorption spectrometry, d.c. arc spectrometry, x.r.f. spectrometry, neutron activation and spark-source mass spectrometry [77].

The sensitivity for solution samples falls in the range from 0.1 ng ml^{-1} for elements such as Ca, Mg and Sr up to 0.1 μg ml^{-1} for non-metals such as P and S [78]. The linear dynamic range extends from the detection limit over five orders of magnitude making it possible to perform simultaneous analysis for elements at the major, minor and trace levels. The technique is free from chemical interferences, but is subject to additive interferences from spectral-

line coincidences and to multiplicative interferences caused by the chemical matrix. The latter may be non-specific resulting from changes in the physical properties of the solution, e.g., due to viscosity changes between the standards and samples, or may be related to the presence of high concentrations of easily ionizable elements in the sample. Unless cationic concentrations are high ($\geqslant$1%), non-specific interferences are adequately controlled by ensuring consistency of the molarity of the acid medium between standards and samples. Interferences due to the presence of easily ionizable elements are reduced to acceptable levels (1—2%) by appropriate selection of the operating parameters and in concentrated solutions by matrix matching of the standards [79]. In routine use, i.c.p.o.e.s. provides a r.s.d. of the order of 1% for elements in concentrations 100 times greater than the detection limit [80].

I.c.p.o.e.s. is primarily a solution technique and therefore quoted detection limits should be increased by one to three orders of magnitude when analysing solid samples, depending on the dissolution procedure employed [81, 82]. The preparation of solid samples is invariably the rate-determining step in an analysis and this, together with the dilution factor incurred in dissolution, must be regarded as a disadvantage when comparing the i.c.p. with techniques such as d.c. arc spectrometry, x.r.f. and spark-source mass spectrometry that handle the solid directly. These drawbacks, however, are compensated for by the relative ease of standardization of solution techniques, although the time spent on calibration and quality control procedures occupies a significant amount of time.

Applications to soils and plants

The analysis of soil extracts by i.c.p.o.e.s. is mostly straightforward [83—88], although the levels of extracted cations in, for example, neutral ammonium acetate [83, 85] can lead to additive and multiplicative interferences and occasionally to blockage of the nebulizer or torch injector tube. Boron in water extracts of soils is readily determined [89], provided that the co-extracted Fe levels are not too high. The sensitivity for some elements, for example Mo, is inadequate, necessitating the use of pre-concentration procedures such as liquid—liquid extraction [90]. Sensitivity is also the limiting factor in the determination of As and Se, although this can be overcome using the hydride generation technique [91]. I.c.p.o.e.s. is particularly suited for multi-element screening, as in the determination of heavy-metal pollutants in aqua-regia extracts of sewage sludges, prior to disposal to the land [92].

Whole soils may be analysed by i.c.p.o.e.s. [93, 94], but fusions or the use of hydrofluoric acid are required to dissolve the silica fraction. In the case of fusions a flux/sample ratio of ca. 4:1 and the dissolution step impose a considerable dilution; this requires matrix matching of the standards to avoid multiplicative interferences. The use of hydrofluoric acid is best avoided unless resistant materials are used in the construction of the sample introduction system and torch injector.

Plant tissues are widely analysed by i.c.p.o.e.s. following sample preparation by dry ashing or acid decomposition procedures [81, 83, 85, 89, 95]. For some elements, notably Cd, Co, Ni and Pb, the levels in solution are close to the detection limit. The high levels of K in plant tissue may result in salting-up of the nebulizer or torch injector, necessitating frequent cleaning of the sample introduction system. The catalytic Kjeldahl decomposition of plant materials is used in many laboratories in the determination of N and the resulting solution can conveniently be analysed by i.c.p.o.e.s. for major and trace cationic constituents.

Future developments

In recent years much research has been devoted to exploiting the inherent sample introduction versatility of the i.c.p. with the result that solid samples and liquid microsamples can be handled by direct-insertion graphite probes [96], or indirect electrothermal vaporization [97] and for conducting materials by spark erosion [98]. The increasingly important topic of elemental speciation is being addressed by using the i.c.p. as an element-specific detector for chromatography, most notably for high-performance liquid chromatography (h.p.l.c.) [99]. Potentially the most important advance has been the use of the i.c.p. as an ion source for mass spectrometry [100—104]. With this technique sensitivities in the sub-ng ml^{-1} range are attainable and the ability to determine isotopic concentrations and to use isotope dilution internal standardization will greatly facilitate the use of stable-isotope tracers in the biological and environmental sciences.

ATOMIC ABSORPTION SPECTROMETRY

In the three decades since Walsh [105, 106] and Alkemade and Milatz [107, 108] independently realized the analytical potential of atomic absorption spectrometry (a.a.s.), this technique has revolutionized the determination of elements in solution. It is now a ubiquitous and indispensible tool for the analytical chemist in most disciplines. While practical flame atomic absorption spectrometers originated in the designs of Walsh [109, 110], their use of an air/acetylene flame into which a sample solution was nebulized derives from Lundegardh's [111] earlier technique of flame emission spectrography for the analysis of agricultural materials. The first practical applications of flame atomic absorption spectrometry were also agricultural, and were to the determination of magnesium in soil extracts and other agricultural samples [112]. Since this time, flame a.a.s. has found a major role in the soil laboratory.

Initially a.a.s. was based largely on atomisation in an air-acetylene flame of sample material introduced into it by a pneumatic nebulizer. A hollow-cathode discharge lamp is used as the atomic line source with wavelength isolation and detection by means of a grating monochromator and photomultiplier tube. The introduction by Willis [113] in 1965 of the high-

temperature, reducing, nitrous oxide-acetylene flame facilitated the analysis of those elements, such as Al, Ba, Be, Ca, Mo and Si, which form refractory oxides and are incompletely atomised in the lower-temperature air/acetylene flame. A wide range of commercial instruments on this basic format is available and extends from manually operated systems to sequential multi-element instruments operating under computer programme control with automatic sampling and concentration read-out.

In the soil laboratory, flame a.a.s. is used for the determination of the total contents of major and some minor cations in soils. For true total contents, either a fusion with lithium metaborate and dissolution of the resulting glass in nitric acid [114] or a treatment involving the use of hydrofluoric acid, often in combination with perchloric acid [115], is necessary. Another preparative procedure commonly used is the digestion of soil with aqua regia, with or without prior ignition [116] or treatment with hydrogen peroxide [117] to remove organic matter. As this method does not dissolve many silicates and some other minerals, near-total contents are only determined for a limited number of elements but these do include the common pollutant elements copper, zinc and cadmium. The method is thus best used to estimate the extent of soil pollution by environmental contaminants from, for example, industrial sources or sewage sludge applications. Elements for which total contents of aqua regia-soluble contents of soils can be determined by flame a.a.s. are listed in Table 1, which takes into account the sample dilution involved in preparation of solutions and the detection limit of the technique.

The total element content of soils is often of less agricultural interest than the extractable content, and flame a.a.s. has found its main use in soil laboratories for these latter determinations. The advantages of flame a.a.s. over other solution analytical techniques of element specificity, sensitivity, freedom from interference and simplicity of operation, are such that it has dominated this area of elemental analysis for twenty years. A wide range of extractants is used to assess the availability of the different elements to plants, measure fertilizer requirements, assess the extent of soil pollution and determine the cation exchange capacity of soils. They include neutral salt extracting solutions such as $CaCl_2$ or $NaNO_3$, as well as acetic acid, EDTA, DTPA, ammonium acetate, acid ammonium acetate/EDTA, ammonium chloride, hydrochloric acid, nitric acid and aqua regia. Elemental analysis can be carried out in all these media by flame a.a.s. and is limited only by the element concentration in the extracts, as indicated by Table 2 for acetic acid and EDTA extracts. Details of some of the various extractants used are given in [118–121].

It can be seen from Table 2 that, on sensitivity criteria, only Mn and Zn are determinable by flame a.a.s. in both EDTA and acetic acid extracts, while the other elements listed are likely to be present in measurable concentrations only in EDTA extracts and not in acetic acid extracts.

Instruments are also available with graphite furnace (electrothermal)

TABLE 1

Soil total contents by flame a.a.s.

Element	World mean soil content[a] (mg kg^{-1})	Analysis soln. content, $LiBO_2/HNO_3$[b] (mg l^{-1})	Expected analysis soln. content, aqua-regia digest (mg l^{-1})	% Extracted by aqua regia	Flame a.a.s 5× det. limit (mg l^{-1})
Al	6.7%	67	320	40	0.09
Ba	1000	1	270	(50)[d]	0.1
Ca	2.0%	20	150	50	0.01
Cd	0.62	NM	0.007	70	0.004
Co	12	NM	0.2	70	0.04
Cu	26	NM	0.5	80	0.01
Fe	3.2%	32	600	80	0.03
K	1.8%	18	110	20	0.01
Li	31	0.03	0.04	(20)[d]	0.008
Mg	0.8%	8	120	70	0.001
Mn	760	0.76	11	70	0.01
Ni	34	NM	0.3	50	0.04
Pb	29	NM	0.5	70	0.1
Si	33%	330	NM		1.0
Sr	280	0.3	2	(50)[d]	0.01
Ti	0.5%	5	6	(20)[d]	0.25
Zn	60	NM	1.4	90	0.005

[a]Ref. 145. [b]NM, not measurable. [c]Sensitivity limitation at below average contents. [d]Estimated values.

TABLE 2

Soil extractable contents by flame a.a.s.

Element	Normal range (mg l^{-1}) in extract		5× Flame a.a.s. det. limit (mg l^{-1})
	0.05 M EDTA	0.43 M Acetic acid	
Cd	0.002—0.06	0.0003—0.008	0.004
Co	0.01—0.8	0.001—0.05	0.04
Cr	0.02—0.8	0.0003—0.03	0.03
Cu	0.06—2	0.001—0.08	0.01
Mn	1—20	0.1—2.5	0.01
Ni	0.04—1.0	0.003—0.1	0.04
Pb	0.2—2	0.00005—0.1	0.1
Ti	0.2—2	0.003—0.03	0.25
V	0.04—1	0.001—0.03	0.25
Zn	0.6—4	0.05—0.75	0.005

atomisers in place of the flame. These became available commercially in 1970 and were based largely on a design by Massmann [122], which followed pioneering research by L'Vov [123], Woodriff and Ramelow [124], West and Williams [125] and others. While these atomisers offer detection limits 10^3 times superior to those obtainable by flame a.a.s., problems of matrix and vapour-phase interference effects have limited their use in the direct analysis of soil extracts and digests [126].

Atomic absorption spectrometry and its application to soil analysis [127] and agricultural materials [118] have recently been discussed and a review of the literature on applications in agricultural and water analysis has been published by Kalra [128].

Despite the attraction of the much higher sensitivity of graphite furnace atomic absorption spectrometry (g.f.a.a.s.), little use has so far been made of it for the direct analysis of soil extracts or digests largely because of interference effects from the complex soil matrix. In combination with liquid—liquid extraction separation procedures, g.f.a.a.s. has been used to some extent for the determination, for example, of cadmium [129] and cobalt [130] in acetic acid extracts of soils. The decrease of vapour-phase interference effects obtained by the use of the L'Vov platform [131] and the sampling probe [132, 133] in g.f.a.a.s. may make this technique more attractive to the soil analyst. The development of totally pyrolytic graphite-furnace tubes promises to eliminate the progressive change in sensitivity with lifetime that occurs with tubes coated with pyrolytic graphite, to prolong lifetimes, and to increase precision and heating rates [134]. The almost unrivalled high sensitivity of g.f.a.a.s. has found application in the determination of Mn and other trace elements in soil solutions obtained by centrifugation [135], and in speciation of trace elements such as Cd and Pb in solutions derived from polluted soils [136]. In this latter application g.f.a.a.s. is appropriate, because of its sensitivity and small sample requirements, for the analysis of the fractions obtained by h.p.l.c. This hybrid technique offers one of the few avenues to trace element speciation in soil extracts and solutions.

Of other specialised forms of a.a.s., hydride generation methods have found application mainly for arsenic and selenium in soils [137, 138] while the generation of arsine directly from soil has been demonstrated as an analytical technique [139]. Another technique which generates a vapour-phase sample, the cold vapour, reduction-aeration procedure for the determination of Hg, has been widely applied to soils [140], and the technique and its applications have been reviewed [141].

The development of atom-trapping atomic absorption spectrometry (a.t.a.a.s.), which offers an in situ preconcentration procedure in the flame, has provided sensitivities up to 40 times greater than conventional flame a.a.s. This technique has been used for the more volatile elements such as Pb and Cd in soil extracts [142] and, in particular, for Cd in calcium chloride extracts because it does not suffer from the interference problems that occur with this matrix in flame a.a.s. and g.f.a.a.s.

Radical new instrumental approaches being developed include multi-element atomic absorption spectrometers which differ from most previous, brute-force, multiple spectrometer techniques [143] in using a continuum light source and a high-resolution monochromator [144].

In conclusion, flame a.a.s. has become the indispensible work-horse in the soil laboratory and, because of its simplicity, cheapness and element specificity, is likely to remain so despite competition from newer analytical techniques. The uniquely high sensitivity of g.f.a.a.s., not yet fully exploited in soil laboratories, offers still greater opportunities in the future both for research and routine analytical purposes.

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ELECTROANALYTICAL VOLTAMMETRY IN FLOWING SOLUTIONS

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SUMMARY

A review is given of the principles, key developments and representative applications of small electrodes in flow-through electrochemical (ec) detectors having very low effective dead volume (<10 μl) for voltammetric and amperometric detection in flowing solutions. Emphasis is placed on factors contributing to high sensitivity, reliability and selectivity of ec detection as an integral part of larger analytical systems utilizing continuously flowing, unsegmented streams, e.g., flow-injection and liquid chromatographic analyses. Solid and mercury electrodes are considered under potentiostatic and potentiodynamic control. A review is given also of auxiliary chemical and photolytic derivatizations coupled to ec detection. The majority of the literature on the subject relates to liquid chromatography with electrochemical detection (l.c./ec); however, applications of these concepts to specific examples in flow-injection systems, as well as for on-line process control, should be obvious. Details of chromatographic separations and design of total analytical systems are not reviewed.

INTRODUCTION

Several factors have motivated the recent flurry of analytical applications of electrochemical (ec) detection in flowing solutions. These include a tendency in chemical analysis to reject time-consuming methodologies of conventional voltammetric electroanalysis applied to static solutions in macrocells (e.g., 50—500 ml) which are usually associated with manual

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transfer of solutions and lengthy deaeration periods. Although the majority of these procedures undoubtedly will be automated in the future using robotic systems, the recent trend has been toward the automation of discrete analysis of small samples (i.e., 10—1000 μl) with high analytical throughput (e.g., 10—200 h^{-1}) by flow-injection (f.i.) analytical systems. For highly complex samples, of course, incorporation of a separator column into the f.i. system provides for on-line resolution of mixtures by the technique of liquid chromatography (l.c.). Voltammetric methods have never been rightfully characterized as having high resolving power for complex mixtures. Consequently, most of the literature pertaining to ec detection in flowing solutions has been directed to l.c./ec. It is very common, however, that new detection principles and previously unstudied chemical systems are characterized by f.i./ec before application in l.c./ec. Emphasis is given here to the general principles of ec detection in flowing solutions and, in cases cited from l.c./ec, the interested reader should consult the original literature for chromatographic details.

The primary analytical strength of voltammetric detection appropriate for application in f.i. and l.c. systems is the high sensitivity with relative simplicity and economy. To illustrate: a detection current of 1 nA over a period of 1 s is easily measured by a cheap ec system. Yet, for a typical electrolytic conversion efficiency of 10%, this signal corresponds to only 10^{-14} mol of analyte reacting with n = 1 eq mol^{-1}. Furthermore, the effective dead volumes of flow-through ec detectors can be very small (<ca. 10 μl) so that the detection process does not produce appreciable band broadening in either f.i./ec or l.c./ec. The principles of optimum design and operation are considered in this review by S. G. Weber.

The primary electrode material for reductive detection is liquid mercury, and applications of renewable mercury electrodes (i.e., DME, SMDE) are reviewed by A. M. Bond. Coverage of both potentiostatic (d.c.) and potentiodynamic (pulse) approaches are included. Numerous solid materials are applicable as electrodes for ec detection and the unique characteristics of the most useful solid electrode materials are described by R. M. Wightman. Specific applications of potentiostatic (d.c.) detection for solid electrodes are reviewed by R. E. Shoup, including supported mercury-film electrodes, because these electrode surfaces are not easily renewable. In contrast to the renewable mercury electrodes which have relatively inactive surfaces, most solid and non-renewable electrodes exhibit interesting surface phenomena which can significantly influence the reversibility of reactions and the long-term precision of the ec response. Potentiodynamic detection at solid electrodes is treated separately by D. C. Johnson.

One characteristic unique to the use of unsegmented flow systems is particularly advantageous for ec detection. That is the ease of mixing reagent streams, including supporting electrolytes, with the sample stream to promote the detection process. Accordingly, reagents which alter the form of the analyte or mask interferences can render ec detection more sensitive

and more selective. It is of very great significance that on-line derivatizations need not be quantitative nor even at equilibrium. This results because the flow rates (i.e., reaction times) and system temperature can accurately be controlled so that even non-equilibrium processes lead to a highly reproducible detector response. As one unique consequence, photons can be used for on-line derivatizations prior to ec detection even though the photolytic reactions are far from being quantitative. The use of ancillary chemical and photolytic processes to improve ec detection is reviewed by I. S. Krull.

Background information to topics presented here is available. Kissinger and Heineman [A1] have presented an excellent general introduction to electroanalytical chemistry and Chapter 22 of their book introduces flow-through ec detection. An earlier text by Adams [A2] continues to provide useful information on voltammetry at solid electrodes and the subject at mercury electrodes was reviewed more recently by Bond [A3]. A text by Ruzicka and Hansen [A4] provides a useful resource for flow injection analysis, and an excellent introduction to liquid chromatography has been written by Snyder and Kirkland [A5].

B. PRINCIPLES OF ELECTROANALYTICAL VOLTAMMETRY IN FLOWING SOLUTIONS

Electrochemical detection in flowing solutions has been used chiefly as a tool for the quantitation of chemical species. Although its use in f.i. determinations has been reported, by far the largest application has been in liquid chromatography. Recently, qualitative aspects of ec detection have received some attention. The concerns of the designer of ec cells for quantitative applications are the linearity of the response, precision and long-term stability. For qualitative applications, the concerns are for a cell design which allows for accurate control of electrode potential, and a response time that is adequate for the voltammetric or amperometric task. In either case, the sensitivity and detection limit are of concern, as is the selectivity of the detector and the predictability of the signal. These characteristics are interrelated and concern over them has led to a significant quantity of research, mostly in the last decade or so. Work on the effects of the detector on peak shape, its ability to do voltammetry, its efficiency in electrolysis, contributions to imprecision, and its selectivity will be discussed.

Effect on peak shape

From the perspective of l.c./ec, the detector should not worsen a separation. An understanding of dead volume is required to appreciate whether a given detector will affect a given separation. The dead volume of any detector may be less than or greater than the physical volume of the detector, because much of the dead volume in a typical detector results from the connecting tubing between the column and the detector inlet. This

problem has been solved to allow microbore [B1, B2] and high-speed [B3] detection. Comments here are restricted to the effective dead volume in the region sampled by the transducer. In many liquid chromatographic detectors, e.g., u.v./visible absorbance, the physical volume and the dead volume are synonymous. This is not true for electrochemical detectors. The fact that the ec detector only detects that which is near the electrode, i.e. within the hydrodynamic boundary layer, can also be useful. The large-volume wall-jet detector squirts the chromatographic effluent at a high velocity through a quiescent liquid reservoir to impinge perpendicularly on the surface of an electrode. This cell, although it may have a physical volume of $\geqslant 1$ ml [B4—B6], demonstrates an effective dead volume of a few μl, which is acceptable for normal chromatographic applications. This is because the hydrodynamics are favorable and reproducible [B6]. Because the jet of fluid from the column passes through another fluid, one can establish favorable conditions for detection by allowing interdiffusion of species in the two fluid domains. This has been used to add supporting electrolyte to the effluent so that electrochemical detection can be accomplished in normal-phase chromatography [B5].

Some detectors are designed to be 100% efficient. In these cells, every analyte species strikes the electrode surface at least once. Whether or not electrolysis occurs depends on the species and the electrode potential. In these detectors, commonly called "coulometric" because the integrated signal is related to the moles injected by Faraday's law [B7], the average concentration of the species being detected decreases significantly as the effluent passes through the detector. It is therefore seen that the downstream portion of the electrode may contribute nothing to the signal, and nothing to the dead volume. Thus, dead volume for coulometric detectors can be far lower than the physical volume of interstitial liquid [B8].

Detectors have a finite response time, perhaps regulated electronically, and this can contribute to the apparent dead volume [B9]. The electrochemical detector has a transient response limitation due to the establishment of the steady-state hydrodynamic mass transport. Studies in which rectangular concentration pulses have been applied to the detector have been able to quantitate the extent of the dynamic contribution to the dead volume [B9].

The flow-through ec detector as a voltammetric cell

Most applications of electrochemical detectors involve a constant potential and the "d.c." or low frequency signal is measured. Under these conditions, the demands on the design of the cell are few. Many workers have shown that the placement of the auxiliary electrode close to the working electrode extends the linear dynamic range to higher quantities in comparison to remote placement of the auxiliary electrode [B10]. The choice of a reference electrode is usually made based on convenience. Thus, a Ag/AgCl reference electrode is often used, though the calomel electrode is less temperature sensitive [B11, B12].

It often is helpful to voltammetrically augment the resolving capability of chromatography to identify one component of a mixture. In fact, recent reports [B13, B14] have shown that in a complex mixture it is very unlikely that a single peak represents one and only one component of the mixture. These results make even more important the ability to determine, if not a complete voltammogram, then at least a measure of "peak purity". When such measurements are contemplated, more attention must be paid to the design of the cell for accurate electrochemical data production.

Voltammetry is the measurement of current as a function of controlled potential. One can obtain two points on a voltammetric (i—E) curve using two electrodes at two fixed potentials [B15]. That principle is extendible, of course, to many electrodes [B16, B17]. In such cases, appropriate placement of the auxiliary and reference electrodes is more important because of the need for accurate control of the various potentials. Detectors in which the potential is altered as a function of time also have been reported [B18—B25]. A variety of waveforms has been used: square wave [B18, B19], coulostatic [B20], staircase [B21—B23] and triangle wave [B24, B25]. One sacrifices detection limit in order to obtain the voltammogram because of the larger bandwidth required for the voltammetry [B20] and because of the influence of alteration of the potential on the surface processes of the electrodes. Details of instrumentation and problems posed by voltammetry are considered by Reardon et al. [B23].

While voltammetry does not possess the resolving power of chromatography, it is still possible that the extra information in the chromatovoltammogram (i—t—E) will aid investigators in the future, especially when dealing with highly complex samples of variable composition, e.g., fuels and blood sera. In one report [B17], it was clearly shown that the voltammetric information clarified the analysis of human body fluids for a variety of neuroactive species. One might predict that l.c./voltammetry will be useful in chemical research where voltammetric characteristics can be determined for small quantities of compounds that have been purified by chromatography. Conditions of flow that result in flow-insensitive voltammetry have been given [B25], allowing the luxury of comparing data obtained in a flow-through system to theory developed for static systems.

Mass transport rate

No signal is obtained from a solute if the solute does not reach the electrode surface. Most of the analyte in any cell is not located at the electrode surface; thus, the analyte must be transported to the surface. The study of mass transport phenomena is as basic to the understanding of electrochemical detectors as the study of optics is to the understanding of absorbance detectors.

Of the material that flows into a cell, if the applied potential is sufficient to drive the concentration of the analyte to zero at the electrode surface, a certain fraction will be oxidized or reduced. When this fraction is 1.0 the

cell is called "coulometric", even though current, and not charge, usually is measured. These cells have the advantage that, in principle, they require no standards because the total charge passed for a certain number of moles of analyte is given exactly by Faraday's law. Recently, this has been put to practice in the determination of the purity of several materials [B26]. Generally speaking, however, cells with a conversion efficiency of 1.0 have been used less often than their less efficient counterparts, the so-called "amperometric" detectors. One application of the coulometric detector that is gaining acceptance is as an electrochemical "filter" [B17, B27]. Here, a coulometric cell located on the upstream side of another detector is used to remove unwanted electroactive species by in situ pre-electrolysis.

Many workers have solved the mathematical problem of predicting the electrolysis efficiency of cells with efficiencies near 1.0 as a function of conditions of design and operation. Only the most recent comprehensive attempt for open channels [B28], and an extensive review for packed beds [B29] are mentioned. In both cases, the analyte concentration decreases exponentially as the channel is traversed. As a rule, the cell will achieve complete conversion in a smaller volume for a larger value of the surface-to-volume ratio.

Detectors with solid, liquid or amalgamated working electrodes have been used in which the efficiency is much less than 1.0. These cells have been called "amperometric". Many varieties of cell geometries have been explored both theoretically and experimentally. Most hydrodynamic electrochemical systems have been studied over the course of two decades by Matsuda and co-workers. Of particular interest is their work on thin-layer and tubular systems [B30], the wall jet [B31] and rotationally symmetric [B32] systems. The last is relevant to detectors based on the mercury-drop electrode. Because the theory is often borrowed from other fields, usually heat transfer, the interpretation of the applicability of a certain theory is often a difficult one. As a consequence, there has been confusion in the literature about the correct theory to apply to thin layer cells. This problem recently has been reviewed [B33]. There has also been confusion as to the appropriate theory to apply to cells of the wall-jet geometry. Recently, it has been theoretically and experimentally demonstrated that the behavior of the cell depends on whether or not the size of the cell allows for unrestricted natural development of the hydrodynamic boundary layer [B4]. The hydrodynamic theory and applications of the large volume, wall-jet electrode have been discussed [B5, B6]. The three most studied geometries, the tube, thin layer or channel and the wall-jet, all have about the same theoretical efficiency, given the same flow rate and electrode area [B34]. Although much effort has been expended to understand mass transport in flow-through cells, few optimization schemes have surfaced that make use of experimental data. One scheme by Roe [B35] is reasonable because it includes signal, noise and band broadening. Mercury drop [B36] and solid [B37] electrodes have been compared experimentally. In both studies, eluent flow perpendicular to the electrode surface gave the best results.

The electrochemical detector works well because its mass-transfer efficiency is good. The mass-transfer efficiency can be increased in a number of ways to obtain higher sensitivity. Application of pulsed potential waveforms results in higher sensitivity, especially at low flow rates [B38], although an increase in detection limit also occurs [B39]. One can increase also the hydrodynamic assistance to the mass transfer by rotating the working electrode [B40]. Advantage can be taken from lateral or radial diffusion. In conventional electrochemical cells with large electrodes, most of the analyte diffuses to the electrode from a region of solution defined by the imaginary projection of the electrode perpendicularly into the solution. Typically, the amount of current due to material arriving from outside this volume is negligible. If the electrode has a large perimeter-to-area ratio, then lateral or radial diffusion will bring substantial amounts of material to the surface from outside the described volume. This edge effect was first pursued for analytical advantage by using very small electrodes [B41]. The same effect is obtained from composite electrodes, i.e., electrodes made of finely divided conducting material embedded in an insulator, or vice versa. These electrodes, and particularly the Kel-F/graphite formulation, have been shown to offer advantages over ordinary electrodes. Recent works show that these electrodes are less flow sensitive, are easier to use at high potentials, and less noisy at all potentials than ordinary homogeneous electrodes [B42, B43]. An interesting alternative to the plastic/carbon-powder composite electrode is the epoxy-impregnated, reticulated, vitreous carbon electrode [B44].

Noise

Considerations in cell design for low noise performance are not substantially different from general practice in electronics. For general practice, the book by Ott [B45] is short, readable, and informative. Early work in the field [B46] identified several sources of noise, including electronics and the wetting of "pores" in the electrode. The magnitudes of the noise from these sources were assumed to be determined, at least in part, by the area of the electrode. More recent work has shown that the noise in electrochemical cells can be modeled using electronic equivalent circuits [B13, B47]. For frequencies above 1 Hz, the noise is dominated by voltage fluctuation in the current-to-voltage converter that creates a current because the fluctuations are impressed across the interfacial capacitance (and pseudocapacitance, which may be more important for solid electrodes, especially carbon, than for mercury). The larger the capacitance, i.e., the electrode area, the greater the noise. Unfortunately, the most nefarious noises are those at lower frequencies, usually called drift. Variations in environment, especially temperature [B12, B13, B47], cause baseline fluctuations. Changes in the chemical environment, e.g., from very late eluting compounds in l.c./ec, also cause baseline fluctuations. The use of a coulometric guard cell, as mentioned above, can help decrease noise from this source.

The signal-to-noise ratio (s/n) is a maximum for some set of cell conditions and geometry which can only be specified knowing details of the application and the electronics. Qualitatively, the argument for a maximum s/n goes as follows: Consider a large area cell, one for which the efficiency is 1.0. Clearly, increasing the area of electrode adds nothing to the signal, yet adds to the noise. Now consider a very small electrode. The input stage of the current-to-voltage converter always has some bias current. This is an unavoidable fact. This current has a shot noise, also unavoidable. Thus, when the magnitude of the signal approaches the bias current shot noise, further reductions in electrode size will lower the s/n. Hence, an optimum area exists. There is also an optimum temperature for some analytes [B48].

Chemical selectivity

One of the most troublesome natural limitations in ec detection is due to the ubiquity of oxygen. When reductive detection is planned, usually oxygen must be removed from the mobile phase and samples. Several methods have been proposed. Perhaps the simplest and most effective method is to remove the oxygen before the detector by using a column packed with zinc metal [B49]. Other methods for solving this problem are critically reviewed in that paper.

Dual electrode detection can also add selectivity, and in some cases can obviate the necessity of oxygen removal [B16, B50, B51]. In principle, the first electrode of a pair in series acts as an electrochemical reactor, and the second electrode is used to detect the products of the reaction at the first electrode. In this case, only the current from the second (sensor) electrode is monitored. Conditions can be established that will reduce oxygen at the first electrode, but not sense the oxidation of the just-formed hydrogen peroxide at the second electrode; thus the signal from oxygen is effectively eliminated [B50]. Dual electrode detection is also useful when both halves of a redox couple are of interest, as is the case for biologically important thiol/disulfide species [B52].

Electrode modification holds promise for increasing the selectivity of detection. Iodide treatment (resulting in iodine adsorption) of platinum electrodes leading to selectivity for reduction of chromium(VI) was the first application of this principle [B53]. More recently, both chemical [B54] and electrochemical [B55] treatments of detector electrodes have been shown to increase selectivity. The electrochemical treatment of glassy carbon electrodes leads to improved response for hydrazine and related compounds [B54]. Selectivity for arsenic(III) was shown with an electrode coated with a ruthenium analogue of Prussian Blue [B55].

Electrode modifications that allow selectivity for small molecules have been developed [B56]. These are membranes of cellulose acetate cast on the electrode surface. This material naturally has selective permeability for very small molecules. It has been used to selectively measure enzyme-produced hydrogen peroxide [B56]. Base hydrolysis of the membrane leads to a more porous network.

Voltammetric sensors were discussed above. These certainly add a degree of selectivity. One could speculate that the next several years will see the development of a practical voltammetric sensor, something not too cumbersome, and yet flexible. In parallel with this, other investigators will develop electrode materials or modifications that will be robust and provide selectivity. Finally, as a kind of combination of these two areas, arrays of electrodes of various selectivities will be developed to provide a multiparameter view of analytes.

C. ELECTROANALYTICAL VOLTAMMETRY AT MERCURY ELECTRODES IN FLOWING SOLUTIONS

In a stimulating and at times provocative article on ec detection in l.c. and f.i. analyses, Kissinger [C1] comments on the "current" and "potential" status of mercury electrodes in modern voltammetry. In his concluding statements he says: "Unfortunately, the very concept of a dropping mercury electrode (DME) is fraught with problems of both a fundamental (high double-layer charging due to surface area) and a practical nature (clogged capillaries, mercury toxicity). At this date, the minimum detectable quantities achieved with DMEs are not competitive with stationary electrodes. Nevertheless, the renewable surface and the high hydrogen over-potential are decided advantages which may give the DME a competitive edge for some applications. I personally doubt that the DME will last as a competitive analytical device (for any purpose) beyond the turn of the century. Even now, there usually is a better way to carry out analytical electrochemical measurements".

In this review, this author has taken the view that concise presentation of a number of examples, where unique characteristics of mercury electrodes are dominant, will provide a balanced perspective of the status of mercury electrodes in flow-through electrochemical cells. There are too many very important examples where solid electrodes are not appropriate or cannot be used reliably, relative to mercury electrodes, to discard mercury as an important electrode material. By way of example, Fig. C1 shows the direct and simultaneous determination of tetramethyl and tetraethyl lead additives in aviation and automobile gasoline using h.p.l.c. with ec detection at the static mercury drop electrode (SMDE) [C2—C4], which is now frequently used as an alternative to the DME. A reverse-phase C_{18} chromatographic column was used in this application to separate the different forms of lead, with acetonitrile (0.5 M Et_4NClO_4) as the eluent. The highly complex gasoline matrix can be injected directly onto the C_{18} column for the alkyl lead determination only if a mercury electrode is used as a detector. This is not the case with carbon or inert metal electrodes. Kochi and co-workers [C5—C7] have reported the oxidation of tetraalkyl lead compounds at platinum electrodes in acetonitrile and well defined processes are observed. This is also the case at gold and glassy carbon electrodes [C8]. Unfortunately,

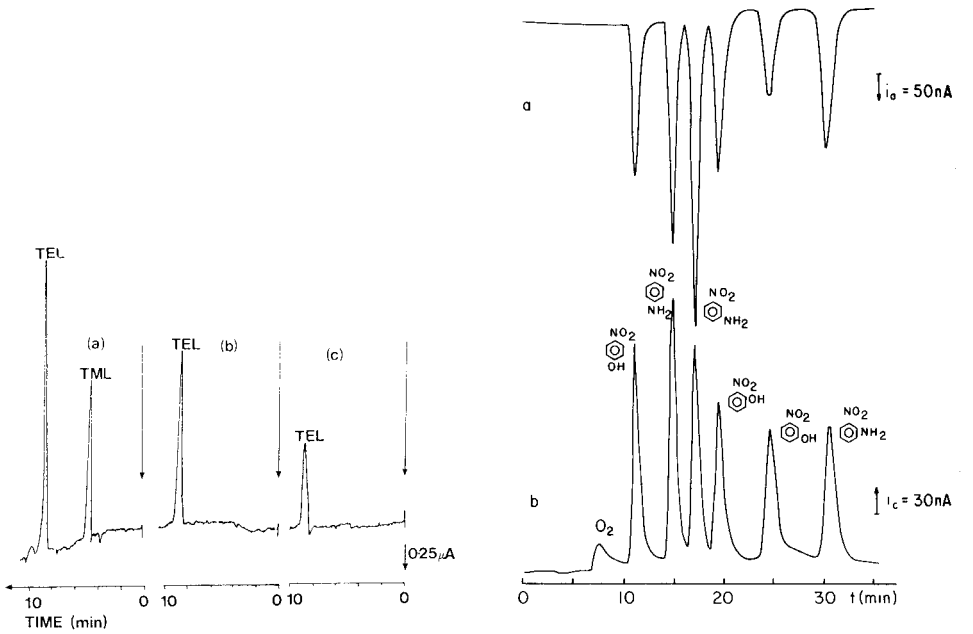
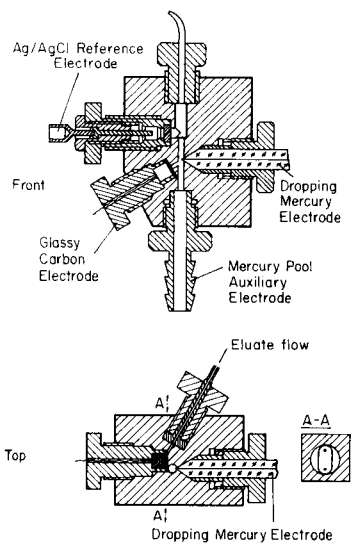


Fig. C1. Determination of tetraethyl lead (TEL) and tetramethyl lead (TML) in gasoline by h.p.l.c. with electrochemical detection at the SMDE. Samples: (a) premium motor gasoline containing 0.84 g l^{-1} TEL and 0.32 g l^{-1} TML; (b) aviation gasoline containing 0.55 g l^{-1} TEL; (c) premium motor gasoline containing 0.30 g l^{-1} TEL. (Direct injection of 5-μl samples onto a Waters Radpak C_{18} Reverse Phase column using acetonitrile, 0.05 M Et_4NClO_4, at 1.0 ml min^{-1} as the running solvent and electrochemical detection with an EG & G PAR Model 310 SMDE in the hanging mercury drop mode at +0.63 V vs. Ag/AgCl.)

Fig. C2. Chromatograms of a mixture of 2 mM each of nitroaniline and nitrophenol isomers recorded simultaneously: (a) glassy carbon electrode, +1.4 V vs. Ag/AgCl; (b) rapidly dropping mercury electrode, drop time = 0.50 s, −1.6 V vs. Ag/AgCl. Chromatographic conditions as per [C12]. Reproduced with permission: Chromatographia, 17 (1983) 322. Copyright, Friedr. Vieweg and Sohn Verlagsgesellschaft MBH.

the oxidation potentials are considerably in excess of +1 V vs. Ag/AgCl. At such positive potentials, numerous constituents of gasoline are also electroactive and sufficient resolution from interfering oxidation processes is not available for determination of tetraalkyl lead compounds. A chromatogram obtained by direct injection of gasoline onto a reverse-phase C_{18} column with these non-mercury electrode materials for detection is the proverbial mess. The oxidation process at mercury is intimately associated with an exchange process with the electrode to form alkyl mercury and trialkyl lead products [C9] and occurs at only ca. +0.5 V vs. Ag/AgCl. Very few constituents of gasoline are oxidized at mercury electrodes at such moderately positive potentials. That is, the unique process at mercury provides a very specific detection method for a particular class of compound in a very complex matrix [C8].

In line with the above discussion, the case for the defense of mercury electrodes now rests. The drawbacks cited by Kissinger [C1] are accepted but the unique and useful characteristics of mercury [C10, C11] which make this the preferred electrode material in a number of instances are acknowledged. In summary, the analytical chemist should ensure that in his or her arsenal of methodology, a wide range of electrode materials is available for use in flow-through electrochemical cells. In particular, for l.c. or f.i. analyses with electrochemical detection, cells incorporating glassy carbon (and other forms of carbon), gold, platinum, silver or copper electrodes, etc., have all found advantageous practical applications as is the case with mercury. Indeed, the simultaneous use of a dropping mercury and glassy carbon electrode in a bi-electrode detector is a very efficient approach to voltammetric analysis in flowing solutions. Figure C2 gives an example of the simultaneous recording of reductive (mercury) and oxidative (glassy carbon) chromatograms for a mixture of nitroaniline and nitrophenol



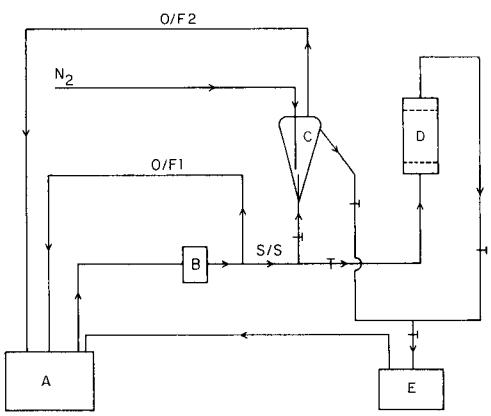


Fig. C3. Bi-electrode (glassy carbon and dropping mercury) amperometric flow-through detector used to obtain data presented in Fig. C2. Reproduced with permission: Chromatographia, 17 (1983) 322. Copyright, Friedr. Vieweg Sohn Verlagsgesellschaft MBH.

Fig. C4. Schematic diagram of a typical analytical system employing a conventional polarographic cell adapted for flow analysis when large volumes of sample are available. The solution is stored in A. Pump B recycles this solution via an overflow pipe O/F1 from which a continuous sample stream S/S is withdrawn. By operating the appropriate valves, T, the sample can be deoxygenated either in vessel C by nitrogen gassing or directed through D, a sulfite bed. After oxygen removal, the sample is determined in the polarographic cell E. Reproduced with permission: Anal. Chim. Acta, 127 (1981) 121. Copyright, Elsevier Scientific Publishing Co.

isomers [C12] using the ec detector cell shown in Fig. C3. Multielectrode detectors and electrochemical or other detectors used in conjunction with, say, spectrophotometric detectors, are becoming increasingly common in modern analytical voltammetry in flowing solutions.

Electrochemical cells for flowing solutions based on mercury electrodes

High-volume flow cells based on adaptations of conventional polarographic cells. Mercury is an ideal choice for the construction of working electrodes. Being a liquid, electrodes can be constructed conveniently in almost any shape or size. The most commonly used mercury electrodes in analytical voltammetry are probably the dropping mercury electrode (DME), the hanging mercury drop electrode (HMDE), the streaming mercury electrode, the mercury film electrode (MFE) and the static mercury drop electrode (SMDE) [C10, C11]. All were initially developed for use in conventional polarographic cells with static solution but have been adapted in some way or another for the flow-through cell configuration.

Early work in flowing solutions using mercury electrodes was frequently associated with relatively large-volume cells. Streams or flowing fluid situations are encountered in many industrial processes, and the use of an on-line polarographic cell as part of the quality control process was a logical extension of polarographic analysis. For example, electrorefining of zinc requires electrolysis of a very concentrated zinc sulfate solution. The presence of trace quantities of cadmium or other metal ions more easily reduced than zinc ions, lowers the current efficiency, thereby increasing production costs and continual checks on the concentration of such species is an essential aspect of plant operation. Off-line polarographic methods obviously work extremely well for the determination of cadmium, lead and other metals in zinc sulfate electrolyte [C13]. A logical extension of this would be direct incorporation of the plant electrolyte stream into a flow-through polarographic detector cell. Figure C4 shows a schematic diagram of a typical continuous flow cell analytical system which has been used in the zinc sulfate problem [C14]. Figure C5 shows cells ensuring that laminar flow can be coupled with detection at a DME or SMDE and examples of polarograms obtained with both kinds of cells are shown in Fig. C6. The use of the differential pulse modes and other transient techniques, in addition to providing data almost independent of flow rate, also provides considerably enhanced sensitivity. Wang et al. [C15] have considered the use of square-wave voltammetry at the DME and rapid scan linear sweep voltammetry has also been examined in detail as an analytical method with a DME in continuous flow systems [C16]. Transient electroanalytical techniques are highly useful alternatives to the d.c. method when using polarographic techniques in flowing solutions.

Fleet and Little [C17] have discussed the difficulties associated with solid electrodes in flow cells. The major problems are linked with maintaining a constant active surface area. Adsorption, film formation, etc.,

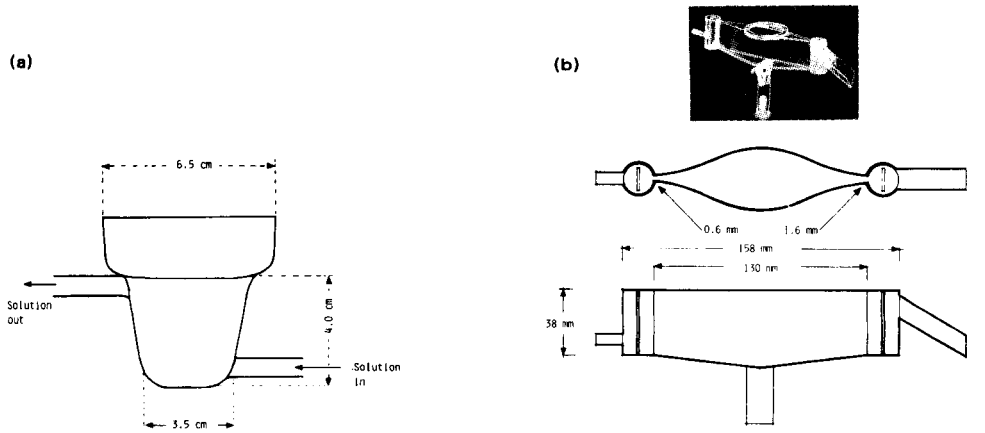


Fig. C5. Diagrams of continuous flow cells ensuring laminar flow: (a) cell adapted from a conventional polarographic cell; (b) cell used in conjunction with a static mercury drop electrode. Reproduced with permission: Anal. Chim. Acta, 127 (1981) 121. Copyright, Elsevier Scientific Publishing Co.

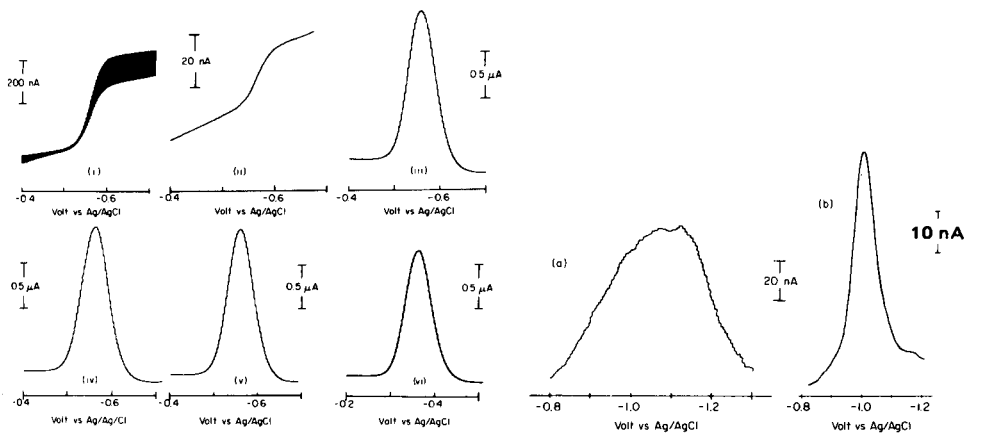


Fig. C6. Typical polarograms obtained in 0.2 M NaCl (pH 2.0) with cells (a) and (b) in Fig. C5. Technique: (i) d.c.; (ii) current sampled d.c.; (iii)—(vi) differential pulse polarography (amplitude —50 mV). Cell: (i), (ii) cell in Fig. C5a; (iii)—(vi) cell in Fig. C5b. Flow rate (ml min^{-1}): (i) 60; (ii) 55; (iii) 100; (iv) 200; (v) 300; (vi) 115. Concentration: (i), (iii)—(v) 1 × 10^{-4} M Cd; (ii) 1 × 10^{-5} M Cd; (vi) 1 × 10^{-4} M Pb. Drop time (s): (i), (iii)—(vi), 0.5; (ii) 1.0. Reproduced with permission: Anal. Chim. Acta, 127 (1981) 121. Copyright, Elsevier Scientific Publishing Co.

Fig. C7. Determination of 1 × 10^{-5} M Zn^{2+} in 0.2 M NaCl (pH 2.0) by differential pulse polarography (amplitude: 50 mV, drop time: 0.5 s) in the presence and absence of oxygen at the SMDE in the continuous flow cell shown in Fig. C5b: (a) oxygen reduction masking the Zn^{2+} wave; (b) Zn^{2+} wave with a sulfite bed to remove oxygen. Reproduced with permission: Anal. Chim. Acta, 127 (1981) 121. Copyright, Elsevier Scientific Publishing Co.

contribute to this problem, and cleaning of the electrodes and their maintenance are often difficult. By contrast, the DME with its constantly renewed surface avoids many surface-related problems and generally the surface area is highly reproducible. However, the well known restrictions with mercury electrodes apply in flow cells as in stationary solutions. That is, in addition to problems noted in the citation from Kissinger [C1], the accessible potential range at mercury is restricted in the oxidation direction, relative to most solid electrodes and, for reduction studies, interference from the dissolved oxygen can be a problem, particularly at fast flow rates and/or when examining low concentrations of electroactive species. Figure C7 demonstrates the necessity for scrupulous removal of oxygen when determining zinc. The problems associated with removal of oxygen were identified in early work in flow cells [C18—C21] and are generally similar to those associated with the polarographic method in stationary solutions. Simple methods for oxygen removal in flowing solutions via addition of a chemical reagent such as ascorbic acid or sulfite [C14], or degassing with nitrogen, argon or helium, have been well documented, although none are universally satisfactory [C22] and special techniques may be necessary in some circumstances (see elsewhere). In addition, vibration or turbulence in flow cells can easily produce unsatisfactory performance at higher flow rates at a mercury electrode where the hydrodynamics become more important.

Low-volume, low flow-rate cells for detection of column effluents. Most recent publications describing applications of mercury working electrodes in flowing solutions have concentrated on detection of species from column effluents, e.g., from ion-exchange columns or reverse or normal-phase columns commonly associated with h.p.l.c., and as detectors for automated methods based on flow injection analysis (f.i.a.). Such studies are generally characterized by use of very slow flow rates and small columns in which oxygen is removed, if necessary, by an inert gas. If an l.c. method is used, a fringe benefit is often associated with separation of oxygen by the column [C23] and the need for its scrupulous removal may not be quite as severe as would otherwise be the case. A number of specialized methods for oxygen removal has been developed [C24—C26]. Electrochemical scrubbing [C24], and the use of a reverse pulse waveform [C25], as well as more generally applied precautions [C26], can all be considered when the presence of oxygen is deleterious to the application being examined.

The use of mercury electrodes as l.c. detectors was pioneered by Kemula [C27], although these early studies still utilized relatively large-volume cells. With the widespread use of modern h.p.l.c., the need for very-small-volume detectors was initiated and a large number of approaches to detection cells with small dead volumes have now been reported. Small-volume cells for l.c./ec using more or less conventional DMEs have been described by Koen et al. [C23], Stillman and Ma [C28], Fleet and Little [C17] and others. Rapidly dropping mercury electrodes have also proved advantageous [C29—C32]. Such DMEs may even be introduced into the cell in a horizontal

rather than vertical position. A host of alternative designs [C33—C39] which also emphasize "growing" mercury electrodes, small dead volumes and minimal turbulence characteristics for the flowing solution have been reported for use as either l.c. or f.i. detectors. Methods based on either the rapidly dropping DME or the SMDE are all likely to give acceptable data consistent with expectations for data available with the stationary solution mode.

Stationary (or quasi-stationary) hanging mercury drop electrodes [C40—C44], as well as thin film mercury electrodes deposited on almost every conceivable substrate, e.g., platinum, glassy carbon, gold, nickel, also are available [C45—C50]. These classes of mercury electrodes obviously have properties akin to the widely used solid electrodes (obviously stationary) and apart from several interesting applications made possible by a unique property of mercury, as described later, this category of electrode will not be considered further. Finally, several useful reviews are available [C51—C53] which may be consulted for numerous other examples of the use of mercury electrodes and their performance in flowing solutions relative to each other and to solid electrodes. Of course, as intimated earlier, cells with dual electrodes [C12] or interchangeable electrodes [C54, C55] are also available and provide a highly desirable flexibility.

Applications involving unique aspects of mercury electrodes

Processes associated with oxidation of the mercury electrode. Many electrode processes of the kind:

$$Hg + 2L^{n-} \rightleftharpoons HgL_2^{2(1-n)+} + 2e^-$$

$$2Hg + 2L^{n-} \rightleftharpoons Hg_2L_2^{2(1-n)+} + 2e^-$$

or related electrode processes exist, where L^{n-} is a species of charge n^- which forms a mercury complex which may or may not be sparingly soluble. By definition, these electrode processes involve oxidation of the mercury in the presence of a species which can form a mercury complex and they occur at potentials less positive than for oxidation of the mercury electrode. If electrochemical detection is based on such a process at a mercury electrode, removal of oxygen is unlikely to be necessary and application of only a moderately positive potential is required. These are both attractive features. At an inert electrode such as glassy carbon or platinum, where direct oxidation of L^{n-} may be required for determination of L^{n-}, very positive potentials may be needed and lack of specificity may be a problem. The direct determination of alkyl lead compounds in gasoline, shown in Fig. C1 and described in the introduction, is an excellent example of the advantage of the use of the mercury electrode when a process involving formation of a mercury compound is available. Other interesting applications of this approach are also available from our laboratories.

Cysteine and homocysteine are biologically important thiols which undergo the reaction:

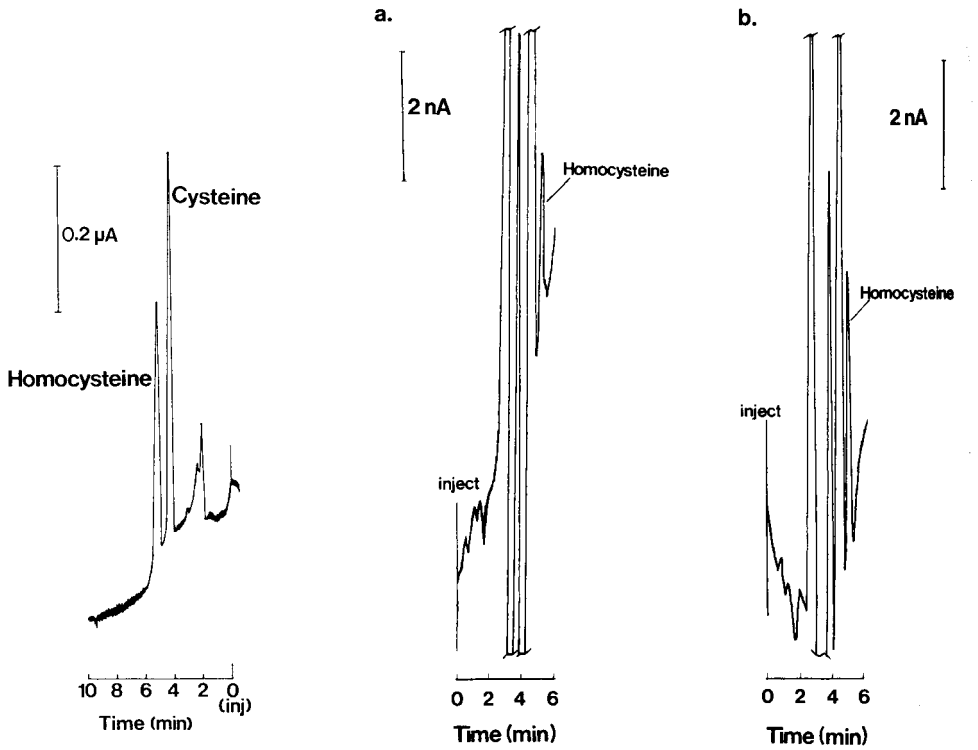


Fig. C8. Cation exchange liquid chromatography with electrochemical detection of 80 μl of a sample containing 5×10^{-5} M cysteine and 5×10^{-5} M homocysteine. Mobile phase: 0.02 M NaH_2PO_4 (pH 2.2) at 0.7 ml min^{-1}. Detection: mercury-coated gold electrode at +0.10 V vs. Ag/AgCl. Reproduced with permission: Anal. Chim. Acta, 156 (1984) 33. Copyright, Elsevier Scientific Publishing Co.

Fig. C9. H.p.l.c. profile obtained using a Partisil SCX-10 column followed by ec detection at a hanging mercury drop electrode at —0.15 V vs. Ag/AgCl. Samples: (a) 10 μl of a rat urine sample (diluted 1:1 with water); (b) 10 μl of a rat urine sample (diluted 1:1 with water) spiked with homocysteine to give an effective amount of 6.75 μg in the injected sample. Mobile phase: 0.02 M NaH_2PO_4 (pH 1.8) at 1.0 ml min^{-1}. Reproduced with permission: J. Chromatogr., 338 (1985) 201. Copyright, Elsevier Scientific Publishing Co.

$$R{-}SH + Hg \rightleftharpoons Hg(RS)_2 + 2H^+ + 2e^-$$

at mercury electrodes. Figure C8 shows the simultaneous determination of both cysteine and homocysteine via ec detection at a mercury-plated gold electrode in the chromatographic effluent after separation by cation-exchange l.c. [C56]. Figure C9 shows the determination of homocysteine in rat urine at a stationary mercury electrode again via l.c./ec [C57].

Synthetic steroids used in oral contraceptives have acetylenic groups which distinguish them from naturally occurring steroids. The proton

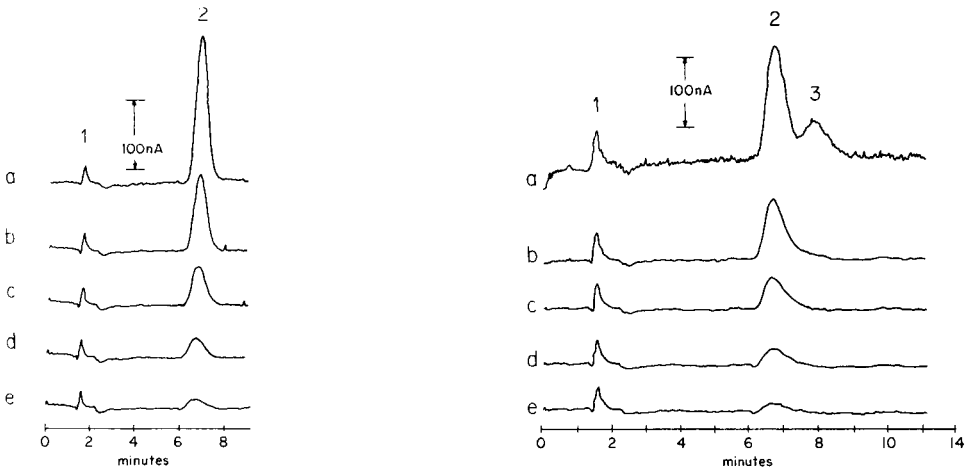


Fig. C10. Simultaneous recording of five chromatograms using pulse polarographic detection with different pulse widths. Response is for a 20-μl injection of a 25 μg ml^{-1} ethynyloestradiol solution after a pulse is applied at the end of a 2-s mercury drop (SMDE) for: (a) 20 ms; (b) 40 ms; (c) 60 ms; (d) 80 ms; (e) 100 ms. Mobile phase: 60% (v/v) methanol/water, 0.01 M $(NH_4)_2HPO_4$ at 2.0 ml min^{-1}. Pulse potential: —0.25 to 0.00 V vs. Ag/AgCl. Peaks: (1) solvent front; (2) ethynyloestradiol. Reproduced with permission: Anal. Chem., 54 (1982) 1702. Copyright, American Chemical Society.

Fig. C11. Simultaneous determination of two steroids in oral contraceptives using pulse polarography in different time domains. Response is for 15 μg ml^{-1} ethynyloestradiol and 25 μg ml^{-1} laevonogestrel at pulse widths of: (a) 20 ms; (b) 40 ms; (c) 60 ms; (d) 80 ms; (e) 100 ms. Mobile phase and detection conditions as in Fig. C10. Peaks: (1) solvent front, (2) ethynyloestradiol, (3) laevonorgestrel. Reproduced with permission: Anal. Chem., 54 (1982) 1702. Copyright, American Chemical Society.

associated with the acetylenic group SC≡CH (S = steroid backbone) is acidic and enables the reaction:

$$SC{\equiv}CH + Hg \rightleftharpoons HgS + H^+ + e^-$$

to occur [C58—C60]. Figures C10 and C11 show results for a multi-time domain l.c./ec, with pulse polarographic detection at the SMDE, for determination of two synthetic steroids commonly used in oral contraceptives. Note the vastly different sensitivities at different pulse widths. Multi-time domain methods offer attractive approaches in voltammetric analysis and are readily applied at mercury electrodes as demonstrated by the steroid example. The determination of sulfide based on the reaction:

$$Hg + S^{2-} \rightleftharpoons HgS\downarrow + 2e^-$$

and cyanide based on the reaction:

$$Hg + xCN^- \rightleftharpoons [Hg(CN)_2]^{(2-x)+} + 2e^-$$

have also been reported [C61] using ion-chromatography with ec detection at the SMDE.

Stripping voltammetry at mercury electrodes. Techniques of stripping voltammetry are inherently very sensitive. Many of the electrode processes used in stripping voltammetry involve reduction of a metal ion to the metal which then dissolves in mercury (amalgam formation) as a preconcentration step

$$M^{n+} + Hg + ne^- \rightarrow M(Hg)$$

followed by oxidation of the amalgam in the detection step

$$M(Hg) \rightarrow M^{n+} + Hg + ne^-$$

Extremely large sensitivities ($\mu A\ M^{-1}$) are obtained with this technique which is usually referred to as anodic stripping voltammetry.

Highly sensitive automated methods of anodic stripping voltammetry can be developed by using f.i.a. with the hanging mercury drop or thin mercury film electrode [C14, C62–C65]. Other extremely sensitive stripping methods based on cathodic or adsorptive stripping voltammetry or potentiometric stripping analysis are also highly sensitive and have been examined with considerable success in flowing solutions [C66, C67]. Many stripping techniques utilize a specific property of mercury not available with other electrode materials and again frequently make mercury the electrode material of choice in this area of voltammetry in flowing solutions.

Tensammetry at mercury electrodes and the use of transient electrochemical techniques in flowing solutions. Analytical use of tensammetric (non-faradaic responses) have been well documented at mercury electrodes for over forty years [C68, C69]. This class of electrode response is ideally suited in many cases to the determination of surfactants (detergents) and polymers.

Tensammetric techniques are not usually highly selective and use of h.p.l.c. for chemical separation prior to tensammetric detection at a DME or SMDE offers considerable scope for reemergence of this voltammetric method, especially when modern transient detection methods are used [C70]. A detailed examination of the use of f.i. or h.p.l.c. with tensammetric detection is provided by Bos and co-workers [C71, C72]. This group has considered various aspects of tensammetric flow-through detectors [C71] and applied this approach to the determination of detergents by f.i.a. [C72]. Studies on cholaric acids [C73], polyethylene glycols and phenol ethoxylates [C70] illustrate the combination of h.p.l.c. with tensammetric detection. Whereas the work of Bond and Jones [C70] utilizes the tensammetric response itself, Kemula and co-workers [C73, C74], as well as Lankelma and Poppe [C75], use effects of the change in double-layer capacity at the DME.

All these tensammetric studies employ pulsed potential, a.c. or other transient ec techniques with considerable advantage at mercury electrodes. At the DME and SMDE, transient techniques in general have played a very

significant role in advancing the sensitivity and versatility of ec detection in flowing solutions [C15, C70, C67—C83] as has the advent of computer-based electrochemical methods [C70, C76, C78]. This does not appear to be equally true for work with solid electrodes where steady-state d.c. methods are dominant. That is, amperometric detection at a fixed potential is used in a majority of instances at glassy carbon electrodes because excellent sensitivity is already available and instrumentation required for this technique is extremely simple in contrast to that required for application of transient voltammetric techniques at a DME. At a DME, the faradaic-to-charging current ratio is not particularly favorable with d.c. techniques because of the use of an expanding mercury electrode. Transient methods are particularly useful to improve the sensitivity and selectivity of voltammetric analysis using mercury electrodes in flowing solutions, as is the case in conventional polarography with stationary solutions [C84].

Conclusions

This section on mercury electrodes, while far from being exhaustive, has highlighted the novel features of mercury as an electrode material for voltammetric detection in flowing solutions. A natural emphasis on the use of oxidative processes involving formation of mercury compounds, stripping voltammetry and tensammetric detection which can be coupled with use of transient voltammetric techniques, emanates from this approach. Traditionally, the dropping mercury electrode or other forms of mercury electrode are associated with the determination of organic compounds or metal ions, etc., via reductive processes. Kissinger [C1], as noted in the introduction, may well be correct in his assertion that solid electrodes are likely to replace the use of mercury electrodes in some situations, but it is highly probable that the mercury electrode will continue to survive and indeed thrive well into the next century. Like solid electrodes, mercury electrodes have a niche in practical analytical voltammetry in flowing solutions.

D. SOLID ELECTRODE MATERIALS

Solid electrodes have seen extensive use as amperometric and coulometric detectors for compounds separated by liquid chromatography. The working electrode for l.c./ec should be stable for an infinite period of time, simple to prepare, and have an excellent *s/n*. The *s/n* can be maximized by working with materials which have a minimum amount of residual current and yet yield maximal current in response to electroactive species. In fact, many solid electrodes approach this ideal behavior, especially carbon electrodes. Many of the advances that have been made in the selection of solid electrodes for l.c. detectors are empirical; materials have been found initially to be practical and then have been used in a variety of applications. For example, carbon paste was one of the first electrode materials used for

l.c./ec detection [D1]. This material has been found to be especially useful for anodic oxidations in aqueous mobile phases. Various composite carbon materials have been used successfully. Perhaps the most popular electrode material is glassy carbon because detectors fabricated from this material are easy to assemble and give reproducible results. However, glassy carbon detectors tend not to give such a favorable *s/n* compared with carbon paste or composite electrodes. Metal electrodes such as platinum or copper have been used in specialized applications. In this section, the different types of solid electrodes which have been used in l.c./ec will be evaluated and their relative features described.

Carbon paste electrodes

The term carbon paste refers to a mixture of graphite or carbon powder and an organic liquid which is immiscible with the solution phase [D2]. Carbon paste electrodes have long been used for anodic oxidations because they exhibit a low residual current and a wide positive potential range. The first use of carbon paste electrodes for l.c./ec was for the detection of catecholamines by anodic oxidation [D1]. The detection limits obtained by this method (less than pg amounts injected on the column) were much lower than for other established methods for the determination of these neurochemicals. Detectors fabricated from carbon paste can be used for months without the need for resurfacing. The use of carbon paste electrodes for the detection of neurochemical compounds separated by l.c. was commercialized in 1974 by Bioanalytical Systems. The utility of carbon paste electrodes for l.c./ec led directly to the success of this Company.

The favorable properties of carbon paste are a result of the purity of the components which are used to prepare the paste. The graphite powder should be of high purity and materials that are commonly used are also used for spectroscopic analysis (e.g., Ultra-F purity carbon, UCP-1-M, Ultra Carbon Corp., Bay City, MI). This powder is thoroughly mixed with an organic liquid such as Nujol. A satisfactory composition for l.c./ec applications using aqueous mobile phases is 25% Nujol by weight. When organic modifiers are present in the mobile phase, a more satisfactory pasting liquid is silicone grease (35% by weight, Dow Corning, Midland, MI). Because the optimum carbon paste composition depends on the application, a variety of other compositions for carbon paste have been explored. The use of fluorocarbon liquids and bromoform has been compared to Nujol [D3]. As will be discussed, several solid materials have also been used to bind the carbon particles together.

Detection limits at carbon paste electrodes are among the best of those reported for l.c./ec detectors. In 1976, it was shown that 2 pg of the neurotransmitter dopamine could be detected by l.c./ec [D4]. However, in that application, the chromatography was not very well developed. Using microbore h.p.l.c., Caliguri and Mefford [D5] have shown that detection limits of 100 fg can be obtained for catecholamines using carbon paste electrodes.

This improvement in detection limits is obtained because, as is well recognized, less dilution of the sample occurs during efficient separation on the microbore columns.

A major reason for the favorable detection limits at carbon paste electrodes is the low residual current. The organic liquid is thought to fill holes which contribute to the residual current as well as binding the carbon particles together. It has been shown that there is an inverse correlation between the amount of pasting liquid and the amount of residual current [D6]. However, the rates of heterogeneous electron transfer reactions are also found to decrease with an increase in the amount of pasting liquid [D7]. Rapid rates of electron transfer are desirable for l.c./ec applications because they allow better defined voltammetric waves and, therefore, easier selection of the applied potential [D8]. However, because variations are found from preparation to preparation of carbon paste, determination of the optimum ratio of graphite to organic liquid is essentially an empirical process.

Despite the favorable detection limits obtained with carbon paste electrodes, they do have several drawbacks. One of these, which has already been mentioned, is the variability obtained when preparing formulations of paste. A second major problem is that they are only suitable for anodic oxidation. The carbon paste contains oxygen which can be reduced at potentials negative of 0.0 V vs. SCE leading to a large residual current [D9]. Carbon paste electrodes also tend to be soluble in organic modifiers in the mobile phase which is especially a problem when the mobile-phase organic modifier concentration exceeds 20%.

Composite carbon electrodes

In an attempt to minimize the problem of dissolution of the paste in organic liquids, several solid binders have been evaluated for use in carbon composite electrodes. Polyvinyl chloride, chloroprene rubber and cerasin wax have all been used with graphite powders to form a more rigid paste [D10—D12]. Carbon black has been shown to give superior values of *s/n* over graphite powder in an evaluation where both forms of carbon were placed in 50% polyethylene [D13]. This electrode material was found to be more stable than other carbon composites for very positive potentials. Detection limits for the detection of phenol at the carbon-black polyethylene electrode were approximately 50 pg. This detection limit, although higher than that cited earlier for carbon paste electrodes, was obtained at a much more positive potential where noise sources are greater, and thus is quite good.

An electrode material fabricated from Kel-F and graphite, the "Kelgraph" electrode, has been evaluated for use as an l.c./ec detector. This material is fabricated by pressing graphite powder (UCP-1-M) and Kel-F into a pellet [D14]. The Kelgraph electrode is found to have an optimum *s/n* for l.c./ec when the electrode is composed of 15% graphite. Under these conditions it has a lower absolute sensitivity to electroactive compounds than solid

electrodes, however, the noise is reduced even further [D15]. Detection limits at this electrode are reported to be 6 pg for the determination of phenol [D16].

Glassy carbon electrodes

The most popular material for amperometric detection following liquid chromatographic separation is glassy carbon. The utility of this material was predicted early on in the development of l.c./ec by Fleet and Little [D17]. The widespread use of glassy carbon as an electrode material has been reviewed [D18]. Glassy carbon is a gas impermeable material which is formed by the heating of phenol–formaldehyde resins in an inert atmosphere. The residual current is higher than at carbon paste electrodes [D19]; however, the electrode can be used at both positive and negative potentials and has a range in aqueous solution of ca. 2 V. The detector response has been shown to be relatively constant with time, in contrast to polished platinum, which exhibits a decreased response to repetitive injections of potassium ferrocyanide [D20]. Furthermore, if the electrode does become fouled it can be reactivated by electrochemical treatments without the need for cell disassembly [D21, D22].

Glassy carbon can be formed in a variety of shapes and also can be machined to optimum dimensions. Thus, glassy carbon electrodes have been employed in coulometric cells [D23] as well as tubular [D24] and thin-layer amperometric detectors. Miniaturized detectors suitable for 0.6-mm i.d. columns have been designed from glassy carbon [D25].

Other forms of solid carbon

Several other forms of carbon have been evaluated as detectors for l.c./ec. One of these, Pyrolyte, is an isotropic pyrolytic carbon. Its electrochemical properties have been found to be similar to carbon paste, although it can be used at more negative potentials [D8]. Like glassy carbon, it has the advantage that it can be machined flat which facilitates the construction of very thin flow-through amperometric cells.

Highly oriented pyrolytic graphite is a sufficiently ordered form of graphite that can be resurfaced by peeling layers with the aid of adhesive tape. The basal plane of this material has been evaluated as an amperometric detector [D26]. This surface has the advantage of "flatness"; however, electrochemical rates are extremely slow resulting in an insensitive detector. Anodic oxidation of the surface in citrate buffer causes a microscopic roughening of the surface with a concomitant acceleration in electrochemical rates. The oxidized material is stable and has a sensitivity approaching that of carbon paste for detection of catechols. The high degree of flatness enables this material to be incorporated in a low-volume cell (0.1 μl) suitable for use with microcolumn l.c. [D27]. This material also has been found useful for cathodic reductions of aromatic nitrocompounds [D28].

Reticulated vitreous carbon is a material with high surface area that in

many ways resembles a sponge. Because of the methods of fabrication, it has a shiny appearance and, electrochemically, has lower residual currents than other forms of porous carbon. This material has been investigated as a coulometric detector for f.i./ec [D29] and l.c./ec [D30]. Detection limits were found comparable to those at other types of carbon amperometric detectors. With coulometric operation, calibration is simplified. This material also has been used with the pores filled with epoxy [D31]. The surface of this material resembles that of carbon paste, because it consists of islands of carbon surrounded by an insulating material. Therefore, it may be of use as an amperometric detector.

Carbon fibers are microscopic carbon materials formed from the pyrolysis of pitch or polyacrylonitrile. These fibers have been used to form an array of disks, each of 10 μm diameter [D32]. At potentials sufficient to oxidize catechols, this array detector has a sensitivity similar to glassy carbon. However, the detection limits improve relative to glassy carbon at more positive potentials (e.g., 12 pg for phenol) because the background signal at the array electrode is less dependent on fluctuations in the velocity of the mobile phase. Strands of carbon fiber have been used as amperometric detectors for conventional l.c. [D33]. Single carbon fibers are an important advance for amperometric detectors with open tubular columns (15-μm i.d.) [D34]. In this application, the fiber is simply inserted in the end of the column. Because of the low flow rates, the detector operates in a coulometric mode.

Metal working electrodes

Metal working electrodes have not been as widely used as carbon electrodes because they tend to exhibit higher detection limits. This is primarily because higher residual currents are found arising from surface oxides when used at positive potentials. In a comparison of a tubular platinum electrode with glassy carbon and carbon paste electrodes, similar detection limits were found [D10]. However, the reported detection limits at carbon were higher than have been reported elsewhere, so other variables may have been operative. Platinum electrodes have been used successfully with capillary columns with diameters less than 20 μm. Detection of less than 1 pg of hydroquinone has been obtained [D35].

Various approaches have been used to improve the performance of platinum electrodes. Johnson and co-workers have made extensive use of potential cycling to improve the detection of carbohydrates [D36], amines and amino acids [D37]. The waveform consists of three pulses: the first to oxidatively clean the electrode, the second to reduce the electrode, and the third for detection. Detection limits with this technique are in the nanogram range; however, these compounds cannot be determined directly at carbon electrodes. Cellulose acetate membranes have been used to improve detection limits for hydrogen peroxide at a platinum electrode [D38].

Other metals have seen limited use. Nickel oxide on nickel anodes

facilitates the amperometric detection of amines and amino acids [D39]. Copper electrodes can be used in a similar manner for the detection of these compounds [D40]. Detection limits are in the nanogram range for these compounds. Cadmium has also been evaluated as an l.c. detector. Although it has a very limited potential range, the cadmium tubular electrode is useful for the determination of nitrate and nitrite [D41].

E. POTENTIOSTATIC (DC) DETECTION AT SOLID AND MERCURY FILM ELECTRODES IN FLOWING SOLUTIONS

There are numerous possible applications of f.i./ec systems which are attractive for simple samples because of high sample throughput. However, for trace determinations of analytes for which the injected amounts remain in the low picomole range, two trends must be considered in the development of a suitable hydrodynamic electroanalytical technique. First, the probability of electroactive interferences derived from the sample matrix increases rapidly for decreasing concentrations of analyte; second, injection artifacts contribute proportionately more to the overall system void-volume response. For these reasons, l.c. separation prior to ec detection is preferred even if just for the separation of a single analyte from the sample matrix.

Oxidative detections

Typical of these techniques are those involving the biogenic amines and metabolites, a group of phenolic phenylethylamines which are readily oxidized at solid electrodes, including carbon and platinum [E1—E3]. The electrode reactions are well defined. The separation of all compounds and primary metabolites in physiological samples is achieved using hydrophobic stationary phases; capacity factors are easily manipulated by adjusting mobile phase pH, the nature and mole fraction of any organic solvents, and ancillary ion-pairing equilibria.

The synergistic selectivity of column and electrode often permits very crude sample extraction procedures. Brain tissue is homogenized in dilute acid, centrifuged, and the supernatant is injected directly. Because the functional groups present on the molecules are too diverse to satisfy one extraction scheme anyway, the column must deliver an adequate separation. Blank et al. [E3] thoroughly examined the high-speed format and recommended the use of modified thin-layer cells, small injection volumes, and fast electronic current amplifiers to make optimum measurements. Separations of eighteen biogenic amines and metabolites could be realized in 4—7 min from brain tissue (see Fig. E1). Obviously, because many of these compounds possess the same electrophores, any selectivity based purely on electrochemistry would be inadequate.

Unlike optical detection, where the *s/n* depends on path length, electrochemistry is a surface technique. Electrochemical transducers for l.c. are distinguished by very low dead volumes. A thin-layer cell, by appropriate

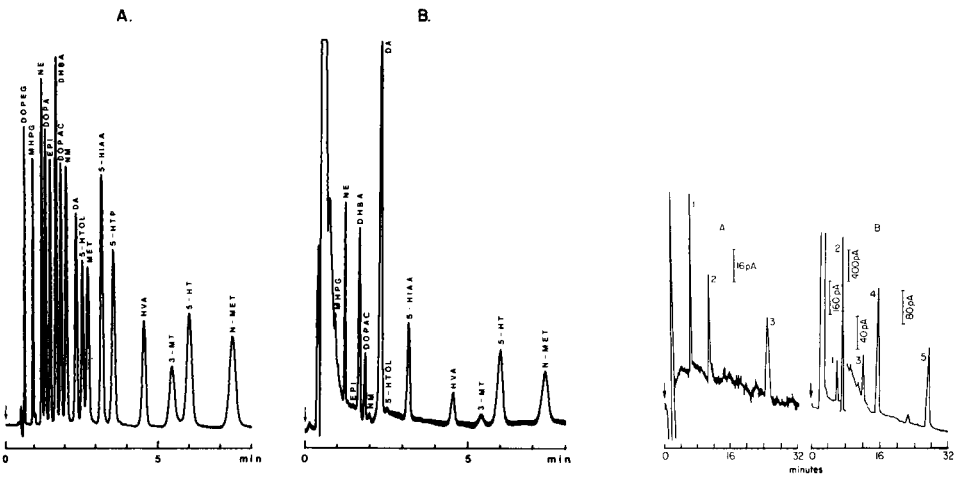


Fig. E1. High speed separation of 18 tyrosine and tryptophan metabolites. (A) Typically 5 nmol of standard injected. (B) Whole rat brain homogenate, 5 μl. Mobile phase: 0.10 M citric acid/0.225 mM octylsodium sulfate/0.06% diethylamine/0.05 mM Na_2EDTA with 5% CH_3CN; pH adjusted to 2.25. Flow rate: 2.0 ml min^{-1}. Perkin-Elmer C_{18} 3-μm (100 × 4.6 mm). Detection: BAS thin-layer cell, carbon-paste electrode at +0.80 V vs. Ag/AgCl. Reproduced with permission: J. Liq. Chromatogr., 7 (1984) 509. Copyright, Marcel Dekker Inc.

Fig. E2. Separation of biogenic amines using microbore l.c./ec. (A) Standards: (1) 5.0 pg NE; (2) 3.0 pg EPI; (3) 4.5 pg DA. (B) Catecholamines in 270 μg of rat hypothalamus: (1) 155 ng g^{-1} DOPAC; (2) 1630 ng g^{-1} NE; (3) 41 ng g^{-1} EPI; (4) DHBA (int. std.); (5) 286 ng g^{-1} DA. Stationary phase: C_{18} 5-μm (250 × 1 mm). Mobile phase: 0.1 M sodium acetate/0.02 M citric acid/50 mg l^{-1} Na_2EDTA, 100 mg l^{-1} sodium octyl sulfate, with 4.5% CH_3CN at 40—50 μl min^{-1}. Detector: BAS thin-layer cell, +0.6 V. Reproduced with permission: Brain Res., 296 (1984) 156. Copyright, Elsevier Scientific Publishing Co.

choice of gasket thickness and channel dimensions, can contain less than 0.3 μl. Hence, minimizing cell volume for microbore l.c./ec is not difficult [E4, E5]. At flow rates of 50 μl min^{-1}, amperometric transducers are over 60% efficient, and they reach 100% efficiency at flow rates of 20—30 μl min^{-1} [E6]. Also, for the same volume injected, a microbore column delivers more concentrated eluting bands, due to the inverse-square dependence of concentration on column diameter. These two factors combine to enable extraordinary detection limits of 1—5 fmol to be achieved (Fig. E2). Very small brain tissues (typically 200 μg) can be assayed with excellent *s/n* [E5].

The logical conclusion of these neurochemical assays would be to sample the brain or peripheral tissues directly, in awake and unrestrained animals. This, in fact, has been achieved using in vivo voltammetry. Discrimination among similar compounds is poor, however, and confirmation via chromatographic separation is preferred. Sampling of the extracellular fluid (rather than gross tissue homogenate) would be advantageous, because this "pool" truly reflects neuronal activity and metabolism. The body samples

these pools via the circulation, and a tiny implanted loop of microdialysis tubing has provided a useful synthetic "blood vessel" [E7—E9]. Artificial cerebrospinal fluid is pumped through the loop at a slow rate (1—10 μl min^{-1}) to permit sufficient solute transport across the membrane for detection. The dialysate is passed directly into the l.c./ec system. Because dialysate is sampled every 5—10 min, a repetitive, multivariate l.c./ec profile in an awake and unrestrained animal over several hours is now feasible to determine the effects of a behavioral or pharmacological stimulus.

Reductive detection

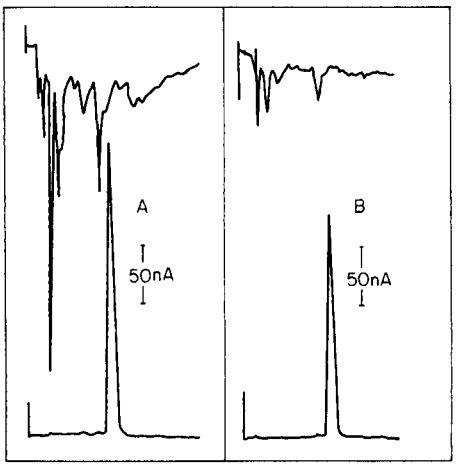
Direct potentiostatic detection of reducible solutes is hampered at more negative potentials by the presence of dissolved oxygen in both samples and the mobile phase [E10—E12]. Standard mobile phase sparging must be accompanied by mild heating to decrease the background current caused by oxygen reduction. Samples must be sparged by moist helium. Typical of direct reductive detection is the determination of various explosives in blast debris and ground water [E13]. These compounds possess highly electronegative nitro, nitrate-ester, and nitrimine electrophores which are readily reducible. Both glassy carbon and supported mercury films have been used for ec detection; the hydrogen overpotential is greater for the latter but only with new films. A comparative study was described by Bratin and Kissinger [E10]. Blast debris could be characterized as to probable source and amount by an l.c./ec profile of common explosive substituents using tandem photometric (u.v.) and ec detectors. An oxidative profile for diphenylamine, a contaminating by-product, could confirm the nature of the original explosive for forensic purposes. Because most of these electrophores are reduced with a large number of electrons, high sensitivity is obtainable. For this reason, nitrophenyl compounds are ideal as ec derivatizing agents [E14—E16]. Other reducible electrophores include benzodiazepines [E17], transition metals and organometallic complexes [E18, E19], naturally occurring quinones [E20] and nitropolyaromatic hydrocarbons [E21].

Specialized phenomena

The selectivity of an l.c./ec analysis can be further improved in certain applications by using electrode materials or surfaces which mediate or participate directly in the redox reaction. The detection of cyanide ions on silver or mercury surfaces is an example. While the anion is not oxidized directly, a silver electrode poised at 0.0 V vs. Ag/AgCl is capable of sensing cyanide via the oxidation of the electrode:

$$Ag + 2CN^- \rightarrow Ag(CN)_2^- + e$$

Cyanide has been determined in industrial effluents and biological matrices using this scheme. Brimer and Dalgaard [E22] elegantly applied it to the detection of cyanogenic glycosides in crude plant tissue homogenates (Fig. E3) by using an immobilized glucosidase column to split the conjugate,



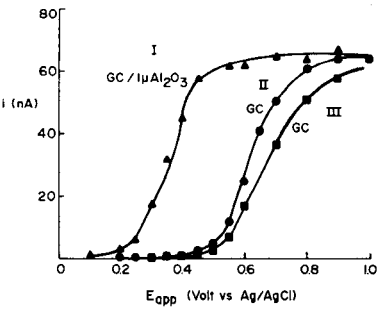


Fig. E3. Ultraviolet absorbance (top) and amperometric detection at silver electrode (bottom) of leaf extracts of *P. lauracerasus L.* Mobile phase: 15% methanol/85% 0.05 M phosphate (pH 5.0) at 1 ml min^{-1}. Detection: u.v. at 254 nm; ec at 0.0 V vs. Ag/AgCl. The major ec peak is prunasin (β-D-glucosylmandelonitrile). The enzyme column contained immobilized glycosidases from *H. pomatia*. Reprinted with permission: J. Chromatogr., 303 (1984) 77. Copyright, Elsevier Scientific Publishing Co.

Fig. E4. Hydrodynamic voltammograms of NADH on: (I) aluminum oxide-modified glassy carbon; (II, III) bare glassy carbon.

addition of base to catalyze release of cyanide, and detection of cyanide at a silver anode:

$$R_1R_2C(CN)(O\text{—}Gly) \xrightarrow{\text{glycosidase}} R_1R_2C(CN)OH + GlyOH$$

$$R_1R_2C(CN)(OH) + OH^- \rightarrow R_1R_2CO + CN^- + H_2O$$

$$Ag + 2CN^- \rightarrow Ag(CN)_2^- + e$$

The same pattern of detection has been followed for halides on silver [E23] and thiols and EDTA-type compounds on mercury [E24, E25].

Chemically modified electrodes

Tailoring electrode surfaces to exact the desired electrocatalytic response is now intensely investigated in electrochemical circles for use in optoelectronic display devices, fuel cells, and electro-organic syntheses. For amperometry, these "chemically modified" electrodes may eventually decrease the likely requirements for kinetically hindered redox reactions (for a review, see [E26].) In Fig. E4, for example, a substrate capable of being oxidized only at high potential on a bare surface (e.g., NADH at +800 mV) may be detected at +400 mV on alumina-modified glassy carbon [E27, E28]. In flowing solutions, mediators on modified electrodes must

react rapidly with the analyte of interest on the time scale of the mass transport process and yet remain immobilized over a period of weeks for the sake of reproducibility. Baldwin and co-workers have examined modified electrodes utilizing organometallics blended into carbon paste [E29, E30]. Cobalt phthalocyanine was selected for its known electrocatalytic properties and its poor solubility in common l.c. mobile phases. The electrodes were capable of catalyzing the oxidation of hydrazines and sulfhydryls at lower than expected potentials. Lifetimes for the mediated detections were highly dependent on applied potential.

Multi-potential detection

For many situations in trace analysis, it would be advantageous to obtain a l.c./ec chromatogram at several values of applied potentials, thereby obtaining a more complete "fingerprint" (i—t—E) of the eluting substances. For d.c. detection at a single electrode, however, such a desire necessitates making repeated injections of sample at each potential. This procedure usually is not considered too time-consuming for an occasional sample, but it is very inconvenient when such data must be obtained routinely for many samples. With multiple sensors at the various potentials, all chromatograms could be obtained simultaneously. Analysis times would be no longer than for the single potential experiment and a "fingerprint" of each solute would be generated. Furthermore, with a single electrode, only one electrochemical reaction can be used for detection of each solute. For electrochemically reversible or quasi-reversible species, however, "follow-up" reactions could be used to detect the original solute indirectly, if multiple sensors were used. Some of these options are summarized in Fig. E5. Multiple electrode

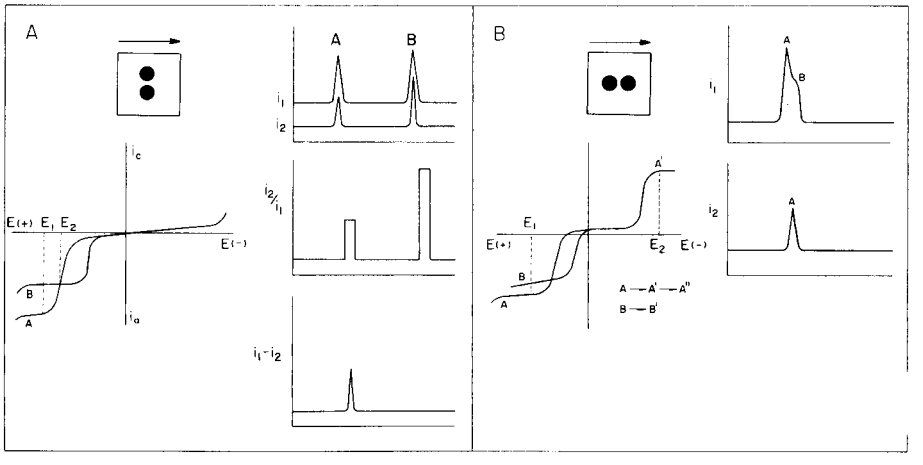


Fig. E5. Multielectrode l.c./ec. (A) Two parallel electrodes (W_1 and W_2) may be operated simultaneously and independently to yield difference and ratio chromatograms. (B) With series electrodes (W_1 before W_2), irreversibly reacted B is excluded from interfering with A (via its W_1 reaction product) by the latter's detection at W_2.

transducers [E31] have been conveniently classified according to the spatial arrangement of the working electrodes with respect to flow as parallel-adjacent, series and parallel-opposed configurations.

Parallel-adjacent configuration. Consider the current-potential curves in Fig. E5A for two oxidizable solutes, A and B. If one operates twin parallel-adjacent electrodes on the most positive plateau (E_1) and selects an additional potential (E_2) less positive than E_1, different current outputs are expected (i_1 and i_2). In the case where the peak cannot be collected for alternate confirmation by other methods, the current ratio (i_2/i_1) provides an in situ two-point hydrodynamic voltammogram which may be compared to calibration values for standards. All studies to date have used thin-layer cells. The ratios are quite precise (<1% r.s.d., see [E32, E33]), even for very low concentrations. Surprisingly, the ratios are also quite sensitive to small changes in molecular structure [E32]. Peak confirmation by ratio has been used to identify catecholamines in brain tissue (Fig. E6), benzene metabolites [E34], toxic azo dye metabolites [E35], pterins [E36] and guanine nucleotides in brain [E37].

Independent, parallel electrodes also permit detection at different, optimum potentials. If detection potentials chosen for the compound are widely

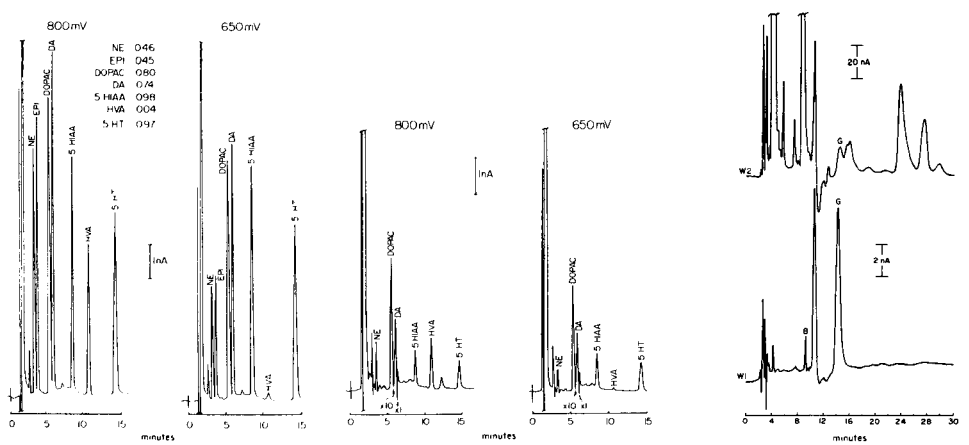
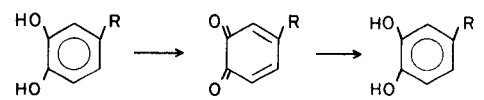


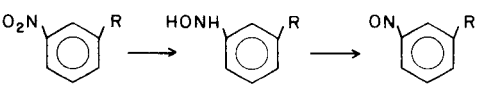
Fig. E6. Parallel dual-electrode chromatograms of a standard mixture (left pair) and brain tissue homogenate (right pair). Calibration peak-height ratios for standards are listed (i_{650}/i_{800}). Mobile phase: 1.9 parts THF/3.5 parts CH_3CN/96.5 parts 0.15 M monochloroaceate (pH 3) with 200 mg l^{-1} sodium octylsulfate and 0.25 g l^{-1} Na_2EDTA at 1.5 ml min^{-1}. Stationary phase: Biophase ODS 5-μm (250 × 4.6 mm). Reproduced with permission: J. Chromatogr., 255 (1983) 533. Copyright, Elsevier Scientific Publishing Co.

Fig. E7. Parallel dual-electrode detection of urinary pterins at +400 mV (W_1) and +800 mV (W_2). Chromatograms obtained simultaneously for a single sample using two detectors at different potentials (E_1 + E_2). Peaks: (B) tetrahydronepterin; (G) tetrahydrobiopterin. Mobile phase: 0.1 M phosphate buffer (pH 2.5)/3 mM sodium octylsulfate/5% methanol at 1.0 ml min^{-1}. Reprinted with permission: Anal. Chem., 55 (1983) 1458. Copyright, American Chemical Society.

separated, it is convenient to use parallel electrodes. For priority pollutan phenols, for example, the chlorophenols and alkylphenols may be detecte at +0.98 V while the nitrophenols are reduced at −0.75 V. Parallel-adjacen electrodes also can render simultaneous chromatograms at high and lo detector potentials. With a single electrode all peaks must be monitore at a potential which is sufficiently positive (or negative) to detect all com ponents, often at the expense of selectivity for the more reactiv components. With twin parallel electrodes, a more selective low potentia chromatogram may be generated, often with better detection limits. Lunt and Kissinger [E36] exploited this advantage in the detection of pterin (Fig. E7) where the most easily oxidized tetrahydro forms could b detected in urine at lower potential than the dihydro forms. The techniqu has also been used to profile acetaminophen metabolites and conjugate phenolic acids and dye metabolites [E35].

Series configuration. In this arrangement, electrodes monitor the mobil phase sequentially. For reversible or quasi-reversible analytes, the products of the initial reaction at an upstream electrode can be detected at subsequent downstream electrodes, perhaps to provide better selectivity or detection limits [E38—E42]. For example, both the catecholamines and aromatic nitro applications described for single electrodes can be transferred to series multiple electrodes due to the stability of the initial products and their electrochemistry:





For series electrodes, the efficiency of recapture of upstream electrode reaction products at downstream electrodes must be addressed. If collection efficiency is high, the signal at the downstream electrode will provide a *s/n* comparable to or better than for the first electrode. Collection efficiency is highly dependent upon the size, shape and the configuration of the electrodes with respect to one another. Even in a thin-layer cell, where single electrode efficiencies are of the order of 3—10% at conventional flow rates, the collection can be 40—50% using dual electrodes. The apparent contradiction is readily understood from a consideration of the position of the diffusion layer created by the first electrode. As the inter-electrode spacing approaches zero, the product-rich diffusion layer from electrode 1 remains well defined as it passes over electrode 2. Without allowing substantial

remixing, the product is therefore effectively trapped downstream. If upstream conversion efficiency is substantial, inter-electrode spacing is less critical.

A routine application of series dual electrodes has been the translation of reductive problems to oxidative ones at less negative potentials. If the reduction product of an analyte is oxidizable, rigorous deoxygenation is avoided, because the products of oxygen reduction (H_2O_2, H_2O) are not oxidized on glassy carbon until a high (+1.0 V) positive operating potential is applied. Thus, reducible nitroaromatic compounds and other quasi-reversible species can be detected by measuring the hydroxylamine reaction product at an electrode downstream. The potential is usually quite moderate (−0.1 to +0.5 V), thereby improving the *s/n* in most cases. This scheme has been applied to nitrophenyl derivatives of amino acids [E43], organometallics [E40] and vitamin K_1 and other quinones [E20]. To improve selectivity, MacCrehan and Durst [E40] modulated the upstream generator electrode potential about the half-wave potential for the analyte. The downstream signal was processed with a lock-in amplifier to discern the a.c. signal due to the analyte.

Series dual electrodes have also permitted the simultaneous measurement of both forms of a redox couple (e.g., thiol and disulfide) from detection of the reduced species only [E25]. Two mercury film electrodes were used in series (Fig. E8). An eluting disulfide is forced to undergo two redox steps: first, conversion to the reduced thiol, and then subsequent oxidation of the mercury surface in the presence of the thiol. An eluting thiol is unaffected upstream but detected downstream. This novel post-column "reactor" is uniquely activated by an electronic switch. Preliminary identification of an unknown peak as resulting from disulfide may thus rely on the disappearance of the peak when the upstream electrode is "off".

For oxidizable analytes, the selectivity of the series electrode measurement is most often advantageous. As an example, a pharmacological investigation [E44] lacked an efficient method for the detection of a drug capable of forming certain *O*-methyl and *O*-sulfate metabolites in vivo (see Fig. E9A). All of the compounds can be oxidized, albeit at high potentials where electrode passivation would occur. Because the oxidation reactions of the parent compound and these metabolites all yielded the corresponding quinone, series dual electrodes with downstream detection was a useful simplification of the instrumental assay. Figure E9B demonstrates the versatility of the system for these methoxy metabolites.

Parallel-opposed configuration. If the redox forms of a solute can be recycled between an anode and cathode indefinitely, the possibility of achieving greater than 100% conversion of analyte with the concomitant improvement in *s/n* can be realized [E45]. The critical parameter is the diffusion distance between these electrodes relative to the transit time through the cell. With microbore l.c./ec, for example, the relatively long residence times in the active cell volume are advantageous. Goto et al. [E46]

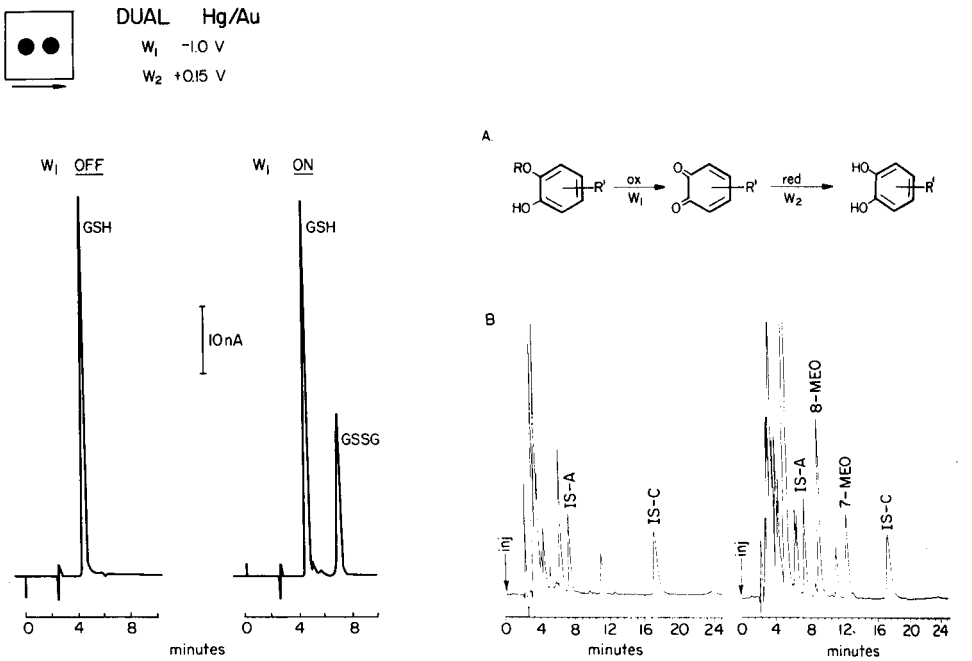


Fig. E8. Response at downstream electrode held at 0.15 V vs. Ag/AgCl after glutathione (GSH) and glutathione disulfide (GSSG) are injected. Left: upstream generator "off"; right: upstream "on". Stationary phase: Biophase ODS 5 μm (250 X 4.6 mm). Mobile phase: 99% 0.1 M monochloroacetate (pH 3.0)/1% methanol at 1.5 ml min^{-1}. Reprinted by permission: Anal. Chem., 55 (1983) 8. Copyright, American Chemical Society.

Fig. E9. (A) Redox scheme for dual series electrodes for selective downstream detection of high oxidation potential analytes (R = CH_3, $C_6H_9O_6$ or SO_3^-). (B) Human urine chromatograms obtained with downstream detection before and 24 h after ingestion of 100 mg of fenoldopam. The 7-methoxy and 8-methoxy metabolites are measured as their quinone oxidation products. Reprinted with permission: J. Chromatogr., 317 (1984) 463. Copyright, Elsevier Scientific Publishing Co.

utilized 1.0-cm long X 0.2-cm wide electrodes separated by 30—50 μm and amplified the signal up to 20X over the expected 100% conversion by using low flow rates. The system was successfully applied to detect catecholamines in ultrafiltered human serum. These same types of regenerative transducers have been studied also for conventional flow rates using much larger electrodes [E45, E47, E48] but the detection limits were not significantly better than amperometric detection at limited conversion efficiency at a single electrode.

Difference mode of detection using discrete electrodes

By incorporating two or more electrodes into the transducer, several options exist for detection in the differential mode [E49]. For parallel adjacent thin-layer cells, the electrodes can be poised at potentials encompas-

sing the region where the current response is markedly dependent on detector potential (i.e., the region of $E_{1/2}$). For example, an enhanced response for the dopamine metabolite, homovanillic acid (HVA), over more easily oxidized compounds can be achieved by operating two parallel-adjacent electrodes along the rising portion of the hydrodynamic voltammogram for HVA (Fig. E10A). In Fig. E10B, chromatograms are shown for potentials of +800 mV, +700 mV and 800—700 mV. The peaks for serotonin (5-HT) and 5-HIAA are diminished relative to that for HVA. Because the differential output is recorded, only compounds displaying a significant current change between the selected potentials produce signals. If the electrodes are 100% efficient, the electrodes can be arranged in series and operated at equally spaced intervals of potential. Each electrode in the set detects only those compounds which could not be removed at the previous electrode set at lower potential [E42]. In simplest form, only two electrodes need be used; the first removes all of the more easily detected solutes at the foot of the wave for the solute of interest. The second electrode is set at a higher potential to detect the solute of interest [E50].

Overall improvement in the signal quality of these detector arrangements depends on the appropriate spacing of operating potentials. Major sources of baseline interference in ec detection are fluctuations in flow rate and temperature, static discharge, and changes in composition of the mobile phase. Because these common-mode interferences are discriminated in this detection mode, this can be an effective technique for improving signal quality. Figure E11 shows an expanded view of the baseline response of a

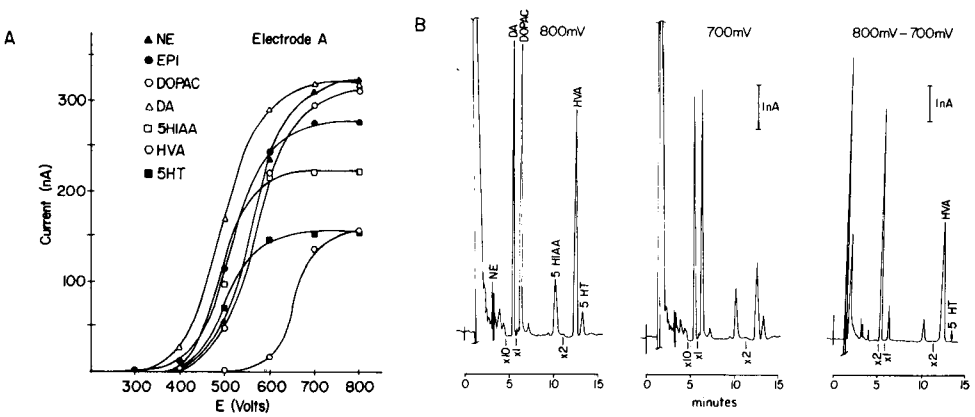


Fig. E10. (A) Hydrodynamic voltammograms for biogenic amines and metabolites obtained on glassy carbon. Mobile phase: 1.9 parts THF/3.5 parts CH_3CN/96.5 parts 0.15 M monochloroacetate (pH 3) with 200 mg l^{-1} sodium octylsulfate and 0.25 g l^{-1} Na_2EDTA at 1.5 ml min^{-1}. Stationary phase: Biophase ODS 5 μm (250 × 4.6 mm). (B) Simultaneously obtained parallel dual-electrode chromatograms at +800 and +700 mV and the difference mode output. Reprinted with permission: Anal. Chem., 54 (1982) 1417A. Copyright, American Chemical Society.

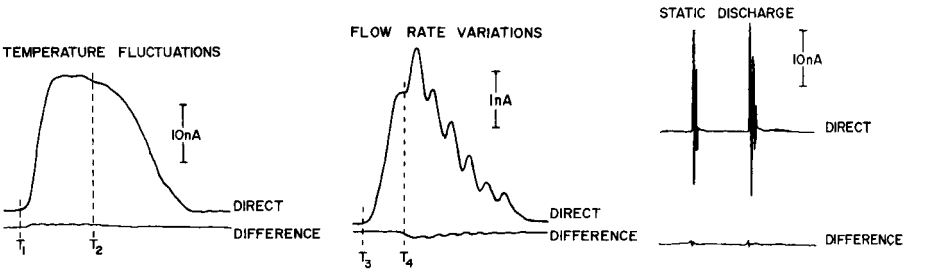


Fig. E11. Parallel electrode difference-mode response (700—600 mV) and single electrode direct detection (+700 mV) for various baseline artifacts: (T_1/T_2) turn on/off heat gun; (T_3/T_4) flow rate change from 1 to 2 and from 2 to 1 ml min^{-1}. Reprinted with permission: Anal. Chem., 57 (1985) 1541. Copyright, American Chemical Society.

single thin-layer electrode operated at +700 mV and parallel-adjacent, thin-layer electrodes operated at 700—600 mV. The difference chromatogram is largely immune to these causes of drift. Naturally, the greatest reduction in baseline interference occurs when both electrodes are operated at the same potential. Unfortunately, no response from an analyte would be observed in this situation. The optimum spacing of potentials typically extends from the base of the hydrodynamic voltammogram to the plateau. Taking the difference accentuates true noise; however, most detectors are limited by environmental factors rather than noise, so improvement still is observed.

Operation in the difference mode is also possible in the series thin-layer configuration [E51]. Suppose two closely spaced thin-layer electrodes are operated at the same potential. The difference response is not zero as it is with the parallel configuration. The net current is appreciable because the flux of analyte is different at each electrode surface. The flux is greatest at the leading edge of the upstream electrode and then decreases in the downstream direction due to the accumulated depletion of analyte in the diffusion layer. In the brief transit time between series electrodes (ca. 10 m), the diffusion layer is not adequately remixed with the bulk solution, and the response of the downstream electrode, therefore, is diminished. The difference-mode response is about 35% of the upstream response alone at conventional flow rates and approaches 100% below 100 μl min^{-1}. The *s/n* improves dramatically as the potential difference is decreased to zero, because contributions to baseline interference are drastically decreased. For all these reasons, the causes of baseline interference cannot be attributed to the same factors of mass transport depletion attributed to the analyte. Rather, other contributors, such as the bulk properties of the eluent or flow rate stability, must determine the difference noise. In essence, the downstream working electrode acts as a reference signal for drift and can be incorporated into the cell without introducing additional dead volume. Because the phenomenon is based on mass transport rather than electrochemistry, the improvement in the *s/n* is obtained for all solutes detected at or below the applied potential. Also, because the two electrodes are sensing

the same flow rate inconsistencies, mobile phase contaminants, and temperature changes, the reference signal is much more indicative of these artifacts than would be possible with the separate flow channels proposed earlier by Brunt and Bruins [E52].

F. POTENTIODYNAMIC DETECTION AT SOLID ELECTRODES IN FLOWING SOLUTIONS

Potentiodynamic techniques are classified here as voltammetric or amperometric. Potentiodynamic voltammetry corresponds to techniques in which, regardless of the general character of the potential-time (E—t) waveform, a record is obtained of the faradaic response of the analyte(s) as a function of electrode potential (i—E) over an appreciable range of E. This includes, for example, the techniques of linear sweep, normal pulse, staircase, square wave and differential pulse voltammetry. Potentiodynamic amperometry corresponds to techniques in which, regardless of the E—t waveform, the faradaic response is sampled for the same value of E in repetitive applications of the waveform. Accordingly, normal pulse (n.p.) voltammetry becomes n.p. amperometry when the pulse amplitude (ΔE) is no longer varied with time. Differential pulse (d.p.) voltammetry and square wave (s.w.) voltammetry become identical as d.p. amperometry. In potentiostatic amperometry, the electrode potential remains constant throughout the analysis and the faradaic signal is monitored continuously.

Mercury electrodes are characterized by the desirable attributes of being easily renewed (dropping mercury electrodes), relatively free of problematical surface phenomena, and atomically smooth so that the charging current background is minimal. Consequently, principles of hydrodynamic voltammetry and amperometry frequently are taught and remembered within the context of the nearly ideal characteristics of mercury. Hence, initial expectations for comparisons of potentiostatic (d.c.) amperometry vs. potentiodynamic amperometry usually are based on experience with mercury electrodes. For that reason, the two forms of detection were considered collectively for mercury electrodes (Section C). Comparisons are far more complex for solid electrodes and the topics are considered with more detail here and elsewhere in this review (Sections D—F).

Potentiodynamic voltammetry

Linear sweep. The voltammetric response (i—E) by linear sweep voltammetry is virtually the same whether for a flow-through ec detector, with continuous flow of the sample solution, or a hydrodynamic electrode submerged in a large volume of the solution in a conventional voltammetric cell. Consideration here is restricted to possible applications for f.i./ec and l.c./ec analyses. Under conditions of uninterrupted flow, it is necessary to apply a fast sweep rate to allow execution of one complete waveform during the brief time period when the peak concentration of the sample is

contained within the detector cavity (i.e., a pseudo steady-state concentration). But, with high sweep rate at conventional electrodes in ec detectors, comes the loss of detectability because background current increases with scan rate, faster than does the analytical signal. Wang and Dewald [F1, F2] suggested a subtraction procedure to correct for background i—E response. In addition to charging-current background, this is effective also in correcting for the presence of dissolved oxygen, provided the oxygen level is identical in the presence and absence of the analyte. Discrimination against background current should be obtained also using twin thin-layer detectors in the series configuration by recording the difference current (Δi) as a function of potential for the simultaneous sweep of the two electrodes. Since the flux of analyte at the downstream detector is diminished by the detection upstream, Δi for the analyte is not zero. Double-layer charging will be identical, provided the two areas are the same, and is eliminated in computing Δi. Correction for oxygen background will not occur.

Stopped-flow injection was applied by Tougas and Curran [F3] so that moderate sweep rates could be used. For a very thin detector cavity, this technique can correspond to voltammetry with a fixed diffusion boundary, as recently considered theoretically by Aoki et al. [F4]. Oldham and Zoski [F5] have recommended the addition of the two branches of current for a triangular sweep i—E curve to eliminate the contribution of double-layer charging in voltammetric trace analysis in a quiescent solution. This technique should have some applicability for mercury film electrodes in stopped-flow f.i./ec detection.

A distinct advantage comes for stripping voltammetry when applied in a f.i./ec system [F6—F8]. Preconcentration (deposition) of the analyte is achieved at constant potential, with linear-sweep stripping voltammetry after passage of the sample. This corresponds to the desirable condition of stripping voltammetry with media exchange.

The signal-to-background (s/b) consideration is optimized for voltammetry at microelectrodes, as demonstrated by Wightman and coworkers [F9, F10]. The s/b increases as the electrode area is made smaller. Furthermore, the total current is small, minimizing iR drop, and the technique can be applied in highly resistive media. Howell and Wightman [F10] applied sweep rates up to 20 000 V s^{-1}. For aqueous solutions having good conductivity, currents can be made larger with the same benefit of high s/b by use of arrays of microelectrodes [F11—F13].

This reviewer concludes, as have others [F14—F16], that a sacrifice in detection limit is always made when linear-sweep voltammetric detection is chosen over d.c. amperometric detection. However, there undoubtedly will be instances for which detectability can be satisfactory and the use of fast sweep voltammetry, especially at microelectrodes, will be important for f.i./ec and l.c./ec.

Pulsed sweep. Pulsed voltammetric waveforms are preferred over the linear sweep technique because of improved discrimination against back

ground current, especially from double-layer charging [F14—F16]. Sokol and Evans [F17] demonstrated a modified version of d.p. voltammetry in which currents were measured twice for each pulse and the difference plot vs. E. The peak response was decreased somewhat from that obtained by conventional d.p. voltammetry; however, the s/n was improved significantly. Ashley and Levine [F18] applied d.p. voltammetry at glassy carbon electrodes to scan rates of 0.5 V s^{-1}. Alexander and Akapongkul [F19] used scan rates to 0.1 V s^{-1} for d.p. voltammetric detection of cadmium and zinc in f.i./ec. Wang and Dewald [F20] demonstrated rapid d.p. voltammetric f.i./ec for determination of acetaminophen, dopamine, caffeic acid, chloropromazine, and copper, lead and cadmium with a sample throughput of ca. 20 h^{-1}. Ŝtastuý et al. [F21] described a double pulse train with semi-differential evaluation of current in a single sweep of potential at ca. 0.1 V s^{-1}.

Of greatest interest is fast-sweep pulse techniques at microelectrodes. Dayton et al. [F9] applied triangular-sweep and d.p. voltammetry and simple pulse chronoamperometric techniques to carbon fiber microelectrodes (ca. 5×10^{-7} cm^2) in quiescent solutions. The diffusion distance is large, relative to the electrode area for these microelectrodes, and current response is time-independent. Ewing et al. [F22] reported that the current response on the back step of the chronoamperometric experiment is predominately due to double-layer charging and can be used for background correction. O'Dea and Osteryoung [F23] had reported on the greater selectivity available at a Hg electrode for l.c./ec using s.w. voltammetry with sweep rates of ca. 0.25 V s^{-1}. Recently, O'Dea et al. [F24] gave preliminary results for rapid-sweep, s.w. voltammetry at microelectrodes made from graphite and gold. Pulse frequencies to 60 s^{-1} were used with a step height of 5 mV (i.e., 0.3 V s^{-1}). The application of fast-sweep d.p. and s.w. voltammetric detection at microelectrodes is particularly appealing for capillary l.c. columns, because the electrode can be inserted directly into the end of the column, as demonstrated by Knecht et al. [F25].

Potentiodynamic amperometry

Sensitivity and selectivity. The improvement of polarographic sensitivity resulting from use of n.p. waveforms, as well as the increased resolving power (i.e., selectivity) from s.w. and d.p. waveforms, are well documented for mercury electrodes [F23, F26, F27]. It was only natural to expect similar improvements for potentiodynamic amperometry at solid electrodes in comparison to d.c. amperometry. As expected from theory, sensitivity ($\mu A\ M^{-1}$) for n.p. or d.p. detection is greater than for d.c. detection [F28, F29]. However, except for one report [F30], applications of n.p. or d.p. amperometry have not been observed to give improved detectability [F31—F36]. Background currents and noise are considerably larger at solid electrodes for pulsed waveforms in comparison to mercury [F33—F35]. A predominant source of background current is faradaic surface transformations [F14—F16], e.g., formation/reduction of oxides at noble electrodes and

oxygen-containing functional groups at carbon (e.g., phenolates and carboxylates). These currents decay very slowly, in comparison to charging current at mercury electrodes, and the pulsed techniques have significantly lower success in discriminating against these processes [F33—F35]. In addition, double-layer charging at glassy carbon electrodes decays slowly [F37], probably because of the existence of micropores in the material.

As expected, selectivity is improved as a result of d.p. amperometry [F14, F15, F31, F33, F37, F38]. However, because detectability suffers, differential d.c. amperometry at twin ec detectors in the parallel configuration is recommended for lowest detection limits in l.c./ec (see Section E).

For a short delay time prior to sampling of current in n.p. amperometry, the signal is unaffected by the hydrodynamic boundary-layer thickness at the electrode—solution interface. The elimination of flow rate dependence in flow-through detectors has been claimed by some workers [F31, F33], but not by others [F34, F35]. Since dispersion of small samples in f.i. and l.c. systems is flow rate dependent, pulsed detection peaks in f.i./ec and l.c./ec will be flow rate dependent. Only for continuous flow of the sample solution, e.g., process control or f.i. systems with very large dispersion, can the need for careful control of flow rate be decreased substantially using pulsed detection.

Amperometric detection with potentiodynamic cleaning. The rapid loss of electrode activity is commonly reported for conventional voltammetric and amperometric detection at solid electrodes. Surface oxides at noble metals interfere with some anodic detection reactions at large positive potential. For example, the anodic d.c. detection current for arsenic(III) at platinum electrodes at 1.0 V vs. SCE has been observed to decay by 50% of the mass transport-limited value over a 10-min period [F39]. The signal can be restored after a brief (i.e., 100 ms) negative pulse to cathodically dissolve the oxide layer [F40]. Trace levels of certain metal cations can result in significant surface coverage by the electrodeposited metal [F14, F15]. Noble metal surfaces are renowned for their tendency to adsorb a multitude of inorganic and organic species, even if present in solutions at only trace levels [F41, F42].

Because platinum is an effective hydrogenation catalyst, a consequence of its ^9d-electronic structure, it is likewise an efficient dehydrogenation catalyst. Hence, many organic molecules can be weakly adsorbed followed by dehydrogenation to produce the more strongly adsorbed carbonaceous free radicals [F43, F44]. Adsorbed radicals and ions can have a severely detrimental effect on reaction kinetics for the analytes of interest by producing changes in the double-layer structure as well as by occlusion of surface sites necessary to support favorable reaction mechanisms. Many anodic detection reactions of organic compounds produce free radicals which, at sufficiently high concentrations, can polymerize in the diffusion layer to produce adherent surface films [F45] which interfere with diffusion of analyte to the electrode surface.

Rigorous pretreatment routines are recommended in conventional voltammetric analysis for obtaining reproducible i—E data [F41, F42, p. 205f in F45]. The routine usually begins with mechanical abrasion of the electrode. This is followed by alternate anodic and cathodic polarizations in supporting electrolyte solution at potentials for evolution of molecular oxygen and hydrogen, with concomitant formation and dissolution of surface oxides on noble metal electrodes. Finally, the particular waveform of interest is applied repeatedly in the presence of the analyte until the i—E data is reproducible. The voltammetric response then is recorded. Adherence to such an involved regimen is consistent only with the leisurely affair of traditional analytical voltammetry in moderately large cells from which the electrode is easily removed. Mechanical resurfacing of miniature electrodes in flow-through cells for f.i./ec or l.c./ec is not conceivable on a routine basis, even though Fleet and Little [F46] claim their wall-jet configuration is easily disassembled for mechanical treatment.

Surface fouling by sample impurities and detection products is diminished in f.i./ec and l.c./ec because the very small samples injected result in relatively brief exposure of the electrode to the sample. Pulsed detection decreases further the exposure to detection products, because of detection pulses with short duration, and reports appeared quickly of improved reproducibility for pulse amperometry for glassy carbon [F46, F33] platinum [F47] and mercury film electrodes [F38]. In addition, analytical applications of flow-through ec detectors are usually for lower concentrations than in conventional voltammetry and rates of second-order dimerization reactions leading to film formation can be negligible. Supporting electrolytes and chromatographic eluents should, of course, be of high purity. If necessary, adsorption columns can be applied in flow systems for removal of certain highly contaminating elements of the sample matrix. All of these factors forestall but do not prevent the inevitable decline or, in some cases, complete loss of electrode activity. A probable major source of contamination is the plasticizers which bleed slowly from the plastic components of some detection systems [F48].

Virtually all adsorbed ions, organic radicals, etc., are cleaned effectively from platinum and gold surfaces by oxidative processes catalyzed by the surface oxide formed upon application of a large positive potential step [F41, F42, F49]. Dissolution of the surface oxide is achieved quickly by a subsequent cathodic polarization and the clean metal surface is restored. In 1972 Clark et al. [F50] reported greater reproducibility for anodic detection of ethylene at platinum electrodes through the use of cleaning pulses. The same was reported by MacDonald and Duke [F30] for anodic detection of p-aminophenol. Štulík and Hora [F47] described the improved cathodic detection of iron(III) and copper(II) at platinum in sulfuric acid when the sampled d.c. detection was interrupted by brief anodic cleaning pulses at a frequency of 0.25 s^{-1}. The reduction of oxygen at platinum electrodes occurs by a preadsorption mechanism which is extremely sensitive

to adsorbed impurities and the d.c. amperometric current quickly decays to zero even in solutions of analytical reagent-grade acids [F51]. Voltammetric i—E curves and amperometric detection peaks for f.i./ec are reproducible and transport limited with intermittent application of a positive cleaning pulse to a value for oxide formation [F52]. It is readily apparent that repeated alternation of anodic and cathodic polarizations of platinum electrodes rather quickly leads to loss of reflectivity of a well polished surface, and it is concluded that formation and dissolution of surface oxide produces a surface covered with disoriented platinum (platinum black) [F42]. Johnson et al. [F53] demonstrated that a small quantity of platinum(II) is dissolved from a platinum electrode during reduction of the surface oxide in sulfuric and perchloric acids. Chialvo et al. [F54] applied x-ray and surface microscopy to a platinum electrode surface following extensive square wave polarization. They reported that surface modification results from repeated formation and dissolution of oxide leading to a reproducibly high surface area.

Fleet and Little [F46] reported in 1974 that precision at carbon electrodes is improved by use of cleaning pulses. In 1977, a patent was issued on behalf of Fleet to EDT Supplies Ltd., England, for the use of cleaning pulses at a wall-jet electrode [F55]. Specific examples given were for anodic detection at glassy carbon of methyl-4-hydroxybenzoate and n-propyl-hydroxybenzoate in 20% methanol/water, and 4-methoxyphenol, 4-aminophenol, 4-nitrophenol and 4-chlorophenol in 5% methanol/water. Detection was at +1.1 V vs. SSCE with cathodic cleaning at −0.2 V at a frequency of 5 s^{-1}. Berger, who was involved in the original investigations with Fleet [F56], has continued to study pulsed-cleaning detection with a two-step waveform at wall-jet electrodes and a recent patent was issued to Hewlett-Packard Co. on behalf of Berger [F57]. Tenygl [F58] recommended also the application of cleaning pulses but cautioned that "polarization to very positive potentials causes corrosion of the electrode itself (formation of oxide films, disintegration of carbon) and should not be considered". No guidelines were given for choosing appropriate values of cleaning potentials. Application of a voltage pulse train (0.5 Hz) for 5 min was demonstrated by van Rooijen and Poppe [F59] to restore the response of a glassy carbon electrode which had been deactivated by exposure to organic compounds.

Pulsed electrocatalytic detection. Electrocatalytic detections are all those based on reaction mechanisms in which intimate interaction between the electrode and analyte is essential. Included are indirect as well as direct detections based on surface-controlled mechanisms. This consideration is not necessarily exclusive of those detections cited previously which benefited from potentiodynamic cleaning, because the need for surface cleaning frequently is indicative of the electrocatalytic nature of a reaction. Indeed, the cleaning phenomenon probably occurs for all electrodes with concomitant changes in the physicochemical nature of the surfaces which enhance favorable mechanistic pathways for detection reactions. Emphasis

here is focused at anodic detections based on surface processes associated with the oxide-catalyzed cleaning of platinum and gold surfaces.

In conventional linear sweep voltammetry, the most convenient and sensitive indicator of the presence of solution impurities is the suppression of the cathodic and anodic peaks for adsorbed hydrogen at platinum electrodes [F41, F42]. Nearly as sensitive is the change observed in the anodic wave for oxide formation during the positive potential sweep; onset of the wave is suppressed and the height of the wave is increased by adsorbed impurities. Because the presence of dissolved oxygen interferes with analytical applications of the hydrogen peaks, Johnson and co-workers [F49, F60—F67] have utilized the observed change in the i—t curve for a positive step to the region of oxide formation for detection of adsorbed analyte. The technique generally uses a three-step waveform: E_1 (100—200 ms) corresponds to the detection potential with current being sampled near the end of the period; E_2 (100—200 ms) corresponds to a large positive potential, usually at a value for oxygen evolution, where the oxidative cleaning of the surface is very rapid; E_3 (200—500 ms) corresponds to a large negative potential step, usually to a value for hydrogen evolution, at which the surface oxide is reduced quickly (<50 ms) and the clean surface is regenerated. Typical repetition rates are ca. 1—2 Hz. Analyte adsorbed at E_3 is detected with the subsequent application of E_1. The analytical response results primarily from adsorbed analyte. Hence, sensitivity is a function of the flux of adsorbing analyte, the adsorption period (t_3) and ultimately, for long t_3, is limited by the corresponding adsorption isotherm. In applications to f.i./ec and l.c./ec with small sample volumes, low concentrations and short t_3, peak signals are mass transport controlled and i_p—C plots are linear with zero intercepts. For isotherm control (e.g. Langmuir), plots of i_p—C exhibit negative deviation from linearity; however, $1/i_p$—$1/C$ plots are linear with a small but non-zero intercept and a slope related to the adsorption equilibrium constant. Careful calibration is recommended for quantitative applications [F49].

The pulsed electrocatalytic detection has been demonstrated at platinum electrodes for alcohols [F60], polyalcohols and carbohydrates [F61, F62], amines and aminoacids [F63], aminoglycoside antibiotics [F64], organic and inorganic sulfur [F49, F65, F66] and even non-electroactive adsorbates, by indirect detection [F67]. The general conclusion is very optimistic. If a molecule or ion is adsorbed, it can be detected by this pulsed amperometric method. Detection limits for 50-μl samples in f.i./ec are in the sub-μg ml^{-1} range ($\leqslant$50 ng). Successful extension of this technique to gold electrodes for chromatographic detection of sugar alcohols and carbohydrates has been made by Edwards and Haak [F68], and Rocklin and Pohl [F69] at Dionex Corp. The sensitivity for sulfur detection at gold is even better than for platinum [F65] with detection limits below 100 pg observed for many compounds, including thiophosphate [F66].

The future of pulsed electrocatalytic detections is expected to include applications at carbon electrodes. The observation of benefit from cleaning

pulses was noted already [F46, F55—F59]. Although still of great analytical use for ec detection, many organic oxidation reactions do not occur at transport limited rates for carbon electrodes [F70]. Numerous reports have appeared describing increased activity resulting from application of extremely positive polarization potentials [F22, F70—F77]. Examinations of carbon surfaces revealed increased surface roughness (pitting) [F76, F77] and an increase in surface bound oxygen groups, e.g., groups at the activated surfaces which can catalyze some ec reactions [F76]. Gonan et al. [F73] reported that activating pulses to +3.0 V were successful; however, pulses to +4.0 V were not beneficial. Wang and Hutchins [F77] applied a ±6 V square wave at 35 Hz for 5 min in sodium chloride and observed significant improvement in reversibility for eight electroactive species. The use of sampled d.c. detection with intermittent cleaning/activation pulses might be useful for maintaining reproducibly high activity at carbon ec detectors.

Coulostatic detection

From the name, coulostatic techniques of detection [F78] do not appear to be potentiodynamic or potentiostatic because instrumental control is of electrical charge ($q = i \times \Delta t$) and not potential. Yet the technique merits inclusion here because the galvanostatic pulses of fixed charge content are applied so as to maintain the electrode potential according to a prescribed waveform. Charge pulses of μs duration are applied to the electrode. Within such a short time span the only process occuring is charging of the double layer with concomitant change of electrode potential. The potential relaxes following the pulse as faradaic reactions neutralize the charge in the double-layer region. The potential is monitored between pulses when net current is zero and *iR*-drop is non-existent [F78, F79]. Last [F80], and Barnes and Nieman [F81], have demonstrated the usefulness of coulostatic voltammetric and amperometric detection for l.c./ec with carbon paste and glassy carbon electrodes. Last reports a sweep rate of 3 V s^{-1}.

G. CHEMICAL AND PHOTOCHEMICAL DERIVATIZATIONS FOR IMPROVED DETECTION IN FLOWING SOLUTIONS

Chemical and photochemical derivatizations have become very commonplace in l.c. within the last two decades to facilitate u.v.-visible and fluorescence detection [G1—G23]. However, only recently have publications appeared emphasizing any type of derivatizations for improved ec detection in l.c. Almost all of the approaches in l.c./ec have involved off-line, pre-injection chemical reactions, as opposed to on-line, pre- or post-column methods, and the reaction mixture of desired derivatives, unreacted sample components, and usually excess reagent(s) is injected. Post-column, on-line, photochemical derivatizations in l.c./ec have begun to appear within the past 2—3 years [G24—28]. There are also a large number of reports describing on-line, dual electrode detection, wherein the series upstream electrode

performs an electrochemical derivatization, and the newly generated product is then detected immediately downstream [G29—G36]. However, because of space limitations, a review of this particular derivatization approach in l.c./ec is not included here.

Off-line, pre-injection derivatization is perhaps the most inefficient, laborious, error-prone, and least desirable approach for improved detection in l.c. Most recent work with chemical derivatizations in l.c. with u.v.-visible or fluorescence detection deals with on-line, post-column, real-time methods, which seem to offer significant advantages over off-line, pre-injection or pre-column derivatizations. Excellent discussions comparing the various approaches to chemical derivatization in l.c. have appeared [G1—G4, G7, G16, G20]. It is suspected that electrochemists involved with l.c./ec have tended to emphasize off-line, pre-column approaches because those have been rather straightforward, simple techniques to show the final improved detection of starting analytes. Post-column, on-line, continuous derivatization approaches in l.c./ec may prove more difficult, in view of the separate operational requirements for the chemical derivatization and electrochemical detection steps.

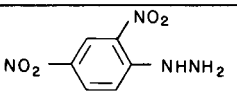
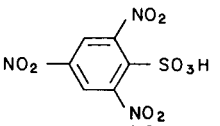
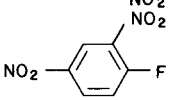
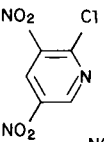
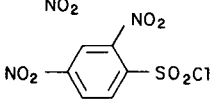
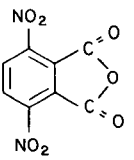
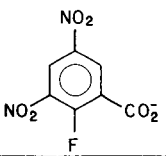
Most of the chemical derivatizations described for l.c./ec have been oxidative (anodic) as opposed to reductive (cathodic) detection. Both oxidative and reductive detection are discussed here, and also the rationale is indicated for selecting derivatizations for analytes in both detection modes. On-line, post-column photolytic derivatizations in l.c./ec allow for oxidative and reductive detection, dependent on the starting analyte and photolytically derived species.

Chemical derivatizations for reductive detection

The literature and numerous possibilities for the use of derivatizations in l.c./ec were first reviewed by Kissinger et al. in 1979 [G37]. Several nitroaromatic compounds were suggested as potential derivatization reagents for nucleophiles, including alcohols, primary and secondary amines, amino acids, carboxylic acids and keto aldehydes, all to be followed by reductive detection. Several nitroaromatic reagents utilized for reductive ec and/or u.v. detection in l.c. are shown in Table G1. Some of these have now been used as pre-column derivatization approaches for oxidative, photolytic l.c./ec [G38]. Jacobs and Kissinger [G39, G40] described the use of various nitroaromatic reagents for carbonyl compounds, amines and amino acids. The use of 2,4-dinitrophenylhydrazine for derivatization of aldehydes or ketones leads to the 2,4-dinitrophenylhydrazone. The product is expected also to be detected oxidatively by photolytic ec [G24, G26, G38]. Detection limits for l.c./ec were less than 100 pg for several aldehydes and ketones, which is ca. 20× lower than possible with u.v. detection for the same derivatives, and significantly better than for the underivatized carbonyl compounds. The determination of amines and amino acids by reductive l.c./ec was described using trinitrobenzenesulfonic acid (TNBS),

TABLE G1

Summary of reagents for chemical derivatization prior to reductive detection

Name	Structure	Substrate
2,4-Dinitrophenyl-hydrazine		carbonyl cpds. [G39, G40, G52]
Trinitrobenzene-sulfonic acid		amines, amino acids [G39, G40, G41—G43]
2,4-Dinitrofluorobenzene (Sanger's reagent)		amines, amino acids [G39, G40]
2-Chloro-3,5-dinitropyridine		amines, amino acids [G39, G40]
Dinitrobenzene-sulfonyl chloride		amines [G44]
3,6-Dinitrophthalic anhydride		peptides [G46]
2-Carboxy-4,6-dinitro-fluorobenzene		peptides [G45]

2,4-dinitrofluorobenzene and 2-chloro-3,5-dinitropyridine [G40]. Use of these three reagents was compared with regard to minimum detection limits, minimum production of interfering by-products, ease of derivatization, stability of derivative, etc. TNBS was found to be superior in all respects for amines and amino acids. As expected, more nitro groups on the aromatic moiety lead to lower detection limits, since the number of electrons transferred per molecule increases.

A similar approach was described by Wightman and co-workers [G41—G43] using trinitrobenzenesulfonic acid for the pre-column derivatization of alkylamines and amino acids for reductive detection in l.c./ec. The

authors concluded that TNBS can be an ideal pre-column reagent for primary and secondary amine derivatives, since the reaction is often quantitative, trinitrophenyl derivatives are fairly stable at room temperature, or below, and final detection is possible by u.v. and ec. γ-Aminobutyric acid was determined in rat brain by derivatization with TNBS, using reversed phase l.c. with reductive ec. Picomole detection limits for the final derivatives and amino acids were possible from brain tissue. Although these derivatives were studied by reductive ec, they are clearly candidates for oxidative, photolytic l.c./ec or l.c./photolytic-ec [G38]. Wightman et al. [G44] have also shown that dinitrobenzenesulfonyl chloride derivatives of amines can be detected at the pmol level using reductive l.c./ec.

Meek [G45] recently described novel derivatizations for the ec detection of peptides at the pmol level, using the nitroaromatic reagents 3,6-dinitrophthalic anhydride and 2-carboxy-4,6-dinitrofluorobenzene. The derivatives could be detected by reductive ec as well as by u.v. absorption. Detection limits were improved in comparison with the underivatized peptides by 50—500×.

The methods described above have used pre-column, off-line chemical derivatizations prior to l.c. with reductive ec. No reports were found for on-line, post-column derivatizations for reductive detection in l.c./ec, though this should be possible. Experimental difficulties would be increased, especially due to the presence in the eluent of unreacted reagent which may be electroactive. However, the use of solid-phase reagents for post-column, on-line derivatization might circumvent this problem, and work is being done in that area [G46—G48].

Chemical derivatizations for oxidative detection

Pre-column derivatizations. Though reductive ec detection for l.c. has now been in use for at least the past five years, it does not appear to have gained an overwhelming number of devotees. This may be due, in part, to operational difficulties, the need to exclude oxygen from the mobile phase and sample, decomposition of the mobile phase and buffers at the working electrode surface, and other parameters. It does not appear that reductive l.c./ec will ever become as popular and applicable as oxidative approaches [G49—G51].

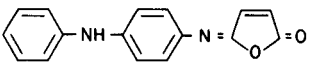
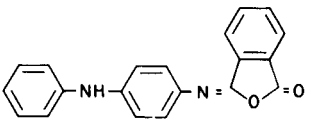
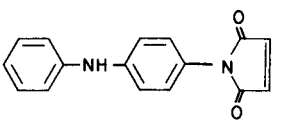
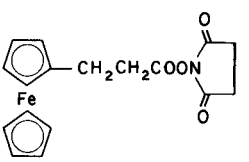
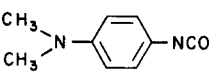
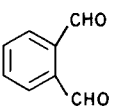
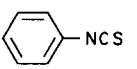
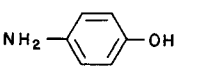
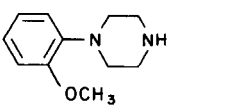
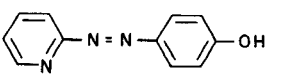
Phenylhydrazine, *p*-nitrophenylhydrazine, and 2,4-dinitrophenylhydrazine have all been used as off-line reagents for carbonyl compounds, followed by oxidative detection at a glassy carbon electrode after reversed phase l.c. [G52]. These authors reported additional derivatization approaches for primary and secondary amines in l.c./ec [G53—G56]. The phenylhydrazones of various steroids were prepared off-line, characterized, and evaluated as standards for optimal detector conditions and detection limits. The *p*-nitrophenylhydrazones showed the highest sensitivity with a detection limit of 200 pg/injection. The *p*-nitro- and 2,4-dinitrophenylhydrazones can be

used both for reductive and oxidative detection, but it would appear that reductive detection limits are lower [G40, G52, G57].

These workers recently described other off-line derivatization approaches for l.c./ec. These were derivatization of amines with *N*-(4-anilinophenyl)-

TABLE G2

Summary of reagents for chemical derivatization prior to oxidative detection

Name	Structure	Substrate
N-(4-Anilinophenyl)-isomaleimide		amines [G58, G59]
N-(4-Anilinophenyl)-isophthalimide		amines [G58, G59]
N-(4-Anilinophenyl)malemide (APM)		thiols [G59]
N-Succinimidyl-3-ferrocenylpropionate		arylalkylamines [G61]
p-Dimethylaminophenyl-isocyanate		arylhydroxylamines [G61]
o-Phthaldehyde		amines, amino acids [G1—G7, G64—G69]
Phenylisothiocyanate		amino acids [G65]
p-Aminophenol		isocyanates [G71, G72]
1-(2-Methoxyphenyl)-piperazine		aromatic and aliphatic isocyanates [G72]
4-(2′-Pyridylazo)resorcinol		metal ions [G74]

isomaleimide (APIM, Table G2) and *N*-(4-anilinophenyl)isophthalimide, and derivatization of thiols with various *N*-substituted maleimides [G58, G59]. Amines and thiols can function as nucleophiles towards unsubstituted maleimides, leading to stable, easily prepared derivatives having excellent oxidative ec properties. Derivatization of typical amines with the indicated reagents was complete within 20 min at room temperature in acetonitrile/0.5 M borate buffer (1:1, pH 9.0). The detection limit for the phenethylamine-APIM adduct was ca. 0.1 pmol, and the ec response was linear in the range 0.1—10 ng of phenethylamine [G58]. Thiols, such as cysteine, *N*-acetyl-L-cysteine, glutathione and D-penicillamine, were readily derivatized with *N*-(4-anilinophenyl)maleimide, and pg amounts of these thiols were separated by reversed phase l.c. with oxidative ec detection [G59].

Amines and amine derivatives have been the most studied class of substrates for pre- and post-column derivatizations, oxidative and reductive, and work continues. Tanaka et al. [G60] reported on the quantitative condensation of arylalkylamines with *N*-succinimidyl-3-ferrocenylpropionate at room temperature for 20 min in acetonitrile/0.05 M borate buffer (1:1, pH 8.0). A detection limit of 0.2 pmol was realized. Final detection of these derivatives was done using an oxidative-reductive series (upstream-downstream) dual electrode system.

Musson and Sternson [G61] described off-line derivatization of arylhydroxylamines using *p*-dimethylaminophenylisocyanate, which leads to stable hydroxyureas. These could be separated by reversed phase l.c. and detected spectrophotometrically and amperometrically. Optical detection offered a detection limit of 9×10^{-7} M, while ec detection provided a limit of 1×10^{-8} M.

Kester and Danielson [G62, G63] reported on the determination of hydrazine and 1,1-dimethylhydrazine as their salicylaldehyde derivatives. Detection limits were less than 5 ng per injection. A variety of other aromatic aldehyde or ketone derivatizing reagents could be useful for this class of analytes.

o-Phthaldehyde (OPA) has been one of the most popular reagents for amines and amino acids, and has been especially popular for u.v. and fluorescence detection [G1—G7]. It has also been used in l.c./ec, and probably more papers have appeared involving OPA than any other reagent [G64—G69]. Leroy et al. [G64] analyzed a series of sympathomimetic drugs, heptaminol and related compounds, by derivatization with OPA followed by u.v. spectrophotometry and oxidative amperometry. It was assumed that the OPA-amino drug adducts were derivatives of isoindoles, by analogy with the known structure of amino acid-OPA adducts and new structural data. Liquid chromatography was carried out on a reversed phase column with a phosphate buffer/methanol mobile phase. Detection limits were lower by oxidative amperometry, usually in the pmol range for original analytes [G64].

Allison et al. [G66] described the use of OPA-amino acid and OPA-amine

adducts for high-speed l.c./ec. Derivative stability could be vastly improved by going to a t-butyl thiol reagent together with OPA, with half-lives in excess of several h. The thiol used for formation of the isoindoles had little effect on the electrochemistry of the derivative. Detection limits were 30—150 fmol for l.c. in the isocratic mode.

In a similar vein, Joseph and Davies [G67, G68] described use of OPA derivatization for amino acids, followed by fluorescence and ec detection for analysis of plasma. Using this series detection method, confirmation of the amino acids could be obtained in a single l.c. run. Improved selectivity for certain amino acids was possible at lower working potentials. OPA derivatization of amines was an example of a reaction in which the product is electroactive at a lower potential than the reactant. It was suggested that peptide and protein OPA adducts should be electroactive.

Granberg [G65] described the use of phenylisothiocyanate reagent for amino acids, together with reverse-phase l.c. and combined u.v. and oxidative ec detection. Separation and detection of amino acids from a calibration standard and an insulin hydrolysate were achieved. Picomole detection limits were possible for all amino acids by both u.v. and ec detection.

Amino acids were also derivatized by Mahachi et al. [G70] with aromatic isothiocyanates such as *p*-*N*,*N*-dimethylaminophenylisothiocyanate to form substituted phenylthiohydantoins. Such approaches have long been used in the ion-exchange determination of amino acids followed by post-column reactions and u.v. detection. These workers demonstrated that these same adducts could be reversibly oxidized at a glassy carbon electrode at pH 2. A mixture of 21 amino acids was separated with 85—90% recovery, and a linear response was observed for 1—150 ng per injection. Detection limits were 0.5—1.0 ng [G70].

Isocyanates have been derivatized for l.c./ec, according to at least two reports [G71, G72]. Meyer and Tallman [G71] utilized *p*-aminophenol as the reagent for toluene diisocyanate, which unfortunately formed more than one product. The separated products were detected oxidatively at a Kel-F/graphite electrode following reverse-phase l.c., and the detection limit for toluene diisocyanate was about 94 pg/injection. Because the isocyanate function can react readily, with similar ease, at both the free amino and alcohol functions of *p*-aminophenol, several products were obtained. For various reasons, in any l.c. study, ideal derivatizations should lead to a single, well established product, in 100% yield, with good chromatographic and ec properties, of high stability, and which forms with relative ease under mild off-line or on-line reaction conditions.

A second approach for aromatic and aliphatic isocyanates in l.c./ec was by Warwick et al. [G72]. They used a series of piperazine analogs as reagent, the best being 1-(2-methoxyphenyl)piperazine, which formed ec- and u.v.-active derivatives. This would appear to be an improvement over the scheme for similar compounds described earlier [G71]. A comparison was made between ec and u.v. detection. As expected, ec was more sensitive for deter-

mination of compounds such as phenyl isocyanate, toluene diisocyanate, hexamethylene diisocyanate, and 4,4′-diisocyanatodiphenylmethane.

Much more work has been reported for organic than inorganic derivatizations, and other than for the work with metal complexing reagents, only the report by Mayer describes derivatizations for oxidative l.c./ec [G73]. A recent development, by Dionex, of post-column reactions for metals uses u.v. rather than ec detection, although it is possible that ec could be applied to at least some of their derivatives. In the Mayer report [G73], cyanide was derivatized with *p*-benzoquinone in the presence of dimethyl sulfoxide to form 2,3-dicyanohydroquinone. Attempted reaction of *p*-benzoquinone with chloride, thiocyanate and thiosulfate did not produce ec-active products. The detection limit was calculated at 0.25 μg ml^{-1} KCN (74 pmol CN^- injected). Preconcentration of the final derivatization solution could lead to lower detection limits. The method has appeal as an alternative l.c. approach for cyanide because the derivatization is simple, it is very reproducible leading to a single product in high yield, and the product is stable with good ec properties.

Pre-column and some in situ derivatizations of metals have been described for l.c./ec, and the elegant studies of Roston, and Bond and Wallace [G74—G78] deserve mention. Though a large amount of work has been described with such reagents for metal ions in l.c. with detection by u.v.-visible or fluorescence spectrometry, little was known about the application of these derivatizations in l.c./ec. Roston [G74] has described the applicability of 4-(2′-pyridylazo)resorcinol (PAR) as a pre-column chelating agent for l.c./ec multielement determinations. Fixed-wavelength u.v. absorption and oxidative thin-layer amperometry were used for detection of PAR chelates. Studies showed that determination of the copper, cobalt, nickel and iron(II) as PAR chelates could be achieved at ng ml^{-1} levels. These results suggested that l.c./ec and l.c./u.v. separation of PAR chelates, formed pre-column, on-line, using a PAR-containing mobile phase into which the metal ions are injected, can be a viable approach to the analysis of multielement matrices.

Bond and Wallace [G75—G79] have established a series of complexing reactions for various metal cations (Cu(II), Ni(II), Co(II), Cr(VI), Cr(III) and Pb(II)) for pre-column or in situ derivatizations. In a typical approach, a copper-diethyldithiocarbamate can be prepared by using a mobile phase containing the chelating reagent. Injection of aqueous solutions of copper onto the column then forms the copper-dithiocarbamate complex ($Cu(dtc)_2$) on-line, which can be considered a form of pre-column derivatization [G78]. These workers have shown that $Cu(dtc)_2$ can be formed off-line, pre-column, in a more conventional manner, and then injected onto the h.p.l.c. system. The $Cu(dtc)_2$ can undergo a reversible one-electron reduction at platinum, gold and glassy carbon working electrodes in acetonitrile/water mobile phases. Detection limits were reported as 1 ng per injection of copper. It was assumed that a large number of other metal ions that form such dtc chelates

could also be detected. A subsequent paper showed that nickel, cobalt, chromium(III) and chromium(VI) also formed dtc complexes that could be detected using reverse-phase l.c./ec. Detection limits substantially below 1 ng per injection of metal were achieved. Other dialkyldithiocarbamates were studied, such as the pyrrolidine analog [G75].

Bond and Wallace also described an automated determination of nickel and copper by l.c./ec and l.c./u.v., again making use of in situ generation of dithiocarbamate complexes [G76]. All the e.c. detections involved oxidative reactions at a glassy carbon electrode. In 1984, these authors described an automated, microprocessor-based system for multielement determination of "real" samples, with spectrophotometric or electrochemical detection [G77]. Limits of detection of 1 ng per 10 μl injected were realized with spectrophotometric detection, and depending on the particular metal, higher or lower limits were possible by ec detection.

Post-column, on-line photochemical/photolytic derivatizations. Photochemical derivatizations leading to improved detection in l.c./ec have been described to a limited degree, and this area has been reviewed [G24, G80–G82]. Several different approaches have been described for the use of light as a post-column reagent together with l.c./ec. Notable in these areas have been Johnson, Weber and Krull [G21, G23, G24, G80–G86]. The work of Krull and co-workers [G21, G23, G24, G80–G82] has been divided into both post-column photolysis between the end of the l.c. reverse-phase column and ec detector, and l.c./photolysis-ec as well as direct irradiation of the working electrode surface in a thin-layer, flow-through, amperometric cell. The present author pursued l.c./photolysis-ec as an outgrowth of Snider and Johnson's earlier report [G83] that on-line, post-column photolysis could be used to generate nitrite from *N*-nitrosamines. The earlier approach used light to generate nitrite, which was then pre-concentrated and collected on an anion-exchange column. It was quantitatively desorbed from this pre-concentration column and detected oxidatively. This approach has been extended, the photolysis unit was placed directly on-line, between the column and a conventional amperometric detector that can be operated reductively or oxidatively [G24–G28, G38, G80]. Figure G1 illustrates the overall system [G80]. This allows the direct, often quantitative formation of

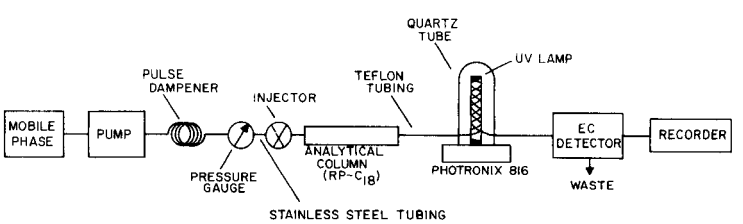


Fig. G1. Schematic diagram of the instrumentation for l.c./photolysis-ec. Reprinted with permission: J. Forensic Sci., April (1984) 449. Copyright, American Society for Testing and Materials.

new ec-active species from often non-ec active analytes, during the photolysis step, and these are transferred directly to the conventional, thin-layer, amperometric, single- or dual-electrode cell. A variety of analyte classes has been studied via these techniques, which appear to offer definite advantages over classic chemical derivatizations. Classes already studied include organic nitro compounds (nitrate esters, nitrites, aromatic nitro and *N*-nitro compounds), organothiophosphates (malathion, parathion, etc.), β-lactams (penicillins and cefoperazone), barbiturates, benzodiazepines, cocaine and aromatic esters and amides. Though initially applied to organic compounds, there is reason to suspect that l.c./photolysis-ec could be used for inorganic species. Such work is in progress, especially with regard to detection of anions. Many classes of compounds not ec active have been converted into new species, on-line, showing different degrees of ec response.

Not all compounds are amenable to l.c./photolysis-ec techniques. In part, to demonstrate possible off-line, pre-column derivatizations possible for such analytes, a study has been completed on the use of nitroaromatic reagents for amines, amino alcohols and amino acids [G38]. These approaches have utilized some of the nitroaryl reagents described in the literature, previously used for reductive l.c./ec. Such derivatives are just as useful for oxidative l.c./photolysis-ec, thus circumventing some of the problems discussed in the use of reductive methods. Amines have been derivatized with 2,4-dinitrofluorobenzene, and injected into the l.c. system. Detection limits were generally in the 1—10 ng ml^{-1} range, and calibration was linear over several orders of magnitude. Applications have been completed for spiked water, beer and spiked beer samples [G38]. All of the nitroaromatic derivatization schemes and final derivatives described for reductive l.c./ec should be adaptable to oxidative l.c./photolysis-ec. It is hoped that others will adopt and adapt existing off-line or on-line derivatization schemes for oxidative l.c./photolysis-ec.

The other major approach for post-column irradiation in l.c./photolysis-ec is irradiation within the thin-layer ec cell [G82, G84]. Weber and co-workers [G84, G86] have irradiated the working electrode surface of a wall-jet electrode, using an intermediate ruthenium-bipyridyl chelate to carry out the derivatizations of analytes. This is a redox system, with one of the intermediate species necessary for detection generated photochemically. It is quite different from the approach of Krull et al. who have recently used a direct irradiation of the working electrode surface in a thin-layer, flow-through, amperometric cell. Their approach involves irradiation of the analytes as they pass across the working electrode surface, as well as the surface itself, and has been limited to carbonyl compounds [G81, G82]. It is possible that some, as yet undefined, intermediate species, derived from the carbonyl analytes injected, has been formed photochemically, and this species is detected oxidatively. Specific applications to samples are nearing completion, such as the determination of benzaldehyde in almonds, liquors and other foods [G87, G88]. However, the nature of the species generated

and perhaps detected at the irradiated electrode appears to be different from the species generated in photolysis-ec detection. Those compounds amenable to one technique are not usually suitable for the other, wherein one appears to involve photochemical excitation/promotion/detection, and the other may involve photolysis and cleavage to stable inorganic or organic anions and hydrolysis products. More work is needed to establish the mechanism(s) operative in each post-column derivatization approach.

Post-column, on-line chemical derivations. Post-column, on-line derivatizations in l.c. have become increasingly commonplace, and there is a burgeoning interest in new and novel reagents and reactions that can be used in this mode [G89]. There are some distinct advantages realized in post-column derivatizations, when preparative recovery of the starting materials is desired, when the derivatization adversely affects the chromatography, e.g., the separation of amino acids by ion-exchange techniques, when the derivative is unstable, or when the derivative is very bulky and not readily chromatographed.

King and Kissinger [G90] described the use of l.c./ec with electrogenerated reagents, wherein a generator electrode, coulometric or amperometric, could be used post-column to generate new reagents from the mobile phase. An example is the reaction of unsaturated organic compounds with molecular bromine, wherein the concentration level of bromine added post-column could be determined before and after elution of the reacting analyte. Direct generation of the reagent needed for the post-column reaction had some inherent advantages and appeal over the more customary post-column addition of the same reagent. The technique was used to detect ng levels of fatty acids, prostaglandins and phenols following separation.

In a similar approach, Watanabe and Inoue [G91] described the ec detection of reducing sugars by use of the copper(II)-1,10-phenanthroline chelate added post-column. The reagent was added in an alkaline solution, and reacted with any eluted reducing sugars at a high temperature in a reaction coil placed after the column and reagent introduction-T, to produce the copper(I) chelate, which was detected anodically. In addition to high sensitivity, there is selectivity only for those compounds capable of reducing the copper(II) chelate. Glucose was determined at levels down to 1 pmol (0.2 ng per injection).

More recently, Honda et al. [G92] described another l.c./ec method for detection of reducing carbohydrates by post-column derivatization with 2-cyanoacetamide. This reagent reacts by addition to the sugar, rather than by a redox reaction, and the final derivatives possess u.v., fluorescence and oxidative ec properties. Glucose was monitored in this manner with a detection limit of 20 pmol and a linear calibration range of 0.05—2 nmol.

Elchisak [G93] described another type of post-column derivatization in the ion-paired l.c./ec for dopamine conjugates, such as dopamine-3-*O*-sulfate and dopamine-4-*O*-sulfate, which were hydrolyzed to free dopamine immediately after elution via an acid catalyzed incubation in a reaction coil at

an elevated temperature. Each isomer could be detected as free dopamine by oxidative ec techniques. This technique resulted in a 15-fold improvement in *s/n* for each isomer, as compared with u.v. detection. This concept can be applied to related compounds, such as carbamate pesticides, which release a primary amine, e.g. methylamine, from the carbamate, followed by derivatization of the amine with OPA. The final derivative could be detected by u.v. absorption or fluorescence, and such isoindole products have been shown also to be ec active [G89].

The inclusion of dual electrode l.c./ec as an approach to post-column derivatizations has been avoided, except for one typical example. Langenberg and Tjaden [G94] reported on the determination of vitamin K_1 in human plasma using l.c., wherein they used coulometric reduction post-column to generate a new species having strong fluorescence. It was also possible in this approach to make use of dual ec detection in the reduction/reoxidation mode for coulometric/coulometric as well as coulometric/amperometric detection. Such dual electrode approaches were more sensitive and selective towards the plasma background than simple reductive ec. Normal phase h.p.l.c. was also possible in this study, but only when supporting electrolyte was added post-column. Higher detection limits resulted.

The use of immobilized reagents, usually enzymes, for post-column derivatization in l.c./ec has been reported [G95, G96]. Kamada et al. [G97] described the use of an immobilized 3-α-hydroxysteroid dehydrogenase column post-column, together with oxidative ec, for the determination of individual bile acids in serum and bile. Bile acids eluting from the column reacted with NAD pumped to the enzyme reactor column to generate NADH. The NADH was reacted downstream after mixing with phenazine methosulfate solution, and the final product detected electrochemically. Each bile acid could be detected at the 20-pmol level.

Post-column enzymatic reactions have been described by Dalgaard et al. [G98] for the detection of phenolic or cyanogenic glycosides. This work utilized immobilized β-glucuronidase on porous glass beads packed in a short column and placed post-column to the separation. Enzyme-catalyzed cleavage of the glycosides occurred at room temperature, and the newly formed phenols could be readily detected downstream at oxidative working potentials. Detection limits for the various phenolic glycosides were in the range 3—23 pmol.

Enzymes have also been used in solution post-column in l.c./ec, such as in the determination of acetylcholine (ACh) and choline (Ch) in neuronal tissue [G99]. Acetylcholine and Ch were separated by reverse-phase l.c., and the effluent was mixed with acetylcholinesterase and cholineoxidase. Endogenous Ch and that formed by hydrolysis of ACh were both hydrolyzed by the enzyme to the betaine (non-ec active) and hydrogen peroxide. The peroxide was monitored oxidatively downstream. Detection limits were 1 pmol for Ch and 2 pmol for ACh. The selectivity of the method was based on l.c., two specific enzyme reactions, and ec detection of the peroxide.

Dual-electrode detection methods could improve selectivity further. The procedure for acetylcholine and choline, using solution enzyme reactions post-column, with oxidative ec detection, has been commercialized by Bioanalytical Systems [G100].

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THE PRIMARY EVENTS IN CHEMICAL SENSORY PERCEPTION
Olfaction as a Model for Selective Chemical Sensing

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SUMMARY

Early theories of olfaction are summarized after brief introductions to the anatomy of the human olfactory system and direct electrical measurements on receptor cells (electro-olfactography). The problems of odourant convection-diffusion, and transduction are discussed in terms of the physical chemistry of interfacial partitioning and membrane-receptor behaviour, respectively. Speculation is presented regarding the significant features of receptor structure required for analytical selectivity. Stimulant—receptor interaction at the membrane surface can lead to changes in membrane permeability to ions in a number of distinct ways. Current knowledge regarding the structure and membrane electrochemistry of the important neurotransmitter receptor for acetylcholine, is examined as a role model for the biological activity of olfactory receptors. Finally the olfactory system is compared concisely to existing chemical sensor technology. Although parallels exist, significant differences are obvious; an example is the particular chemistry exhibited by molecular receptors and the hybrid of digital and analogue coding for transduction.

"It is always possible that we may discover that what engineers have to do to make technological devices and systems work, nature has already done to make biological systems work" — Francis Eugene Yates [1].

"When we know enough to describe brain processes capable of giving perception in machines, the problem of consciousness may be solved as the problem of matter is solved in physics" — Richard Langton Gregory [2].

It is apparent that the essence of the statements presented above is attracting considerable attention in current research. One only has to look at recent advances in vision and tactile systems in robotics, artificial intelligence and molecular electronics to appreciate this point. The germination of this progress can, in part, be said to have stemmed from perceptual research in which there are many dimensions of activity. For example, ethologists deal with sensory information and species survival, physiologists with brain processing, and psychophysicists with the quantitative aspects of the stimulus situation. Here, we shall discuss the initial sequence of events in chemical sensory perception, which is largely the domain of physical chemists, biochemists and biophysicists.

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The sensitivity of animal life to chemicals is multifaceted. Biological species respond internally to messages in the form of transmitters and hormones, as well as externally through receiving sites (receptor cells) for gleaning information about the external environment. In this respect, there is a tremendous amount of variety in the engineering and location of such cells. For example, insects sense chemicals with their antennae, certain invertebrates have chemical receptors in their feet, and selected land vertebrates such as snakes, rabbits and dogs have a special structure called the vomeronasal organ. Often the distinction between taste (gustation) and smell (olfaction) becomes blurred. Despite these variations there is a great similarity in the application of these senses. Chemical sensitivity is used to assess the proximity of food or other environmental objects, and for communication purposes, through release of pheromones, for mating, alarm, trail marking, aggregation and dispersion, etc. In the present paper, our emphasis is on the human sense of smell where, obviously, the primary receptors are located in the nose. After brief introductions to the anatomical and electrophysiological aspects, we consider our state of knowledge of the appropriate physical chemistry and biophysics, the important acetylcholine receptor system as a potential role model and, returning to the opening theme of this article, possible lessons we can learn with respect to chemical sensor technology. The interested reader is directed to the bibliography as well as the list of literature cited.

ANATOMY OF THE OLFACTORY EPITHELIUM

The olfactory epithelium is made up of an array of 10—20 million receptor cells [3], interspersed with supporting and basal cells, as shown in Fig. 1. A mucous layer (10—55 μm thick [4]) formed from secretions emanating from Bowman's glands (not shown) covers the surface. Each olfactory receptor cell is a bipolar neuron which is composed of the cell body and axon. The dendritic portion (1—3 μm in diameter [4]), which generates electrical potentials as a result of chemical stimulation, terminates at the olfactory rod. From this region, cilia project to the surface of the mucous. These structures are widely believed to bear the molecular receptors for odourous molecules. Based on the size and number of cilia per cell (10 to 20 for vertebrates [3]) and the total number of receptor cells (e.g. 10—20 million for humans vs. 100 million for rabbits [5]), the superiority of different species for odour discrimination has been correlated with overall receptive area, viz. humans 250 cm^2 and rabbits 600 cm^2 [6].

The precise role of the supporting cells remains to be established. For instance, these cells have a secretory property in that granules, 1—4 μm in diameter [7], are released into the surface mucous layer. The purpose of this contribution as compared to the secretion of the Bowman's glands is not known. Other structures to be examined with respect to their purpose [8] are the yellow-brown pigments in the supporting cells and the microvilli which often occupy the free portion (mucous region) of the supporting cell

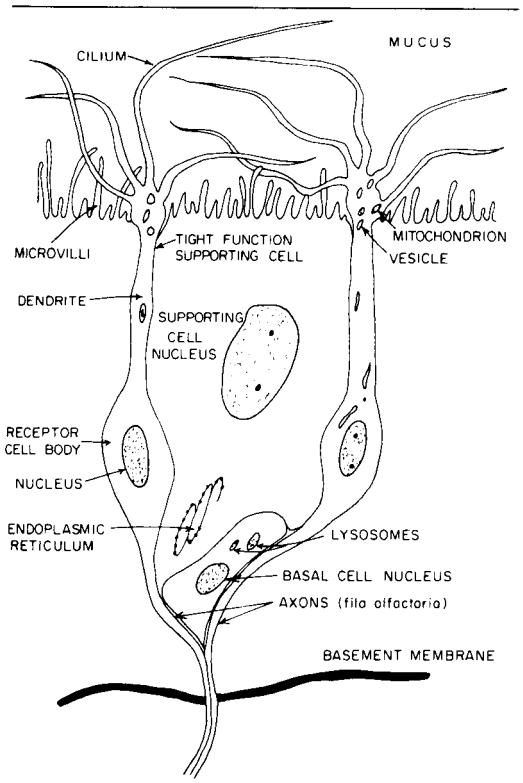


Fig. 1. Schematic representation of the human olfactory epithelium showing two olfactory cells, a supporting cell and a basal cell.

The axons (diameter 0.2 μm [4]) of the receptor cell collect into bundles in an unmyelinated form and make up the filia olfactoria. These nerve fibres continue upwards through perforations in the ethmoid bone and terminate in a strip of brain matter, the olfactory bulb, located in the under surface of the front of each cerebral hemisphere. Thus, not only do the bipolar cells function as receptors, but they also constitute both the transducer and conducting part of the olfactory apparatus. The fact that the receptors are directly connected with the cerebrum is unique because all other senses have relay centres in lower parts of the central nervous system (CNS).

The axons synapse for the first time in a complex structure called the glomerulus that is located in the olfactory bulb (Fig. 2). These synapses are made with mitral cells which connect with regions outside the bulb. A lateral tract is formed from the axons of the tufted and mitral cells, which terminates in the olfactory cortex. In this area it is thought that olfactory discrimination takes place since lesions produce hallucinations of smell [6]. Also, connections with many other parts of the brain occur making contributions to the sensory component of conscious activity. It is thought

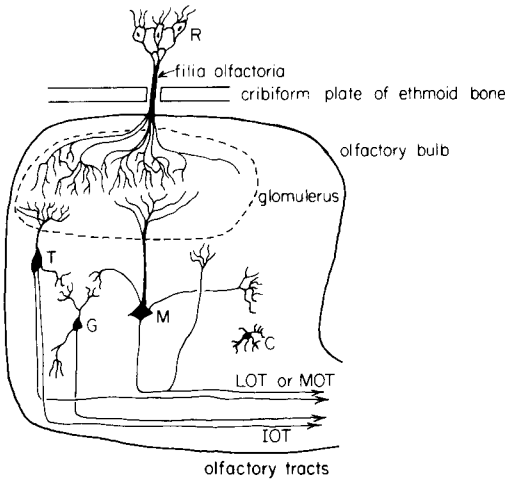


Fig. 2. Schematic representation of projections of the olfactory bulb: (R) receptor cells; (M) mitral cells; (T) tufted cells; (G) granule cells; (C) other cells. Olfactory tracts are: (L) lateral; (M) medial; (I) intermediate.

that a second order of processing occurs in the olfactory bulb involving feedback from the brain, since there are many efferent synapses on the second or third order olfactory cells. Accordingly, there are additional olfactory tracts with connections to various regions of the brain [9].

ELECTROPHYSIOLOGY AND ELECTRO-OLFACTOGRAPHY

Receptor cells are regarded as biological transducers which convert the energy of a given stimulus into an electrical signal responsible for initiation of neural impulses. The transduction process involves the modulation of flow of ion current through electrochemically resistive cell membranes. As expected, there have been many experiments concerned with direct measurement of electrical potentials of the olfactory receptor cells in vertebrates [10, 11] and insects [12, 13]. These techniques are used in the determination of the binding affinity between odourant and receptor site [14], measurement of threshold levels of stimulus on the olfactory system [15], identification of receptor sites [16], and comparative studies of olfactory bulb and receptor cell responses [17, 18]. The variety of measurements that can be made on the olfactory system are summarized in Fig. 3. The electro-olfactogram (EOG) is simply recorded by measuring the potential difference across a reversible electrode placed on the surface of the olfactory mucosa and a reference electrode mounted elsewhere on the animal. The high impedance signals are then fed into an amplifier and displayed on an oscilloscope to observe any changes in polarization. Similarly, the electroantennogram (EAG) is recorded by contacting the insect antenna with a microelectrode in an electrical circuit. The EOG effectively results from a

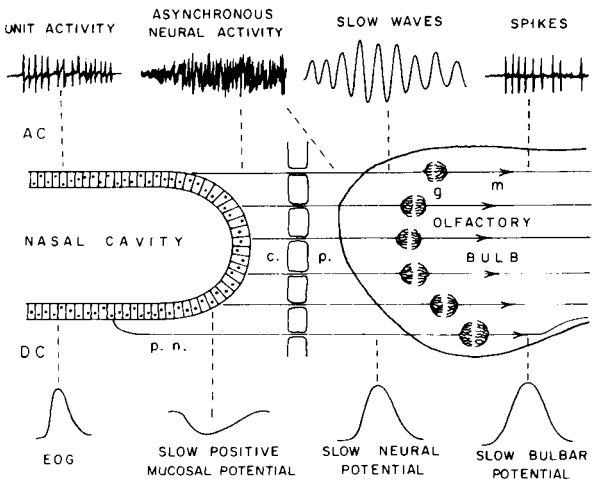


Fig. 3. Schematic diagram illustrating the general form of the potentials which can be recorded from various sites in the olfactory mucosa, nerve and bulb in response to chemical stimulation. Potentials at the top are obtained with a.c. recording; those on the bottom are d.c. (c.p.) Cribiform plate; (m.) mitral cells; (p.n.) primary neurons; (g.) glomerulus; (EOG) electro-olfactogram. (Reprinted with permission of Academic Press, New York [83].)

population average of receptor potentials concerned with olfactory impulse. A typical response to a chemical stimulus is a slow negative monophasic potential with a rapid rising phase followed by an exponential fall. The slow adapting nature of olfactory receptors is based on the steady state response to the presence of stimulus [19, 20]. The fact that response amplitude increases with increasing concentrations of odourant shows that the potential reflects the summed activity of olfactory receptor cells.

The time course of the EOG response is of interest because it reflects the characteristics of the excitatory processes in the receptor system. In the rising phase, the potential rises faster with increasing stimulus concentration. However, the time required for the potential to reach its peak is approximately the same regardless of the nature of the stimulus. This suggests that the excitatory event which generates the receptor potential must be similar in nature in all olfactory systems. The exponential decay following the excitatory phase reflects the elimination of odour molecules from the olfactory mucosa. The quantity of stimulus determines the time duration of the decay. Some substances produce a longer time sensation, which may be attributed to higher binding affinity between stimulant and receptor site.

Electrophysiological experiments can be used to determine the properties of receptor sites. For example, pairs of stimuli which elicit maximal response in an olfactory system can be examined with respect to whether each odourant competes for one site or whether different sites are involved. Cross-adaption studies provide a route to evaluate whether there are different transduction processes associated with different receptors [21, 22]. If two

stimuli share the same olfactory transduction process, phasic olfactory response will be reduced when the olfactory receptors are adapted by one of the stimuli. Olfactory responses relatively unchanged by adaption suggest that stimulation proceeds by different independent transduction mechanisms, i.e., different receptor sites. For example, adaption studies of EOG responses to amino acids have shown that there are three different receptor sites in the olfactory receptors of the catfish [23]. Another area of interest is the deliberate variation of the structure of a stimulus that is known to elicit a maximal EOG response. Often the response is affected, but this is not always the case. For example, studies on the eel show that the response is not diminished on esterification of the COOH group of L-alanine [24, 25]. This observation indicates that the concept of multiple binding sites and allosteric binding may be important.

Receptor cell depolarization is consistent with the fact that the membrane, in which receptor molecules are embedded, apparently becomes more permeable to sodium ions. The depolarization is then propagated down the axon. In order to mimic the primary event, attempts have been made in recent years to reconstitute extracted olfactory protein material in a synthetic bilayer lipid membrane (BLM) [26, 27]. The purpose here is to evaluate the nature of gated ion fluxes occurring at the membrane surface on stimulation with a particular species.

THEORIES AND RESEARCH

Early theories

The earlier research in olfaction was dominated by efforts to classify odours and determine the 'primary' odours in a somewhat analogous fashion to the primary colours. Many of these experiments involved human odour panel assessment or the psychophysical approach which, as one would expect, is highly subjective in nature. Numerous attempts were made to find some unique physical or chemical factor which determines the smell of a class of compounds, without dealing with other important olfactory properties such as the threshold concentration for stimulation. The early theories are summarized in Table 1 and discussed briefly below.

In the early 1900s chemical theories tended to emphasize the importance of unsaturation, associated with possible reaction chemistry [28]. The stumbling block for many of these theories was that paraffins have a perceptible odour. It would therefore seem that a chemical reaction (as opposed to interaction) is unlikely to be the cause of odour sensation.

After inspecting the various classification schemes of the time, Amoore [29, 30] proposed the existence of seven primary classes of odourous compounds. He designed primitive receptor sites for each of the seven classes based on the steric (space-filling) properties of the primary compounds. The correlation coefficients between odourant structure and quality are quite low, typically 0.45—0.66 [31]. It is difficult to explain the large number of

TABLE 1

Some early theories of olfaction and their proponents[a]

Author	Category	Molecular property
Durrans	chemical reaction	addition reaction at membrane
Wright	vibrational	low frequency molecular vibrations
Amoore	steric	size and shape of molecule
Davies	puncture	momentum, ability to puncture membrane
Kurihara	physiochemical	surface potential changes
Kistiakowsky	enzyme	inhibition of enzyme-catalyzed reactions
Mozell	chromatographic	mucous acts selectively like a chromatographic column

[a]Selected references are included in text.

odourants (thousands) that can be perceived based on these classes. From research done later on specific anosmia, he increased the number of receptor types to at least 32 [32].

In the penetration and puncture theory put forward by Davies [33], the odourants are thought to bore a hole in the lipid membrane through which ions can subsequently pass (until the membrane has "healed"). This model provides an explanation for both the differences in odour quality and the transduction mechanism (cause of depolarization). Experimentation has demonstrated that many odourants do not possess the capacity to puncture membranes, but may cause transduction by interaction with membrane lipids. It is possible that the odourants interact directly with the lipid bilayer causing a variety of responses including phase transitions, changes in ion permeability, fluidity, packing and lipid/protein interaction. Odourants have been found to change the surface pressure of lipid monolayer films and these changes have been correlated with the threshold concentrations necessary for odour perception [34]. The role of lipids has been found to be critical in the ATPase activity of olfactory preparations [35]. Also, odourants have been observed to change the ionic conductivity of artificial bilayer membranes made from lecithin (a major lipid component of natural membranes) but a correlation with the olfactory properties of the odourants was not established [36].

Wright [37] proposed that odour quality is determined by the low-frequency vibrations of the odourous molecules. He found correlations between the odour of a compound and its Raman spectra. Certain enzymes have been found in olfactory mucosa tissue (for example, ATPase) and an enzyme theory can explain a number of features of olfaction [38, 39], viz. the great sensitivity and selectivity of the nose, the wide range of compounds that can be detected, and changes of odour quality with concentration. Of significance is the fact that the enzyme and receptor theory can coexist. Some selectivity has been ascribed to the mucous layer behaviour in a manner analogous to a chromatographic column [40, 41]. When the gas

passes along the mucous surface, there is evidence for spatial and temporal partitioning of the constituent odourants which can aid in their identification.

The convection—diffusion problem

An overview of the vertebrate olfaction process can be obtained by following the path of a typical odourant molecule (Fig. 4). Obviously the source of this species must have a measurably high vapour pressure, although it should be mentioned that some compounds can be detected at concentrations of less than 1 ppb. (Substances with a molecular weight greater than 400 generally will not be detected.) After transport by diffusion and convection in the air, the stimulant is inhaled into the nose which takes in about 10–20 l of air per min under normal breathing conditions. Once in the nose, the odourant must be carried past the turbinate bones to reach the 2.4 cm^2 olfactory epithelium with its array of receptor cells. Only a small fraction of the molecules inhaled will actually reach the olfactory epithelium [42, 43] although sniffing will increase this number. A simple calculation yields the number of molecules (n) striking 1 cm^2 of a 'perfect' capture surface per s [44]:

$$n = 3640\, n_a/(M/T)^{1/2} \quad (1)$$

where M is the molecular weight of the odourant, n_a is the total number of molecules per cm^3, and T is the absolute temperature. For $n_a = 10^8$ cm^3 and $M = 100$, n would be 7×10^{11} at 37°C. Near the epithelial surface the odourant will, however, encounter a convection-free layer of air through

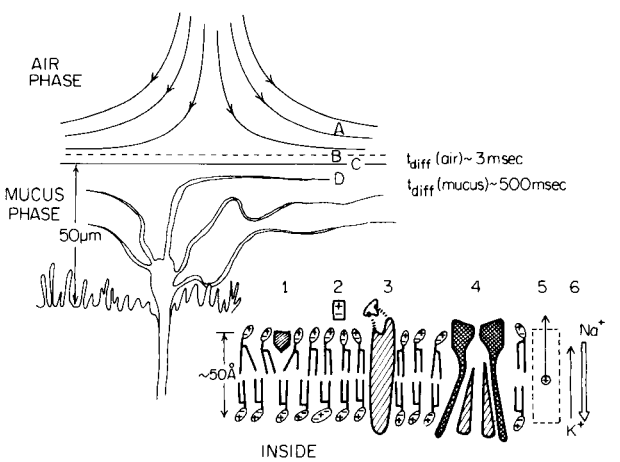


Fig. 4. Path of an odourant molecule (A—D) to the site of reception (1—3) and transduction (5—6): (A) odourant lines of convection; (B) diffusion in stagnant layer; (C) partition at air/mucous interface; (D) diffusion in mucous. Selectivity by: (1) steric perturbation; (2) electrostatic perturbation; (3) receptor-odourant binding with two sites. Transduction process requires Na^+-K^+ ATPase pump (4) and gated channel (5). Nerve impulse initiated by change of Na^+/K^+ permeability (6).

which it must diffuse [45]. The time required for diffusion across this region (t_{air}) is estimated from:

$$t_{air} = \frac{1.44\, l}{U} \frac{[V]^{1/3}}{D_g} \approx 3 \text{ ms} \quad (2)$$

where l is the diameter of stimulant epithelium, U is the fluid velocity toward the epithelium, and V and D_g are the kinematic viscosity and the diffusion coefficient, respectively. Typical diffusion coefficients for odourants in air are in the range 0.05—0.1 $cm^2\ s^{-1}$.

The odourant must next partition into and diffuse through the aqueous mucous layer (estimated to be 30—100 μm thick). The diffusion time for transit through the mucous (t_m) can be roughly estimated from:

$$t_m = L^2/2D_m \approx 120 \text{ ms} \quad (3)$$

where L is the diffusion distance and D_m is the diffusion coefficient of the odourant in mucous. This diffusion coefficient is often taken to be the same as that of water, which is at least four orders of magnitude smaller than that of air. The above calculations show that the time for odourant diffusion in the mucous layer is comparable with the observed latency times (time between stimulation and nerve firing). The mucous layer may be more viscous near the membrane and may contain enzymes such as alkaline phosphatase, mucopolysaccharides and pigment granules [46].

After having travelled some distance through the mucous layer, the odourant molecule will encounter some part of the receptor cell, most probably the cilia where, as mentioned above, it is believed the molecular receptors are located. Here, prior to receptor binding, lateral diffusion along the surface of the membrane, with diffusion coefficient about 10^{-8} to 10^{-11} $cm^2\ s^{-1}$, may be involved [47]. The location of a hypothetical 'receptor plane' and the minimum concentration necessary to initiate signal transmission have been estimated as follows [48]. The concentration of odourant molecules (C) at a location x beneath the air/mucous interface will be time dependent

$$C(x, t) = C_0 K \mathbf{erfc}\, [x]/2(D_m)^{1/2} \quad (4)$$

where D_m is the diffusion coefficient of the odourant in mucous, C_0 is the gas phase concentration, K is the gas/mucous partition coefficient, and **erfc** is the error function complement. The latency time, t_L, required for the odourant concentration to reach a threshold value, C_T (concentration just sufficient to cause a response), can be described by

$$t_L = h^2/4D_m\, [\mathbf{erfc}^{-1}\, (G/C_0)]^2 \quad (5)$$

where h is the depth location of the molecular receptor below the air/mucous interface. From an experiment measuring latency versus odourant (safrole) concentrations, the threshold concentration and the location of the receptor plane were determined to be 21×10^{-9} M and 30 μm, respectively, for a safrole-sensitive unit.

The transduction problem

Differentiation of neurons results in the production of chemoreceptive tissue which is characterized by its ability to bind and interact with certain molecular stimuli. It has been demonstrated that well-defined proteinaceous structures exist which can interact specifically with certain stimuli as in the case of the neural communication system (see next section). Such systems are analytically limited because they seem to be designed to eliminate interference and concurrently maximize sensitivity. The mammalian olfactory system represents the antithesis to such a situation, because it is capable of detecting a very broad spectrum of chemically divergent compounds, albeit often with only moderate sensitivity. Correlation of these processes leads to two hypotheses regarding the primary transduction of olfactory chemical information. One is that olfactory tissue must contain many different receptors (i.e., molecular receptors) to account for the diversity of sensing possibilities. The other is that the receptors involved in the olfactory process may be limited in number, but may employ two-dimensional spatiality and/or reactivity to increase informing power.

Of the first hypothesis, it has been previously postulated that 2 to 30 different receptors may be present in olfactory tissue [32, 49] and therefore chemicals causing the sensation of smell could be divided into distinct classes representing the characteristics of the receptors. Two major theories of olfaction, outlined above, have evolved from this premise and include the concepts of steric shape and vibrationally coupled sensors. Even though both of these theories have had some success in explaining response trends for a limited number of stimulants, no uniform trends have been found among the multitude of compounds which can be sensed [49]. Furthermore, no microscopic shape sensors or vibrationally sensitive structures have been extracted and characterized from olfactory tissue.

Consideration of calculated results from three-dimensional molecular modelling of drug action on selective proteinaceous sites has provided evidence that receptor binding is governed mainly by electrostatic fields and dipole vectors, and local electron density and delocalization [50]. These properties combine to produce the conventional macroscopic features of molecular shape, size, polarity and hydrophobicity commonly used to explain molecular interactions. However, the latter features are only poor representations of the underlying electronic structure of any molecule, and serve to disguise the true nature of molecular interactions. The facts that steric or vibrational features can be employed to define systematic groups of odourants attests to the similar electronic natures of such groupings, but because many exceptions exist in such groupings, a more detailed description of the true molecular electronic nature is required. The work of McGill and Kowalski [49] indicates that chemical trends are a function of electron distribution, delocalization and symmetry. By means of calculation and experiment, these researchers concluded that the most significant electronic features responsible for explaining differences in olfactory

response can be defined by combination of the electron dipole moments of the shortest and longest molecular axes (with molecular weight), and the separation between the top filled and bottom unfilled σ orbitals. The fact that two features are of fundamental importance indicates that (at least) two receptors must be invoked to explain olfactory processes. Given that only two sensors may be required, and may be sensitive to only two features, it is possible to envisage a two-dimensional reactive surface "continuum" which provides an opportunity for pattern recognition, and is limited in resolution by the capability of an observer to recognize a stimulation pattern.

Little is known about actual receptor size, shape or membrane placement. Apparently some examples of proteinaceous olfactory receptors have been observed, and have been related to electrochemical channel-type current conduction through lipid membranes on interaction with appropriate stimulants [27]. Since the orbital distribution and dipolar nature of the stimulant is fundamental in determination of receptor binding, it may also follow that the same electronic nature, or a new electronic distribution created by the complex formed when the stimulant is associated with a receptor, controls the electrochemical properties of the supporting bilayer lipid membrane. Previous experiments with artificial bilayer membranes formed from naturally occurring lipids have demonstrated that ion currents through such structures are tremendously sensitive to lipid molecular packing and dipolar electrostatic alterations [51]. This could represent a perturbation of the ion current through the generally non-permeable membrane, leading to nerve cell depolarization and signal propagation to the nervous system. The puncture theory of Davies, taken in association with electronically sensitive receptor activity and the potential for directed dipolar events, is sufficient to account for the evolution of primary signals in the olfactory process. This scenario is represented in Fig. 4 where olfactory stimulant interaction with receptor can lead to one or more of the following physical changes of the supporting lipid membrane, resulting in transduction of the binding event into an ion current. The contribution of the stimulant dipole to the existing transmembrane electrical field reduces the membrane inherent ion energy barrier [52]. Complexation of stimulant and receptor alters the original electric field contribution of the receptor alone [52]. Either of these electric field changes may locally change the ion conductivity of the lipid membrane matrix, or may locally alter the conductance of a threshold voltage dependent proteinaceous channel or pore [53]. The channel and receptor need not be physically coupled in such a process. The electric field variations may cause a synergic alteration of intermolecular association in the membrane, leading to increased ion permeability of the lipid matrix [54].

The last feature is critical to the understanding of ion permeability of lipid membranes when specialized channels or pores for ion conduction are not invoked. Experimentation has demonstrated that features such as transmembrane dipolar potential and molecular packing are mutually dependent. This is significant because the sensitivity of ion current to electrostatic

potential (exponential relationship [55]) is further increased by large transmembrane ion energy barrier reductions caused by minor molecular packing variations. This latter energy barrier relation is reflected in a 100 meV potential barrier decrease for each 0.01 nm^2 average molecular area increase experienced by molecules in the membrane [51]. Clearly, minor variations in membrane electrostatics due to an olfactory stimulant complexation with receptor can lead to dramatic changes in membrane ion permeability as the first step in the chemical-to-electrical signal transduction process. It should be recognized that membrane physical effects caused by stimulant/receptor interaction occur on a local basis extending to a few nm from the reaction site within the membrane. Such a phenomenon could lead to channel-like ion conductance observations.

THE ACETYLCHOLINE RECEPTOR AS A METAPHOR

The receptor

As discussed above, there has been very little definitive work on extracted olfactory receptor material in order to examine the mechanism of electrical events triggered by chemical stimulation. One system that has attracted a tremendous amount of attention is the acetylcholine receptor (AChR) which is a major neurotransmitter in the post-synaptic membranes of some central and peripheral nerves [56]. When the pre-synaptic nerve is stimulated, it releases the neurotransmitter acetylcholine (ACh), which diffuses across the synapse and binds to the AChR on the post-synaptic nerve. By an unknown process, this binding event opens an ion channel at or near the AChR, causing a small Na^+ flux into the neuron. There is no evidence that a priori analogous chemistry should be expected for olfactory receptors, but there is an excellent chance that the detailed structural mechanistic information that is garnered from research on AChR will be highly valuable in formulating potential mechanisms for membrane events in chemical sensory perception.

Since AChR in mammalian nerves and muscles is relatively scarce, most work has been performed on protein extracted from the electroplax organ of electric eels and rays. Typically, 600 g of tissue yields 50 mg of pure AChR [57], which is incorporated in lipid vesicles [58—60]. Purification involves sucrose density ultracentrifugation and removal of receptor peripheral protein (mostly the 43K protein). These vesicles have 95% of the AChR molecules facing out [57]. Further purification of detergent, lipid and AChR matrices can be achieved by affinity chromatography for the sugar moieties on AChR (using concanavalin A) or cholinergic binding site (using α-cobratoxin).

The AChR is composed of four discrete subunits of approximate molecular weights 40K (α subunit), 50K (β), 60K (γ) and 65K (γ) in a 2:1:1:1 ratio. They contain 4—7% sugar residues by weight, with 25% of the AChR having helical secondary structure [61]. Generally, it is

considered that in vivo the AChR exists in the dimeric form [62], with cluster or aggregate formation being, possibly, associated with the 43K protein. The view is widely held that there are two agonist binding sites per AChR monomer, one on each α subunit, although they are not identical because many affinity agents and inhibitors bind to only one position [63]. Moreover, there is evidence that the α subunit site serves to desensitize the AChR to the presence of agonist, and that the true physiological binding locations are found elsewhere [64]. Electron microscopy and neutron diffraction studies [62] of tissue show that AChR is 11 nm long, protruding 5 nm long and 9 nm wide into the synapse, and 1.5 nm long and 6 nm wide into the cytoplasm (Fig. 5a). There appears to be a central hole which is 2.5 nm wide at the synapse side, 0.65 nm at the narrowest point, and it likely traverses the protein. The hole is thought to be responsible for ion transport in the gated membrane event.

Electrophysiology

Agonist binding can result in current-time records which reflect both opening of large groups of ion channels (Fig. 5b) and single ion channel events. Many semi-empirical observations have been made regarding the properties of these channels. For example, currents are in the range of pA [65]. They are selective for Na^+, but will allow other species to pass through [66]. They are extremely sensitive to the nature of surrounding lipid [67]. Their properties vary with respect to extracted receptor preparations [68]. They spontaneously open without the presence of agonist, at the rate of about 1.4 s^{-1} [69]. Noise is seen for open channel events with characteristic 50 ms closings [70]. Frequency vs. open or closed time duration has been analyzed as the sum of two exponential curves with two different time constants [70].

Not surprisingly there have been several attempts to describe the physical chemical link between agonist binding and the consequent electrical event. Some studies suggest that the channel is narrow near the cytoplasmic surface and is gated there [64] and others have indicated that a conformational change must be involved [71]. From the large amount of biochemical and

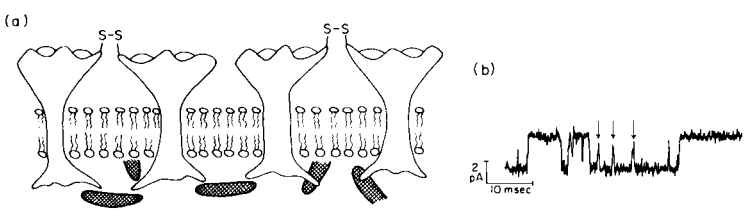


Fig. 5. (a) Schematic representation of proposed structure of acetylcholine receptor in lipid membrane; cross-hatched areas represent associated 43K protein. (b) Typical current/time profile for AChR membrane electrochemistry [70]. Note open states about one-third of normal value (arrows).

electrophysiological work it is clear that there is yet much to do and there are many questions to be answered. For example, possible receptor conformational changes have not been identified and the link between the ion channel effects and eventual nerve depolarization has not been established. If this system provides a model for olfactory membrane electrochemistry, then its complexity provides enormous challenges to us in the light of the huge problems involved in extracting reasonable quantities of olfactory receptor.

OLFACTION AND CHEMICAL SENSOR TECHNOLOGY

There is no doubt that dedicated microsensors are becoming pervasive in many aspects of high technology [72]. It is not surprising, then, that significant expansion in research activities in the chemical sensor and biosensor area is taking place today. Considerable scope exists for such sensors in on-line monitoring for chemical process control, real-time analysis of the environment, automation in clinical biochemistry, feedback control of artificial organs, in vivo analysis of drug levels, robotic control and strategic and tactical monitoring of chemical warfare [73]. Nearly all devices employ a basic union between a selective site for molecular (or ionic) recognition and a transducer which is capable of translating a perturbation of physical chemistry associated with the stimulant/site interaction into a useable signal. Accordingly, it is instructive to distill parallels and divergences from what we have seen above with respect to existing electrochemical, optical and piezoelectric sensor technology, etc.

Several gas sensors incorporate the principle of direct adsorption of the analyte on a surface from ambient atmospheric conditions. The SnO semiconductor device would be placed in this category. In contrast, the TAGA mass spectrometer 'sniffs' deliberately for samples, before a fragmentation pattern is analyzed. The mucous layer in olfaction acts as a pseudo-frontal chromatographic stationary phase, which is extremely versatile in its ability to trap compounds of highly variant polar character. Few existing sensors employ such a layer, however, one comparable system would be the poorly selective microgravimetric piezoelectric sensor [74]. In olfaction, of course, partitioning between air and mucous, and then between the mucous and the chemoreceptive sites occurs, which is not the case for the conventional piezoelectric sensor. However, it has been shown recently that acoustic shear wave transmission from such devices in liquids is indeed affected by the solid/liquid phase interplane [75].

For molecular recognition, the sensor community has almost exclusively turned to the inherent selectivity afforded by the tertiary structure of proteins and polypeptides. Enzyme/substrate, antibody/antigen and complement, and lectin/saccharide combinations have been employed in conjunction with a variety of transducers. Membrane molecular receptors have been used very sparingly, undoubtedly due to the difficulties experienced in their

extraction. However, one direct example would be the electrochemical signal developed from corn coleoptile auxin receptor [76]. Enzyme systems are reactive and immunochemical systems may involve irreversibility in the way of cross-linking, compared with the olfactory and other membrane receptors, which are characterized by rapid and weak binding [77]. As discussed above this type of selective site may respond to elements of a molecular conformation (or a 'reduced molecular representation'). A good example of this type of philosophy is the proposed 'map' of the dopamine receptor which apparently is characterized by two highly directed sites for hydrogen-bond formation [78]. Another important factor is the realization that a component of the free energy of binding, the intrinsic value, could be used by the biochemical system to 'pay' for a required conformational alteration (resulting in a lower observed free energy) [79]. This effect may solve the apparent contradiction between selectivity with strong binding and high reversibility. This exquisite transduction system appears to be highly attractive from a sensor standpoint, but greatly challenging because we do not know the link between binding and electrical event. Moreover, complex ternary structures, aggregation, allosteric binding effects and regeneration are all probably involved.

Intensities of signals in olfaction (and in synaptic transmission) are represented (poorly) by the rate of firing of receptor neurons. In modern sensor technology, there appears to be no parallel with this sort of digital coding. However, we could think of the perceptory system as being a hybrid of digital and analogue processing in the sense that frequency of firing could be represented by an analogue function. In order to understand brain processing we must appreciate the logic rules which is much more difficult than dealing with input-output analogue signals [80]. Directly concerned with this area is the obvious epithelial spatial or two-dimensional pattern recognition system employed in the olfactory process. In this respect, it is interesting to note that sensor technologists are now attracted to the multi-sensor approach, and the use of chemometrics and artificial intelligence in signal processing [81].

Evolution has apparently achieved much in the way of sensor technology, viz. receptor design, sensitivity, calibration, bioelectrochemistry and reasonable ruggedness. With respect to present difficulties, it is particularly encouraging to see somewhat analogous philosophies being employed in molecular electronics and Langmuir-Blodgett technology, engineering of protein tertiary structure and generation of chemical information from electrical 'noise'. Only future research will lead to the answer of the question posed recently: "Biological Chemical Sensing—An Inspiring Model Mechanism for Sensor Development or a Problematical Academic Curiosity?" [82].

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COATED-WIRE ION-SELECTIVE ELECTRODES

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SUMMARY

Coated-wire ion-selective electrodes were first developed in 1971, and comprise a film of polyvinyl chloride or other suitable polymeric matrix substrate containing a dissolved electroactive species, coated on a conducting substrate (generally a metal, although any material with conductivity substantially higher than that of the film can be used). Electrodes of this sort are simple, inexpensive, durable and capable of reliable response in the concentration range of 10^{-1} to 10^{-6} M for a wide variety of both organic and inorganic cations and anions. The principles on which these electrodes are based, as well as their application to a variety of analytical problems, will be discussed.

When faced with the problem of a trace level determination of an inorganic ion, the analyst often considers use of an ion-selective electrode (ISE) for the species of interest. This approach is advantageous because of the speed and ease of ISE procedures in which very little sample is required. Further, an ISE possesses a wide dynamic range, and is relatively cheap. These characteristics have inevitably led to sensors for several ions, and the list of available electrodes has grown substantially over the past two decades. In most cases, the traditional barrel configuration has been utilized. However, the large size of this type of ISE along with the requirement that it be used in a nearly upright position renders it somewhat cumbersome to use and unnecessarily expensive.

In this laboratory, these disadvantages have been overcome with the development of the coated-wire electrode (CWE). This sensor, having response characteristics equal to and occasionally better than conventional types, is only 1—2 mm in diameter (further size reduction can be easily achieved) can be used at any angle, and costs very little to make. Indeed, they can be considered disposable, though with proper handling lifetimes of over six months have been realized. During the course of CWE investigations here the list of analyte species has been lengthened to include not only most common inorganic ions of interest, but also organic species which are anionic or cationic under appropriate solution conditions. This paper will review this research from the inception of the CWE to the present.

The first electrode of this type was based on the Ca^{2+}-didecylphosphate,

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dioctylphenyl phosphonate system [1]. An effective Ca^{2+} selective CWE resulted when a 6:1 mixture of 5% polyvinyl chloride (PVC) in cyclohexanone and 0.1 M calcium didecylphosphate in dioctylphenyl phosphonate was dried on the end of a platinum wire. Favorable comparison of response characteristics of this electrode against the commercial counterpart (Table 1) encouraged further studies with other membrane components. The Ca^{2+} electrode response relied upon the complexation of aqueous Ca^{2+} by didecylphosphate dispersed in the organic, or membrane, phase. In a similar manner, incorporation of methyltricaprylammonium (Aliquat 336S) salts in polymer membranes produced CWEs for their respective anions [2]. A 60% (v/v) solution of Aliquat 336S in decanol was first converted to the desired anionic form via shaking with 1 M aqueous solution of the appropriate Na^+ salt. A 10:1 mixture of PVC in cyclohexanone and this decanol solution was then used to coat copper wires by repeated dipping and drying until a small bead completely encapsulated their ends. Listed in Tables 2 and 3 are anionic species for which Aliquat-based electrodes were prepared. Applications ranged from critical micelle determination using laurylsulfonate sensors [3], determination of atmospheric NO_x with nitrate electrodes [4], and assay of phenobarbital tablets using a phenobarbital anion CWE [5].

In many cases, poly(methyl methacrylate) or epoxy resin could be substituted for PVC with retention of response. This, along with the absence of the traditional internal reference electrode, raised fundamental questions surrounding the charge conduction mechanism occurring in the membrane and at the polymer/substrate interface. Calculation of activation energies from the temperature dependence of conduction suggested that an electronic mechanism was operative, such as that observed in organic semiconductors [6]. Later studies of the pressure dependence of conduction gave strong evidence for ionic conduction because much larger activation volumes, than could be expected from an electronic mechanism, were obtained [7, 8]. As such, the existence of some redox couple at the substrate/polymer interface

TABLE 1

Selectivity coefficients, $k_{i,j}^{pot}$, of various divalent cations [2]

Interferent	Orion electrode	Coated-wire electrode
Ni^{2+}	0.026	0.0039
Cu^{2+}	0.24	0.15
Mg^{2+}	0.033	0.014
Ba^{2+}	0.016	0.0036
Sr^{2+}	0.029	0.021
Pb^{2+}	0.23	1.86
Zn^{2+}	1.44	32.3

TABLE 2

Response characteristics of coated-wire electrodes [3]

Electrode	Slope (mV/log a)	Concn. range of linear response (M)	Useful concn. range (M)
Perchlorate	58	10^{-1}—10^{-4}	10^{-1}—10^{-4}
Chloride	55	10^{-1}—10^{-4}	10^{-1}—10^{-4}
Bromide	59	10^{-1}—10^{-3}	10^{-1}—10^{-4}
Iodide	60	10^{-1}—10^{-4}	10^{-1}—10^{-4}
Thiocyanate	59	10^{-1}—10^{-3}	10^{-1}—10^{-4}
Oxalate	28[a]	10^{-1}—10^{-4}	10^{-1}—10^{-4}
Acetate	50[a]	10^{-1}—10^{-3}	10^{-1}—10^{-4}
Benzoate	53[a]	10^{-1}—10^{-3}	10^{-1}—10^{-4}
Sulfate	28	10^{-1}—10^{-3}	10^{-1}—10^{-4}
Salicylate	53[a]	10^{-1}—10^{-3}	10^{-1}—10^{-3}
Phenylalanine	54[a]	10^{-1}—$10^{-2.6}$	10^{-1}—10^{-3}
Leucine	52[a]	10^{-1}—$10^{-2.6}$	10^{-1}—10^{-3}

[a]Log C.

TABLE 3

Selectivity coefficients, $k_{i,j}^{pot}$, for the coated-wire electrodes compared to those for the liquid-membrane electrodes [3]

Electrode	Chloride	Interfering anion		Miscellaneous
		Nitrate	Sulfate	
Perchlorate	0.004(0.18)[a]	0.028(0.12)	<0.001(<0.001)	ClO_3^-, 0.039(0.20)
Chloride	—	2.0(3.0)	0.12(0.061)	Br^-, 1.2(2.7)
Bromide	0.19(0.40)	2.0(1.1)	0.020(<0.001)	I^-, 14.5(3.6)
Iodide	0.0048(0.064)	0.11(0.23)	<0.001(<0.001)	Br^-, 0.056(0.22)
Thiocyanate	<0.001(<0.001)	<0.046(0.067)	0.001(0.018)	I^-, 0.34(0.42)
Benzoate	1.3(0.17)	1.3(0.48)	0.15(<0.001)	Salicylate, 1.4(0.29)
Salicylate	<0.001(<0.001)	0.42(0.030)	<0.001(<0.001)	m-OH benzoate, 0.22(0.13)
Oxalate	51(≈80)	—	1.3	OAc^-, 11(11)
Sulfate	16(100)	30(820)	—	
Phenylalanine	0.8(1)	2(1.6)	0.04(0.1)	Gly 0.025(0.040) Leu, 0.13(0.40)
Leucine	0.56(0.50)	0.50(0.30)	0.020(0.025)	Gly, 0.032(0.063) Val, 0.25(0.25) Phe, 2.0(1.6)

[a]Value of K_i for liquid membrane electrode is in parentheses.

probably functions as an internal reference. This hypothesis is further reinforced when one considers that conditional standard potentials shift by significant and reproducible amounts from one type of metal substrate to another.

Attention was next turned to the development of cation-selective electrodes in order to develop methods for protonated alkylammonium ions. Initial studies in this area were aimed at improving selectivity among simi-

larly charged cations by utilizing a mobile exchange site, facilitating membrane response to changing counter ions [9]. These membranes comprised dinonylnaphthalene sulfonic acid (DNNS), a lipophilic anionic extractant, dissolved in a PVC membrane plasticized with dioctylphthalate. Extremely high selectivity was observed for alkylammonium ions over the common inorganic ions tested (Table 4). Among organic species, selectivity increased regularly with the number of carbon atoms of the interferents tested. This indicated great promise for sensors of pharmaceutical interest, because many such compounds are high molecular weight protonated amines in the physiologic pH range. Currently, a DNNS-based CWE is made by dissolving the amine of interest in a 5% solution of PVC in tetrahydrofuran (THF) which is also 0.5% in DNNS and 4.5% in plasticizer, usually dioctylphthalate. This solution is used to coat the end of a copper wire which is elsewhere insulated with non-plasticized PVC. Following conditioning in a 10^{-4} to 10^{-3} M solution of the analyte, the electrodes are ready for use.

EMF AND SELECTIVITY MEASUREMENTS

Early on, it was anticipated that many repetitive calibrations and EMF measurements would be done in the evaluation of a large quantity of electrodes. The first microcomputer-based automated titration system utilizing

TABLE 4

Selectivity coefficients for the DNNS-based electrodes [10]

Interferents	Na^+—ISE	TBA^+—ISE	$DoTA^+$—ISE	
			CWE	Conventional
$DoTA^+$[b]	>10^5[a]	5.3	(1)	(1)
TBA^+[c]	>10^5[a]	(1)	0.18	0.21
DTA^+[d]		0.54	0.084	0.085
TPA^+[e]		0.02	0.031	0.038
TEA^+[f]	4.7×10^3	4.4×10^{-4}	1.1×10^{-4}	1.5×10^{-4}
TMA^+[g]	30	1.2×10^{-4}	$<10^{-4}$	$<10^{-4}$
Ag^+	22			
K^+	6.5	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$
NH_4^+	3.1	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$
Na^+	(1)	$<10^{-4}$		
Li^+	0.76		$<10^{-4}$	$<10^{-4}$
H^+	0.46	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$
Pb^{2+}		$<10^{-4}$	$<10^{-4}$	$<10^{-4}$
Ca^{2+}		$<10^{-4}$	$<10^{-4}$	$<10^{-4}$
Mg^{2+}	0.69	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$

[a] K_{sel} varies greatly with concentrations of primary and interfering ions. [b] Dodecyltrimethylammonium. [c] Tetrabutylammonium. [d] Decyltrimethylammonium. [e] Tetrapropylammonium. [f] Tetraethylammonium. [g] Tetramethylammonium.

high level software (CONVERS) hastened these studies [10], as did a more recently constructed minicomputer system (Fig. 1). Typical results are shown in Fig. 2, where a set of five protriptyline CWEs were calibrated simultaneously [11]. Graphical side-by-side comparison of different electrode calibrations was also useful in establishing structure/selectivity relationships.

In liquid-liquid extraction studies of various anions using Aliquat 336S, values of K_{ex} were obtained that showed a one-to-one correspondence between K_{ex} and electrode selectivity coefficients, $k_{i,j}^{pot}$ [12]. The latter value is calculated from e.m.f. responses of the electrodes samples in the presence and in the absence of an interfering ion using the relationship: $E = E^0 + 59.2(a_i + k_{i,j}^{pot} a_j^{n/z_j})/n$, where n and z_j are charges of the primary and interfer-

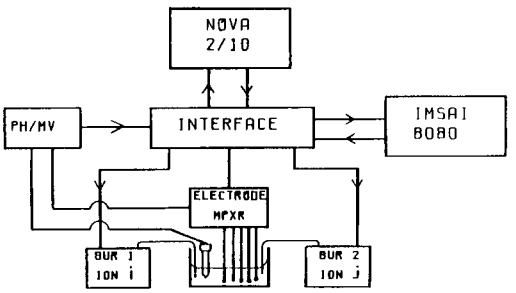


Fig. 1. Computer-controlled potentiometric analysis system.

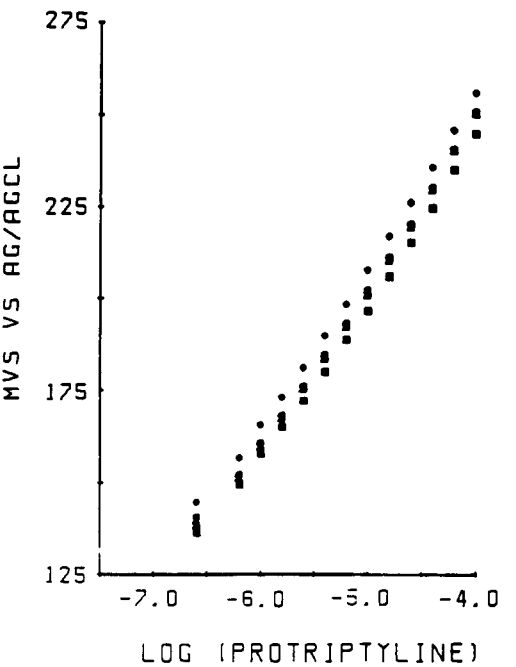


Fig. 2. Calibration graphs obtained with five protriptyline electrodes.

ing ions, respectively, and a_i and a_j are their activities. Experimentally, computer-generated solutions of specific ratios of a_i/a_j are made, followed by correction of the new activity of ion i due to addition of ion j.

Systematic selectivity studies of DNNS-based electrodes were done in this fashion [13]. Using tributylammonium as a primary ion, log $k_{i,j}^{pot}$ values were determined for various substituted alkylammonium ions. As was the case for Aliquat-based electrodes for organic species, selectivity improved considerably with increasing molecular weight. These observations are summarized in Figs. 3 and 4. In Fig. 3, log K_D values, as calculated by the correlation technique of Hansch and Leo [14], are plotted against the ammonium ion carbon number. When compared to Fig. 4, where the corresponding log $k_{i,j}^{pot}$ values for a set of TBA^+ CWEs against these ions are plotted, the utility of considering liquid-liquid extraction parameters in predicting electrode selectivity behavior can be seen. Indeed, plots of log $k_{i,j}^{pot}$ vs. log K_{ex} are linear ($r = 0.98$) though quaternary ammonium ions displayed a greater slope (2.1 vs. 1.1) (Fig. 5) than did the less substituted ions tested. This may be due to the faster increase in steric hindrance as successive methyl groups are added to the more symmetric quaternary ions.

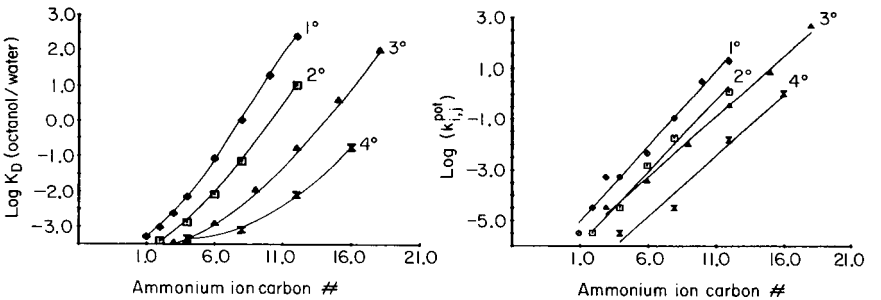


Fig. 3. Log K_D vs. carbon number for various alkylammonium ions.

Fig. 4. Log $k_{i,j}^{pot}$ for tributylammonium CWEs vs. carbon number for various alkylammonium ions.

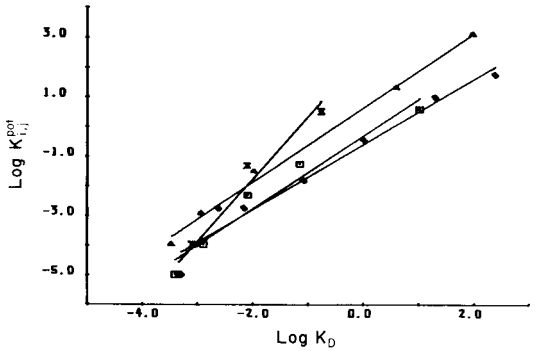


Fig. 5. Log $k_{i,i}^{pot}$ for tributylammonium CWEs vs. log K_D for various alkylammonium ions: (♦) primary ammonium ions; (□) secondary ammonium ions; (▲) tertiary ammonium ions; (×) quaternary ammonium ions.

The effect of increased interference with increasing molecular weight of interferents could be minimized when heteroatoms and polar functional groups are present. Their effect, though, would be to increase the hydrophilicity of the molecule, thereby decreasing the selectivity coefficient. On the other hand, an electrode prepared for a highly-substituted compound may show significant response to a lower-molecular weight less-substituted ammonium ion. These predictions were realized in a study of *N*-ethylcyclohexylammonium ions, where the log $k_{i,j}^{pot}$ values calculated from measured EMF responses of tributylammonium CWEs decreased with increasing hydrophilic character of the added functionality. In Table 5, these values are compared to log K_D values calculated by the Hansch method. These arguments lead to another consideration, namely that among a similar set of interfering ions, the electrode for the primary ion highest in lipophilicity would display greatest selectivity. Electrodes for aliphatic ammonium ions of carbon number twelve were prepared and selectivity coefficients were measured for each set versus the other two. The selectivity order was found to be dodecylammonium > dihexylammonium > tributylammonium (see Table 4).

In addition to the greater magnitude of the primary amine distribution constant, less sterically hindered nitrogens would result in a larger value of K_{IP}, the ion-pairing constant of the protonated ammonium ion with the DNNS anion. Therefore, among various CWEs, the family of curves obtained for tributylammonium (Fig. 4) would shift either positively for more hydrophilic primary ions, or negatively for more hydrophobic ions.

These results were consistent with subsequent studies for the drugs shown in Fig. 6 [15]. Selectivity orders were in excellent agreement with K_D values calculated for these compounds. Addition of hydrophilic groups tended to decrease selectivity of even high molecular weight compounds, though sensitivity seemed to be independent of this effect. In general, though, relative selectivity among a group of CWEs selective to different drugs is readily predicted from calibration curves. As shown in Fig. 6, the most selective electrodes (see Table 6) generally yield a higher value for the *y*-intercept. This supports the previous assertion that more lipophilic ions will produce a higher membrane potential and, therefore, a larger value of $E_{C.W.E.} - E_{REF}$.

TABLE 5

Log $k_{i,j}^{pot}$ and log *P* values for substituted cyclohexylamines at 25°C

Compound	Log $k_{i,j}^{pot}$[a]	Log *P* (Hansch)
N-Ethylcyclohexylamine	−1.97	−1.36
N-2-Hydroxyethylcyclohexylamine	−2.35	−3.98
N-2-Cyanoethylcyclohexylamine	−2.12	−2.16
N-3-Aminopropylcyclohexylamine	−2.22	−3.52

[a]TBA^+ coated-wire electrode. The concentration of interfering ion was 10^{-2} M with a background level of 10^{-4} M TBA^+.

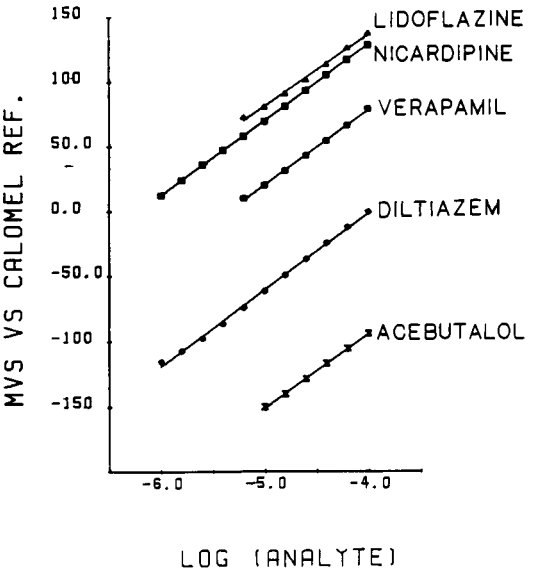


Fig. 6. Calibration graphs for drugs shown in Table 6.

TABLE 6

Log $k_{i,j}^{pot}$ and log P values for substituted cyclohexylamines at 25°C

Drug	Log $k_{i,TBA}^{pot}$ ± s.d.	Log K_D
Acebutalol	2.44 ± 0.2	−0.2
Diltiazem	0.71 ± 0.02	2.3
Lidoflazine	0.30 ± 0.02	5.3
Verapamil	−0.32 ± 0.02	5.9
Nicardipine	−0.39 ± 0.02	6.3

Recent studies by other workers have shown that similar membrane systems give potentiometric response to changes in solution pH [16]. Preliminary investigations in this laboratory have shown that DNNS-based drug electrodes behave differently depending upon the incorporated drug. The magnitude of the pH responses were such that over a sufficiently wide range of solution pH, shifts of 0.02 pH would cause only concentration errors of 1.0%. Future work in this area, besides developing electrodes for additional drugs and compounds of interest, will be aimed at improving the sensitivity of these devices. Current studies of interfacial processes occurring at liquid-liquid interfaces should provide insight into the fundamental mechanisms responsible for CWE behavior with regards to detection limits, selectivity, and varying pH response.

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THE DEVELOPMENT OF BIOCATALYTIC MEMBRANE ELECTRODES

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SUMMARY

Recent developments and future prospects of biocatalytic membrane electrodes are examined in the light of past experience. The influences of scientific, economic and human factors on the course of development is evident.

The past 25 years have seen a vigorous and continuing development of electrochemical biosensors in which enzymes and other biocatalysts are coupled with various sensing electrodes. The technical principles, construction details and operating characteristics of these probes have been extensively documented in comprehensive review articles [1—3] as well as in several excellent monographs [4—6] and need not be restated here. The flow and interplay of ideas and concepts which triggered and sustained the development of this important field are, however, more elusive and merit some examination from the perspective of a quarter century of experience. In retrospect, so many ideas or experiments appear to be straightforward or even obvious that it is easy to forget how daring they seemed at the time.

IMMOBILIZATION OF ENZYMES

The original impetus for enzyme immobilization must have been economic. Isolated or purified enzymes are often expensive, so that analytical procedures which do not reuse enzymes can be prohibitive in cost. Thus, the attachment of enzymes to the surfaces of beads, tubing, glassware, etc. becomes attractive from an analytical point of view, yet it must have taken a leap of the imagination to conceive this idea in the first place. The enhanced stability and change in enzyme kinetic parameters sometimes obtained with immobilization is a bonus for the user. Over the years, numerous techniques for enzyme immobilization have been developed [4] to meet almost any practical analytical need and the entire field has been placed on a firm scientific footing, with a sharp decrease in its empirical content.

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ENZYME COUPLING TO PROBES

The concept of enzyme immobilization, while powerful and useful, still separates the site of biocatalytic action from the analytical detector or sensor. Again, in retrospect, one wonders why it was not immediately obvious that the enzyme should be immobilized directly on or at the sensor. Such a configuration not only serves to concentrate the biocatalytic activity in the micro-environment of the sensor tip but also provides the possibility of making reagentless probes. Under favorable conditions, such probes can offer long lifetimes, rapid response and good analytical selectivity with relatively low cost and easy preparation.

Despite the elegant concept involved, the early development of such enzyme electrodes proceeded rather slowly with primary emphasis on a limited number of model systems. The early use of glass electrodes as sensing elements may also have limited the rate of progress. In consequence, the wider practical acceptance of enzyme electrodes for analytical or clinical purposes has been glacial, with only tentative commercialization of even the most deserving systems emerging from research laboratories. Several persons in different laboratories have made significant contributions to the development of enzyme electrodes, in a truly international effort; an appreciation of both the advances and remaining problems can be gained from Guilbault's monograph [6] which also tabulates most of the enzyme electrodes reported up to 1982, with critical appraisals of the major representative systems.

Possible choices of sensor elements to be used in conjunction with biocatalysts are expanding rapidly. Not only is there a wider selection of possible probes within a given sensor category, e.g., potentiometric membrane electrodes [7], but an increasing use of amperometric [8], field effect, fiber optic, piezoelectric and other sensors. While it is usually feasible to use several sensor/biocatalyst combinations to meet some analytical measurement requirement, it is usually the case that there is a 'best' combination. For example, clinical measurements are almost always simplified by the use of potentiometric gas sensors [9] because of minimal ion or protein interferences, but an amperometric sensing element may be equally satisfactory, or even more attractive, owing to its linear response and sensitivity [8]. As in other experimental techniques, the simplest arrangement usually wins out for practical or routine use.

Because of the large number of adjustable variables inherent to biocatalytic probes, e.g., those of the biocatalyst and of the sensor individually as well as their permutations, it is often difficult to compare the merits of competing possibilities. Very few comparison studies conducted under strictly controlled conditions can be found in the literature [10] and the prospective user of biocatalytic electrode sensors is usually obliged to do additional evaluation or method development when using such sensors for a particular analytical or clinical application. This fact may have retarded the adoption of enzyme electrodes for practical use in many laboratories.

NEWER BIOCATALYSTS

During the 1960s and early 1970s, numerous biosensors were developed using the basic principle of coupling isolated or purified enzymes to electrochemical probes [6]. Some of these sensors were shown to be highly suitable for analytical purposes; others were only of marginal utility, but served to expand scientific understanding of the field.

In the mid-1970s, microbiologists showed that viable whole cells could be collected and kept intact on such inert supports as silica gels and teflon membranes [3]. These findings suggested that bacteria and other cells might be used as alternative biocatalysts in conjunction with membrane electrodes. This expectation was borne out by subsequent research and has expanded opportunities for novel biocatalytic sensors [11].

The use of whole cells in place of isolated enzymes can offer three principal advantages. Some enzymes which are unstable when isolated are stabilized in the environment of the whole cell and biocatalytic activity can be extended to enzyme systems not available in isolated form. Required cofactors or multi-step reactants may already be present in the cell and do no need to be separately immobilized. The cost of whole cells may be substantially lower than that of purified enzymes, especially when cells are regenerated in situ. Whole cells may also suffer disadvantages with respect to isolated enzymes in terms of lower selectivity, mechanical handling and sterility requirements. These disadvantages are real, but recent research has demonstrated that they can often be circumvented or minimized by relatively simple procedures [12].

By 1978 it had become apparent that whole-cell biocatalysts need not be restricted to bacteria and other micro-organisms. Indeed, even early experiments with tissue samples from the organs of pigs [13] and cattle showed amazingly high levels of biocatalytic activity for the deamination of amino acids with excellent selectivity and time stability. By happy coincidence, one of the very first materials studied, porcine kidney [13], yielded superb results and remains one of the best animal tissue biocatalysts to this day.

The use of animal tissue slices as biocatalysts retains all of the positive features shown by micro-organisms and has the added advantage of structural integrity and mechanical ruggedness. Moreover, biochemical knowledge of mammalian metabolic pathways is so extensive that the biocatalytic properties of such tissues can often be sufficiently predicted in advance to permit the proper tissue to be selected for specific measurement applications [3].

As is frequently true for a new field, there was initially wide skepticism regarding the feasibility of this approach. Reservations were expressed regarding the adequacy of enzyme-activity levels in untreated tissue, preservation of the tissue material, and undesirable side reactions. These and other concerns were largely laid to rest by the results of further experimental

studies. Not only mammalian tissues but animal tissues in general were soon shown to possess attractive biocatalytic properties for selected analytical applications of potentiometric [7] and, later, amperometric [14] biosensors. In some cases, membrane electrodes prepared with tissue materials were even found to be superior to those using either enzymes or immobilized bacterial cells [10]. As a consequence, the possible range of biocatalytic sensors could be further extended to new systems both in terms of intrinsic interest as well as for practical application to analytical or clinical measurement.

In part, the success of tissue-based membrane electrodes results from a combination of fortunate circumstances. For example, the relaxation time for translocation of substrates into intact cells is in the 10—1000 s range, which is compatible with the gas-sensing potentiometric membrane electrodes being used as sensing elements. Moreover, the rates of gas diffusion, e.g., for CO_2, in both mammalian and non-mammalian tissues are rather constant [15]. Finally, the input/output characteristics of such biocatalysts display a fairly well defined chemical stoichiometry, so that analytical response proportionality can be achieved.

It is probably fair to say that almost all electroanalytical chemists were surprised that plant tissues could also be used as effective biocatalysts at membrane electrodes. Intuitively, plant tissues were thought of as having a slower metabolism and less biocatalytic activity than animal tissues on a weight basis. The opposite turns out to be true [3], however, for tissues taken from the growing portions of plants or from fruits and vegetables that store nutrients for future growth and propagation. Thus, beans, corn kernels, squash, bananas, onions, etc. have a surprisingly high level of biocatalytic activity [16]; sometimes this activity is concentrated in discrete segments or layers. Similarly, the growing tips of plant roots and young leaves are found to have activity levels suitable for biocatalytic-electrode purposes [17]. Leaves are especially interesting on structural grounds; their arrangement of hydrophobic layers and membrane-bound bioactive portions appears almost tailor made for incorporation at or into electrode phases [17]. The study and use of plant tissues for biocatalytic-electrode purposes is so new that it is difficult to predict what directions this subfield might take in the future, but it is already apparent that there are several interesting and, perhaps, unique possibilities.

HYBRID SENSORS

In addition to the straightforward use of enzymes or cells as biocatalysts, arrangements have been reported in which several bioactive components are "hybridized" at membrane electrodes. For example, sensors have been proposed in which micro-organisms plus an auxiliary enzyme are co-immobilized [18]; the function of the enzyme is to facilitate the overall biocatalytic scheme by conversion of the initial substrate, an intermediate, or the product of the microbial metabolism. Auxiliary enzymes have also been used

in conjunction with both plant and animal-tissue based membrane electrodes [19].

Similarly, sequences of two or three individual enzymes have been immobilized to do multi-step catalysis [20]; even a dual enzyme/bound cofactor enzyme electrode has been reported [21]. The principal limitation for these arrangements, and for hybrid sensors in general, is that the optimal operating conditions for the several components are usually not identical. Thus, some compromise in terms of pH, ionic strength, temperature, etc. is necessary with an inevitable loss of activity or lifetime.

Particularly imaginative sensors have been designed in which multi-component biocatalysts provide for regeneration cycles or for the harmless removal of an interferent or side product [20]. Such arrangements may also permit the effective use of amplification cycles to enhance sensitivity [21] and, thus, offer hope for truly practical and fully reagentless biocatalytic sensors. The concepts involved apply equally to the amperometric and potentiometric electrodes and, perhaps, to optical probes as well.

PROBLEMS TO BE OVERCOME

One might think that biocatalytic sensors would have been largely perfected after more than two decades of investigation and development. The fact that so few probes have been made commercially available and, indeed, are rarely found outside the research laboratory, indicates clearly that there are still major shortcomings compared to other sensing devices or bioanalytical techniques.

The causes responsible for this state of affairs are both technical and human in nature. On the technical side, it is easy to identify some of the factors which serve as obstacles in the wider adoption and use of biocatalytic membrane electrodes.

(i) Enzyme or cell-based electrodes require assembly by the user on an individual basis. Because the construction of such electrodes, even those which employ commercial components only, requires both skill and art, it is difficult to do in a consistent and fully reproducible manner. Most laboratories are not set up for this purpose. In the future, commercialization may facilitate such assembly by providing all the components in kit form.

(ii) Biocatalytic membrane electrodes require frequent calibration both during use and on a day-to-day basis. Every time a new electrode is assembled, new calibration data need to be established and reproducibility has not been impressive.

(iii) The dynamic response characteristics of biocatalytic membrane electrodes can be inconvenient. Even the best electrodes have response times in the order of minutes. More seriously, the recovery times to re-establish baseline response between samples can be extensive. Some of these problems might be overcome with improvements in construction geometry.

(iv) The working lifetime of biocatalytic membrane electrodes is limited,

ranging from a few days to several weeks. However, even those electrodes with relatively long lifetimes will display a steady (and not always reproducible) loss of activity with time.

(v) Biocatalytic membrane electrodes are not "idiot proof" and generally cannot be used by semi-skilled laboratory personnel. They are labor intensive and, at least in their present configuration, not amenable to automation or unattended operation.

Some of these factors also have an impact upon the "human" considerations. As long as enzyme and cellular electrodes are tools of the research laboratory only, there is no scientific or commercial driving force to bring them into routine use and production. For many years now, work on such electrodes has been the province of academic research laboratories with serious effort confined to perhaps four or five centers at most. With scientific curiosity as the principal motivation, progress has been cyclical and leisurely. Some patents have been granted but have not been widely exploited.

In the 1980s, this situation is undergoing a drastic change. With the strong world-wide emphasis on biotechnology as a major scientific area, biosensors have also become a "hot" topic. An entirely new cohort of workers has entered the field and has brought a much more competitive and business-oriented approach. While one can decry the loss of professional courtesy and ethics associated with these events, the new situation nevertheless represents a genuine opportunity for biosensors to move out of the laboratory stage into a more practical and useful role. The needs of the biotechnology area, e.g., monitoring fermentation processes, antibody production, agricultural genetic manipulation, etc., are entirely consistent with the analytical capabilities of biocatalytic membrane electrode sensors and will surely provide a strong impetus for future development. Care must be taken, however, that the scramble for competitive advantage does not result in a lowering of scientific standards or the piracy of partially developed ideas from academic laboratories. An increased emphasis on secrecy is already apparent at recent scientific conferences.

PROSPECTS

So many of the sub-areas of biocatalytic membrane electrodes are currently still in early stages of development, that major new results and their impact may yet lie in the future. Indeed, there is a feeling among workers in the field that the creative possibilities are only now beginning to be realized thanks to some new initiatives and an expanded perspective. It seems highly probable that biosensors will become increasingly sophisticated in terms of biochemical or physiological schemes and, thus, will be applicable to even more challenging measurement problems. In the very near future, sensor probes using enzyme-immuno components and monoclonal antibodies can be expected; in the long run, receptor-based sensors and probes for genetic materials would not be out of the question.

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SOLVENT POLYMERIC MEMBRANE ION-SELECTIVE ELECTRODES

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SUMMARY

The significant role of poly(vinyl chloride) as a support polymer in solvent polymeric ion-selective electrodes is briefly reviewed. This lays emphasis on the way PVC has contributed to the relatively high state of development of conventional ion-selective electrodes, microelectrodes, coated-wire electrodes and ion-sensitive field effect transistors based on liquid ion-exchanger and neutral carrier ionophore sensors. Also discussed are possibilities of using alternative polymer matrices for overcoming some of the shortfalls of the PVC electrodes.

Just over half a century has elapsed since the metal ion-sensitive glass analogue to the glass pH electrode was predicted [1]. Developments in sensor membrane electrodes in the first half of the period since then was slow, but this has been followed by phenomenal advances in the science and technology of the ion-selective electrode (ISE). These were stimulated by the introduction of cation-selective glass membranes which led on to various other electrochemical sensor membranes consisting of precipitate incorporated in a chemically inert material, monocrystals, pressed solids, supported liquid ion-exchanger and neutral carrier ionophores, etc.

PVC MATRICES

The popularization of the polymeric membrane ISE followed on the simple design principle of dissolving poly(vinyl chloride) (PVC) in a solution of liquid ion-exchanger in tetrahydrofuran solvent by slow evaporation at room temperature [2—4]. PVC is by far the most commonly used polymer matrix for ion sensors of the liquid ion-exchanger and neutral carrier ionophore types. Because the composition of around 70:30 percent plasticiser (plus sensor): polymer is essentially a single phase homogeneous mixture, it is sensible to follow precedent [5] and refer to such membranes as "solvent polymeric" types. They generally exhibit liquid membrane properties, although the diffusion coefficients of ca. $10^{-8} - 10^{-9}$ cm^2 s^{-1} for cations through polymer-trapped liquid ion-exchangers are characteristic of diffusion through a very viscous medium [6].

For neutral carrier-based solvent polymeric membranes, where PVC was

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soon adopted as a support matrix [7], diffusion coefficients of the ionophores and their complexes with cations are 10^{-7}—10^{-8} cm^2 s^{-1} which places them even nearer to liquid membranes [8, 9]. However, at very low plasticiser contents, the diffusion coefficients [9] may be as low as 10^{-11} s^{-1}, thus approaching the values for solids.

The mechanism of selective ion-transport through PVC membranes containing trapped liquid ion-exchangers is prompted by the parallel patterns between selectivity coefficients of the electrodes with the ion-pair extraction constants of related solvent extraction systems [10]. This is illustrated in Table 1 for tetraalkylammonium sensors used for nitrate and perchlorate ISEs and in Table 2 for di(2-ethylhexyl)phosphoric acid which, with suitable plasticising solvent mediation, shows selective electrode properties for calcium (see Table 3).

Radiotracer studies with ^{45}Ca and ^{36}Cl demonstrate that PVC matrix membranes containing Orion 92-20-02 liquid calcium ion-exchanger (based on dialkylphosphate sensor and dioctylphenyl phosphonate solvent mediator) are permselective to counter cations [6]. This is important for use in ISEs.

Although the above diffusion data place these membranes in the viscous medium category, such solvent polymeric systems, for what are essentially liquid membranes, permit fundamental ion-permeation study as well as convenience of use as membranes in ion-selective electrodes. Thus, radiotracer studies have shown selective permeation of calcium ions from along alkaline earth cations, through organophosphate sensor membranes [6, 15].

As illustrated in Table 3, it is the solvent mediator that promotes the electrode selectivity features of the organophosphate sensors. With decan-1-ol in place of dioctylphenyl phosphonate, there is little discrimination between calcium and magnesium ions [14] (Table 3). The equal sensing of calcium and magnesium ions by the divalent ion electrode based on Orion 92-20-32 divalent cation exchanger (dialkylphosphate plus decan-1-ol solvent mediator) is reflected in the value of 32×10^{-7} s^{-1} for $d(C''/C')/dt$ for permeation of ^{45}Ca from calcium chloride through the PVC membrane containing the exchanger into counter solutions of calcium chloride and magnesium chloride, respectively [16]. In the $d(C''/C')/dt$ measure of permeation, C'' is the radiotracer concentration of the solution on the initially inactive side of the membrane, and C' is the tracer concentration of the solution on the initially active side.

While permeation and other studies on selectivity have yielded useful data, it is still difficult to reliably predict the selectivity of liquid ion-exchanger based ISEs, partly because the solvent mediator and the primary ion sensor may each contribute. Also, selectivity can depend on the composition of sample solutions. On the other hand, the behaviour patterns of ISE membranes based on neutral carrier ionophores are more amenable to theoretical treatment and have been well discussed in recent reviews [17, 18].

For calcium ISEs, the development of which also matches the use of solvent polymeric membranes, considerable progress has been made with

TABLE 1

Selectivity data for PVC nitrate electrode $(C_{12}H_{25})_3C_{16}H_{33}N^+NO_3^-$ sensor and ion pair extraction constants for $(C_4H_9)_4N^+(Q)$ for nitrate and other anions

B	$k^{pot}_{NO_3^-}$ [a]	Log E_{QB} [b]	E_{QB}/E_{QNO_3} [c]
NO_3^-	1	+1.39	1
OH^-	—	—1.67	8.7×10^{-4}
Cl^-	5×10^{-3} (5×10^{-1})	—0.11	3.2×10^{-2}
Br^-	0.01 for liquid membrane	+1.29	7.9×10^{-1}
ClO_3^-	1.66 (5×10^{-4})	—	—
ClO_4^-	800 (5×10^{-5})	+3.48	1.2×10^2
I^-	17 (5×10^{-5})	+3.01	42

[a]Molarity of B is given in parentheses [11]. [b]Reference 12. [c]$E_{QB} = [QB]/[Q][B]$ where [QB] is the concentration of ion pairs in the organic phase and [Q], [B] are the concentrations of the extractant, Q, and the counter ion, X, in the aqueous phase. Data taken from ref. 10.

TABLE 2

Extraction coefficients of alkaline earth metal cations from 4 M sodium nitrate into di(2-ethylhexyl)phosphoric acid in benzene and $k^{pot}_{Ca,B}$ data from PVC matrix membrane ion-selective electrodes with dialkylphosphate sensors

Counter ion B	Log of extraction coefficient[a]		$k^{pot}_{Ca,B}$ [b]		
	pH = 7.0	pH = 5.2	Ca di(2-ethylhexyl)phosphate sensor with decan-1-ol, 25°C	Orion 92-20-02 liquid ion-exchanger	
				25°C	35°C
Be^{2+}	4.3	4.3	2.6 (5×10^{-5})	12.0 (5×10^{-1})	14.0 (5×10^{-5})
Mg^{2+}	1.2	1.4	1.0	0.01 (4×10^{-1})	0.052 (4×10^{-4})
Ca^{2+}	0.9	2.2	1.0	1.0	1.0
Sr^{2+}	—0.2	0.1	0.5 (4×10^{-4})	0.05 (4×10^{-4})	0.09 (4×10^{-4})
Ba^{2+}	—0.6	—0.5	0.5 (4×10^{-4})	0.002 (4×10^{-4})	0.01 (4×10^{-4})

[a]Based on data from [13]. [b]Numbers in parentheses are the molarity of the interfering counter ion, B. Data from [14].

regard to sensitivity and selectivity by the superseding of the original calcium bis dialkylphosphate sensors. Such advances have been reviewed from the standpoint of the newer di-4-octylphenylphosphate and di-4-(1,1,3,3-tetramethylbutyl)phenylphosphate ion-exchanger type sensors [19, 20] and also of the neutral-carrier neutral-carrier ionophore type [17, 18], especially the calcium ion sensor based on *N,N'*-di[(11-ethoxycarbonyl)undecyl]-*N,N'*-4,5-tetramethyl-3,6-dioxooctane diamide which is very widely used. An even newer range of calcium ion sensors is based on macrocyclic polyether-amides with two polyether rings [21, 22].

TABLE 3

Selectivity coefficient ($k^{pot}_{Ca,B}$) data for calcium ISEs with calcium di-2-ethylhexylphosphate sensor, [B] = 0.001 M [14]

Solvent Mediator / Ion (B)	Dioctylphenyl phosphonate	Dioctylphenyl phosphonate + decan-1-ol (1 + 1)	Decan-1-ol
Mg^{2+}	0.057	0.52	1.0
Sr^{2+}	0.040	0.17	0.5
Ba^{2+}	0.048	0.17	0.5
Mn^{2+}	0.28	1.0	2.0

The characteristics of a large number of solvent polymeric ISEs for cations and anions have been summarised in earlier reviews [23, 24]. These tabulations [23, 24] signify that the polymer matrix makes for convenience in the study and use of liquid membrane-based ISEs. There is no attempt to bring the tabulation up to date in this article, but it is emphasised that the number and range of solvent polymeric membrane ISE types have greatly increased.

Among the desirable features of polymeric membrane ISEs is the longer lifetimes of these electrodes compared with liquid membrane counterparts. This, however, depends on the control of leaching of sensor and solvent mediator components from the membrane. Thus, efficient design of a barium ISE based on a sensor of the tetraphenylborate salt of a barium complex with an appropriate nonylphenoxypolyethoxylate demanded a high viscosity solvent mediator (2-nitrophenyl phenyl ether) in order to reduce the leaching which led to very short electrode lifetimes when low viscosity plasticising solvent mediators, such as 2-ethylnitrobenzene, were employed [25]. The same principle ensures long lifetimes of other solvent polymeric membrane electrodes, including the organophosphate-based calcium electrodes discussed above, which are based on the high viscosity dioctylphenyl phosphonate plasticising solvent mediator. Similarly, the high viscosity of 2-nitrophenyl octyl ether or 2-nitrophenyl phenyl ether as plasticising solvent mediator led to the predicted long life of PVC matrix membrane nitrate electrodes with tri(bathophenanthroline)nickel(II) nitrate sensor [26].

Leaching from surfaces of solvent polymeric membranes is indicated by direct proportionality of the diffusion coefficient to thickness for calcium ions permeating through an organophosphate membrane [6]. This may be attributed to the diffusing species experiencing the same resistances, such as may occur from having a viscous interface at the membrane surface, arising from some loss of plasticising solvent mediator.

Similar leaching seems to be associated with the interference of PVC organophosphate-based calcium electrode by anionic surfactants [27, 28] as has been demonstrated by thin layer chromatographic and x-ray fluores-

cence studies [28]. Such interferences can be overcome by replacing the dioctylphenyl phosphonate solvent mediator with trioctyl phosphate [28], a system which also resists interference by certain biochemical components [29]. However, the real reason for such interferences is more complicated than mere leaching, possibly by the formation of a complex between the surfactant and surface components of the membrane, for the interference is also characteristic of liquid membrane electrodes [30, 31]. The relative freedom from interference by a commercial neutral carrier-based calcium ISE for biochemical components contrasts with the gross interference incurred by anionic surfactants [28, 29], and points to the possibility of reasons in addition to simple leaching.

ALTERNATIVE POLYMER MATRICES

Various reasons, such as leaching of membrane components and the interferences by surfactants, have led to studies of possible alternatives to PVC as the polymer matrix. However, because of surfactant interferences, the use of matrices based on poly(vinylidene chloride) and VAGH copolymer (hydrolysed vinyl chloride/vinyl acetate) for calcium electrodes, offer no advantages [28].

In general, it is desirable that the solvent polymeric membranes should have glass transition temperatures below room temperature [32]. Solvent mediators used in association with the sensor normally have plasticising properties and are effective in reducing the glass temperature which, for PVC, is 81°C. It has been suggested [32] that polymers like silicone rubber and polyurethane are suitable matrices due to glass transition temperatures below room temperature. Of course, silicone rubber is used in precipitate-impregnated membrane electrodes, but these are outside the range for this article.

Poly(methyl acrylate) provides an alternative support matrix to PVC, but the proportion of polymer must be doubled from the normal (ca. 30%) in order to obtain membranes of sufficient mechanical strength for use in an ISE [33]. Poly(methyl methacrylate) is suitable for coated-wire electrodes and has been used as a matrix for a nitrate ISE to determine nitrogen oxides in ambient air [34]. Poly(methyl methacrylate) and polystyrene are said to be less successful because of their higher glass transition temperatures (105 and 100°C, respectively), but low sensor solubility and incompatibility of the components must also be considered [32].

The selectivity profile of an electrode with a poly(vinyl isobutyl ether) matrix with calcium bis di-2-ethylhexylphosphate sensor and decan-1-ol solvent mediator is similar [35] to a PVC electrode containing the same sensing ingredients [14]. Urushi, a natural lacquer of Japanese origin, provides a matrix compatible with various liquid ion-exchangers for divalent ions [36], perchlorate [36, 37], nitrate [38] and other ions. Likewise, membranes comprising vinyl plastic repair cement (Lepage No. 330), 2 drops

of a solvent mediator (dibutyl phthalate or dioctyl phthalate), and metal complex, for example, $Pb[Hg(SCN)_4]$, give electrodes responsive to lead [39].

Matrices of cellulose acetate [32, 40], ethyl cellulose [40], and collodion [40] are totally unsatisfactory owing to their hydrophilic character. The same might also be expected of poly(vinyl alcohol) because of the hydroxyl group network, but controlled amounts of vinyl chloride/vinyl alcohol copolymer with low levels of vinyl alcohol (VAGH copolymer) give good functional membranes with organophosphate sensor and dioctylphenyl phosphonate solvent mediator [41]. The hydroxyl groups form an agency for grafting the sensor to the matrix [41]. Such covalently linked calcium-sensitive membranes required additional PVC support and dioctylphenyl phosphonate solvent mediator [41, 42]. Monoctylphenyl phosphonate covalently bonded to VAGH was used with some additional dioctylphenyl phosphonate solvent mediator and calcium bis di-4-(1,1,3,3-tetramethyl-butyl)phenyl phosphate sensor in electrode membranes [42]. This showed some loss of selectivity over normal calcium ISEs, but the slightly lengthened lifetime was insufficient to warrant synthesis of the phosphonated polymer matrix [42].

Various other approaches have been made to the covalent bonding of selective sensors to polymer matrices in order to promote robustness and extended lifetimes of an ISE. Among these is the immobilisation of calcium ion-exchange sites in a styrene-n-butadiene-styrene (SBS) tri-block elastomer. Good calcium electrodes of lifetimes in excess of six months were obtained although they were of limited selectivity towards calcium over important ions, such as sodium and magnesium [43, 44]. Attempts to phosphonate polystyrene by Friedel-Crafts and free-radical processes resulted in membranes of poor quality in respect of calibration range, brittleness and mechanical strength [42].

Rather more success seems to have been obtained in the covalent grafting of surfactant sensors. Thus, cationic surfactant electrode membranes may be obtained by converting sulphonated PVC to the tetraalkylammonium form [45]. By grafting tetraalkylammonium bromide to PVC and converting to alkylsulphate or alkylbenzene-sulphonate forms, it was possible to obtain anionic surfactant electrode membranes [45]. However, there does not seem to be any subsequent adoption of these systems for routine applications. This suggests that a lot of motivation is required to move away from the very convenient and straightforward design principle of simply mixing readily available PVC membrane components together, a principle which has also stimulated the development of microelectrodes, coated-wire electrodes and ion-selective field effect transistor (ISFET) sensors.

PVC IN OTHER ELECTRODE TYPES

Coated-wire electrodes emanated from the desire to miniaturize, to simplify and to produce cheaper ion-selective electrodes [46, 47]. The first

coated-wire ISE also introduced cyclohexanone as a solvent for dissolving the PVC polymer and various other components as an alternative to tetrahydrofuran [46]. The main feature of coated-wire electrodes is the elimination of the conventional aqueous reference system found in the normal solvent polymer membrane electrodes and the polymer film employed is most commonly PVC. The subject has been well reviewed [48, 49], and it is discussed in the following paper.

The PVC matrix membrane principle has aided the development of conventional micro-electrodes with tip diameters down to 1 μm [18, 50] and these have been produced in single-, double- and twisted-barrelled modifications [18].

With regard to ISFETs, these seem inconceivable for ordinary liquid membrane sensors without the agency of an immobilizing polymer. These, as indeed for any ion-sensing membrane, demand that pinholes are absent from the membranes. Therefore, membrane thickness in excess of 50 μm is required in order to avoid pinholes which fill up with electrolyte and eventually short out the membrane [51]. However, the use of improved membrane application [52] has led to better performance characteristics and to a wide range of ISFETs with homogeneous polymeric membranes [51, 53].

Of course, solvent polymeric ISFETs may be looked on as an extended development of coated-wire electrodes, an intermediate stage having the solvent polymeric membrane applied to the gate lead of a field effect transistor. An example of this approach is that of the valinomycin potassium sensor in urushi as a membrane matrix applied to 2 SK241 as the optimum transistor and using 3 V as the optimum drain source voltage [54].

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NEUTRAL-CARRIER-BASED ION-SELECTIVE ELECTRODES

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SUMMARY

Neutral-carrier-based ion-selective electrodes for the assay of alkali and alkaline earth metal cations have advanced to become the most frequently used potentiometric sensors in clinical chemistry. The major developments since the realization of the first potentiometric cell assemblies utilizing electrically neutral complexing agents (valinomycin and the macrotetrolides) in 1966 are presented.

Neutral ion carriers, a class of ionophores, are electrically neutral, lipophilic complexing agents that have the capability to bind ions reversibly and to transport them across organic and hydrophobic membranes by carrier translocation [1]. Ideally such carriers induce a selective permeation of one type of ion through this membrane. Using such membranes in cell assemblies of the type:

external reference electrode	sample solution	membrane	internal reference system

a wide variety of analytically relevant potentiometric sensors has been realized [1].

The antibiotic valinomycin (a cyclododecadepsipeptide) and the macrotetrolides (nonactin, monactin, dinactin, trinactin) have become classical neutral ionophores. Moore and Pressman in 1964 [2] and Graven et al. in 1966 [3, 4] have shown that valinomycin [5, 6] and the macrotetrolides [7], respectively, induce ion selectivity in rat liver mitochondria. The mechanism of action of these antibiotics was rather obscure at that time. An article in Experientia describes clearly the state of conception in 1965 [8]:

> "Apparently the depsipeptide antibiotics are inducers of K^+ ion transport as the result of interaction with a specialized mitochondrial receptor site controlling this transport."

In 1967 it was still said [9]:

> "Although their effects on passive membrane permeability are mimicked

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by alkali complexing agents, the inducers form no such complexes in free solution."

The first potentiometric cell assemblies based on different neutral carriers were described by Štefanac and Simon in 1966 [10] and the interaction of the macrotetrolide antibiotics with alkali metal ions in "free solution" was corroborated by vapour pressure osmometry. Although the fact of the complex formation of alkali metal ions by neutral organic compounds was quickly accepted, the introduction of neutral carrier sensors had a slow beginning. In 1969 they were credited under the heading "miscellaneous" as follows [11]:

"Even more exotic systems in which electrochemical cells using macrotetrolides (nonactin homologs) on various inert supports as membranes have been described."

This is not surprising because the first electrodes with membranes of sintered glass, which were impregnated with solutions of the neutral carrier antibiotics in an organic solvent, had response times of about 2 h (Fig. 1a) [12]. Not until 1972 did sensors based on valinomycin find their first application in a commercial clinical analyzer (Table 1). Figure 1b displays the current state of the art of neutral-carrier-based electrodes. This arrangement allows the simultaneous monitoring of H^+, Na^+, K^+ and Ca^{2+} in the continuous flow of whole blood in a mammalian extracorporeal bypass with high long-term signal stability and response times of a few seconds [95]. Another recent trend in the development of sensors using neutral-carrier-based membranes combines membrane technology and semiconductor technology to yield highly miniaturized sensors where the ion-selective membrane together with the conventional external instrumentation is packed on a single integrated circuit (IC) chip (Fig. 1c).

A chronological description of the major steps in the development of neutral-carrier-based sensors up to 1980 is given in Table 1. Because so many efforts have been devoted within the last few years to the design, the application and the mechanism of action of this group of ion-selective electrodes, it has become difficult to point out more recent major significant contributions. Although it might be too ambiguous an undertaking to predict future impacts of more recent efforts, Table 2 tries to depict some steps the authors consider significant.

At present, membranes based on neutral carriers are widely applied in sensors for clinical applications and in microelectrodes for intracellular studies. More than 30 manufacturers [14] all around the world produce at least one clinical analyzer each of which utilizes neutral-carrier-based ion-selective electrodes to assay alkali and/or alkaline earth metal cations. All the potassium ion-selective electrodes in these analyzers rely on valinomycin. Among the total number of analyses done in clinical laboratories the assay of K^+ is the most frequently performed. In industrialized countries an

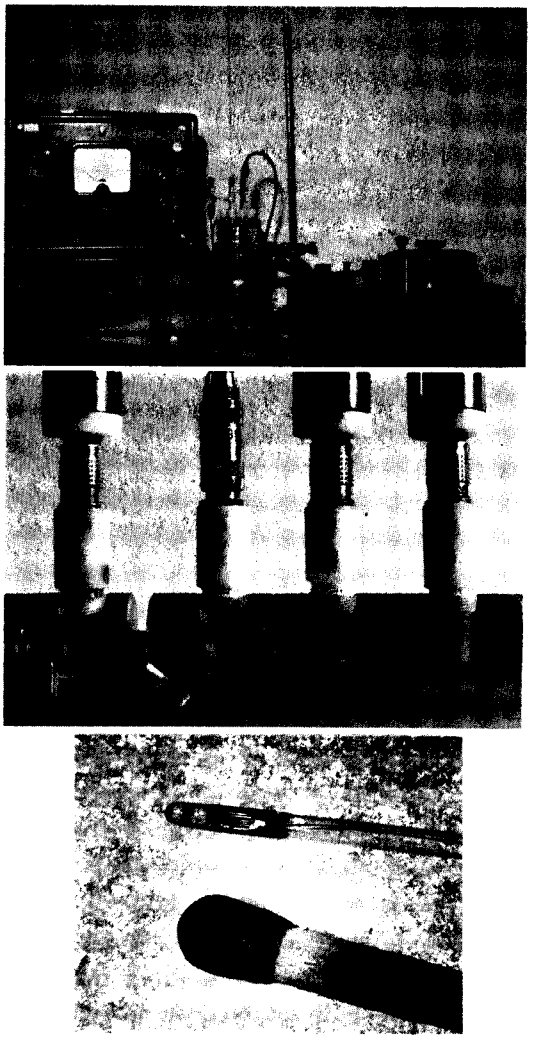


Fig. 1. (a) Cell-assembly (Fig. 1 in [12]) with e.m.f. measuring equipment (Poggendorff compensation [13]) as used in 1966 [10]. (b) Serial arrangement of several ion-selective electrodes for the continuous and simultaneous ion monitoring in a blood bypass. (c) Catheter-type ion-selective field effect transistor (courtesy of N. F. De Rooij, IMT, Université de Neuchâtel, Switzerland).

average of about 0.2 determinations per capita per year is made [15]. It has been estimated for 1984 that in some industrialized countries 60—70% of these assays are done by potentiometric procedures using a K^+-selective membrane electrode based on valinomycin.

Figure 2 illustrates impressively the worldwide efforts particularly during the recent years in designing new synthetic neutral ionophores of different

TABLE 1

Chronology of important contributions to the development, application, and response mechanisms of neutral-carrier-based ion-selective electrodes

Moore and Pressman	(1964) [2]	Discovery that the antibiotic valinomycin induces ion-selective effects in metabolic studies in rat liver mitochondria.
Štefanac and Simon	(1966) [10, 12]	First report on the action of antibiotics as neutral complexing agents, as carriers for cations, and on their use in ion-selective membrane electrode cell assemblies.
Pedersen	(1967) [16]	Discovery of cation-complexing macrocyclic polyethers (crown compounds).
Müller and Rudin	(1967) [17]	Studies on the effect of carrier antibiotics on bilayer membrane potentials.
Lev and Buzhinsky	(1967) [18]	
Kilbourn et al.	(1967) [19]	X-ray analysis of the K^+-nonactin complex.
Pioda et al.	(1968) [20]	Valinomycin/KSCN complex isolated. K^+-selective coated metal electrode based on valinomycin.
Pioda et al.	(1969) [21]	K^+-selective electrode based on valinomycin in diphenyl ether (millipore filter matrix).
Dietrich et al.	(1969) [22]	Development of macroheterobicyclic ligands for cations (cryptands).
Pinkerton et al.	(1969) [23]	X-ray analysis of the K^+-valinomycin complex.
Simon	(1969) [10]	First patents on valinomycin and other antibiotics as components for ion-selective electrodes issued.
Wipf et al.	(1969) [24]	Selective electrodialytic ion transport through bulk membranes induced by neutral ionophores. Elucidation of carrier transport mechanism.
Eisenman et al.	(1969) [25]	Theory of membrane potentials and conductances of neutral carrier membranes (bilayers).
Diebler et al.	(1969) [26]	Kinetic studies of the cation-carrier complexation.
Moody et al.	(1970) [27]	Standard procedure for the preparation of ion-selective PVC membranes.
Scholer and Simon	(1970) [28]	NH_4^+-selective electrode based on nonactin/monactin in tris(2-ethylhexyl)phosphate.
Wipf et al.	(1970) [29]	Selective electrodialytic ion transport through bulk membranes induced by valinomycin. Elucidation of transport mechanism.
Krull et al.	(1970) [30]	Use of collodion or cellulose acetate supports for valinomycin-based membranes.
Kedem et al.	(1970) [31]	

TABLE 1 (continued)

Pioda et al.	(1970) [32]	First application of valinomycin electrodes for K^+ determination in blood serum or plasma.
Wise et al.	(1970) [33]	
Levins	(1971) [34]	Ba^{2+}-selective electrode based on polyethylene glycols as carriers and tetraphenylborate in *p*-nitroethylbenzene.
Schneeweiss and L'Orange	(1971) [35]	Physiological application of K^+-selective carrier membrane electrodes.
Morf and Simon	(1971) [36]	Analysis of the molecular aspects of cation selectivity of neutral carriers.
Eyal and Rechnitz	(1971) [37]	Extended correlation between potentiometrically measured selectivities and complex formation constants.
Ammann et al.	(1972) [38]	Ca^{2+}-selective electrode based on the first tailored synthetic neutral carrier.
Technicon/Photovolt Corp., USA	(1972) [14]	First commercial clinical analyzer using a valinomycin-based sensor (STAT-ION).
Morf et al.	(1972) [39]	Reports on PVC membrane electrodes based on valinomycin.
Fiedler and Růžička	(1973) [40]	
Ryba and Petránek	(1973) [41]	K^+-selective electrodes based on crown ethers.
Pick et al.	(1973) [42]	Use of silicone rubber as matrix for valinomycin membranes.
Guilbault and Nagy	(1973) [43]	Urea sensor consisting of a NH_4^+-selective electrode coated with the enzyme urease.
Morf et al.	(1973) [44]	Analysis of the parameters affecting the monovalent/divalent ion selectivity of neutral carriers in membrane electrodes.
Boles and Buck	(1973) [45]	Studies of the anion interference in cation-selective neutral-carrier-based electrodes.
Morf et al.	(1974) [46]	
Ammann et al.	(1974) [47]	Na^+-selective electrode based on a synthetic neutral carrier.
Cattrall et al.	(1974) [48]	First coated-wire electrode based on valinomycin.
Cram and Cram	(1974) [49]	First synthetic neutral carrier with enantiomer-selective behavior.
Ammann et al.	(1975) [50]	Structure/selectivity studies on synthetic neutral carriers for electrodes of different selectivities.
Güggi et al.	(1975) [51]	Li^+-selective electrode based on a synthetic neutral carrier.
Thoma et al.	(1975) [52]	Potentiometric and ion-transport studies on membranes with an enantiomer-selective carrier.

TABLE 1 (continued)

Thomas et al.	(1975) [53]	First microelectrode (tip diameter $\simeq 1$ μm) based on a synthetic neutral carrier.
Moss et al.	(1975) [54]	First ion-selective field effect transistor (ISFET) based on valinomycin.
Morf et al.	(1975) [55]	Theoretical and experimental study of the dynamic response of valinomycin-based electrodes.
Morf et al.	(1976) [56]	Detailed analysis of the ion transport properties of thick membranes containing neutral carriers.
Růžička et al.	(1977) [57]	First application of valinomycin electrodes in flow-injection analysis (f.i.a.).
Schindler et al.	(1977) [58]	Flow-through system using neutral carrier membrane electrodes for the on-line determination of Na^+, K^+ and Ca^{2+} in whole blood.
Thoma et al.	(1977) [59]	Study of response mechanisms of neutral carrier membrane electrodes using labeled ions and labeled ligand.
Caroni et al.	(1977) [60]	Ca^{2+}-transport studies on biological membranes with synthetic ionophores.
Cammann	(1978) [61]	Electrochemical investigations of interfacial processes at neutral carrier membranes.
Koryta	(1979) [62]	
Šenkyr et al.	(1979) [63]	UO_2^{2+}-selective electrode based on a synthetic neutral carrier.
Corongiu et al.	(1979) [64]	Additive procedures for the reproduction of results of ab initio calculations of ion-carrier interaction energies.
Gresh et al.	(1979) [65]	
Kimura et al.	(1979) [66]	Synthesis of cation-selective bis-crown-ethers.
Schneider et al.	(1980) [67]	Cd^{2+}-selective electrode based on a synthetic neutral carrier.
Oesch and Simon	(1980) [68]	Lifetime studies on neutral-carrier-membrane electrodes.

selectivities. The list is not comprehensive but depicts some proposed chemical constitutions of neutral carriers either with already established relevance for certain applications or, in our opinion, with interesting and promising approaches to offer. Thus, they have been proposed to be selective for H^+ (**1** [72, 81], **2** [82], **3** [82]), Li^+ (**4** [83], **5** [84], **6** [51], **7** [85], **8** [86], **9** [87], **10** [88]), Na^+ (**11** [89, 90, 91], **12** [92], **13** [93], **14** [47], **15** [94]), K^+ (**16** [10, 95, 96], **17** [97], **18** [66], **19** [98], **20** [99], **21** [100], homologues of **18** [100] and **20** [41, 101]), Cs^+ (**22** [102]), NH_4^+ (**23** and homologues of **23** [92]), enantiomers of the type $R—NH_3^+$ (**24** [103], **25**

TABLE 2

Promising recent developments in the field of neutral-carrier-membrane electrodes

Treasure	(1978) [69]	Development of catheter electrodes for in-situ monitoring in whole blood.
Hill et al.	(1978) [70]	
Lanter et al.	(1980) [71]	Availability of microelectrodes for intracellular Mg^{2+} determinations.
Schulthess et al.	(1981) [72]	pH-sensitive PVC membrane electrode based on tri-n-dodecylamine.
Gajowski et al.	(1981) [73]	Use of a macroheterobicyclic compound (crypt-and) in a neutral-carrier-membrane electrode.
Anker et al.	(1981) [74]	Development of an electrode procedure for the measurement of the total Ca^{2+} concentration in blood serum.
Meyerhoff et al.	(1982) [75]	Application of neutral-carrier-membrane electrodes in gas-sensing devices.
Funck et al.	(1982) [76]	HCO_3^--selective electrode using a gas-permeable, pH-sensitive neutral carrier membrane.
Keating and Rechnitz	(1984) [77]	Design of antigen-ionophore-based membrane electrodes for potentiometric antibody measurements.
Wuthier et al.	(1984)	Introduction of tin-organic compounds as neutral carriers for anion-selective electrodes.
Lindner et al.	(1984) [79]	Neutral-carrier-based membrane electrodes with selectivity for Pb^{2+}.
Sibbald et al.	(1984) [80]	Four-function ion-selective field effect transistor for simultaneous on-line measurements of blood K^+, Ca^{2+}, Na^+, and pH.

[103], **26** [104]), Tl^+ (**18** [105], **21** [106], **23** [107]), Mg^{2+} (**27** [108], **28** [108], **29** [71, 109]), Ca^{2+} (**30** [110, 111, 112], **31** [113], **32** [114]), Ba^{2+} (**33** [115], **34** [116]), Cd^{2+} (**35** [67]), Pb^{2+} (**36** [79]), Cu^{2+} (**37** [117]), UO_2^{2+} (**38** [63]) and Cl^- (**39** [78]).

It is interesting to see also that microelectrodes based on neutral carriers have found widespread practical application in physiology soon after their introduction (see Fig. 3).

OUTLOOK

Because there are attractive systems emerging for an assay of Mg^{2+} [108] and Li^+ [123] under clinically relevant conditions, potentiometric sensors for an assay of all the major cations of clinical interest will be soon at hand. Such highly cation-selective electrodes will displace flame photometry in clinical laboratories within a short time, because the use of such electrodes

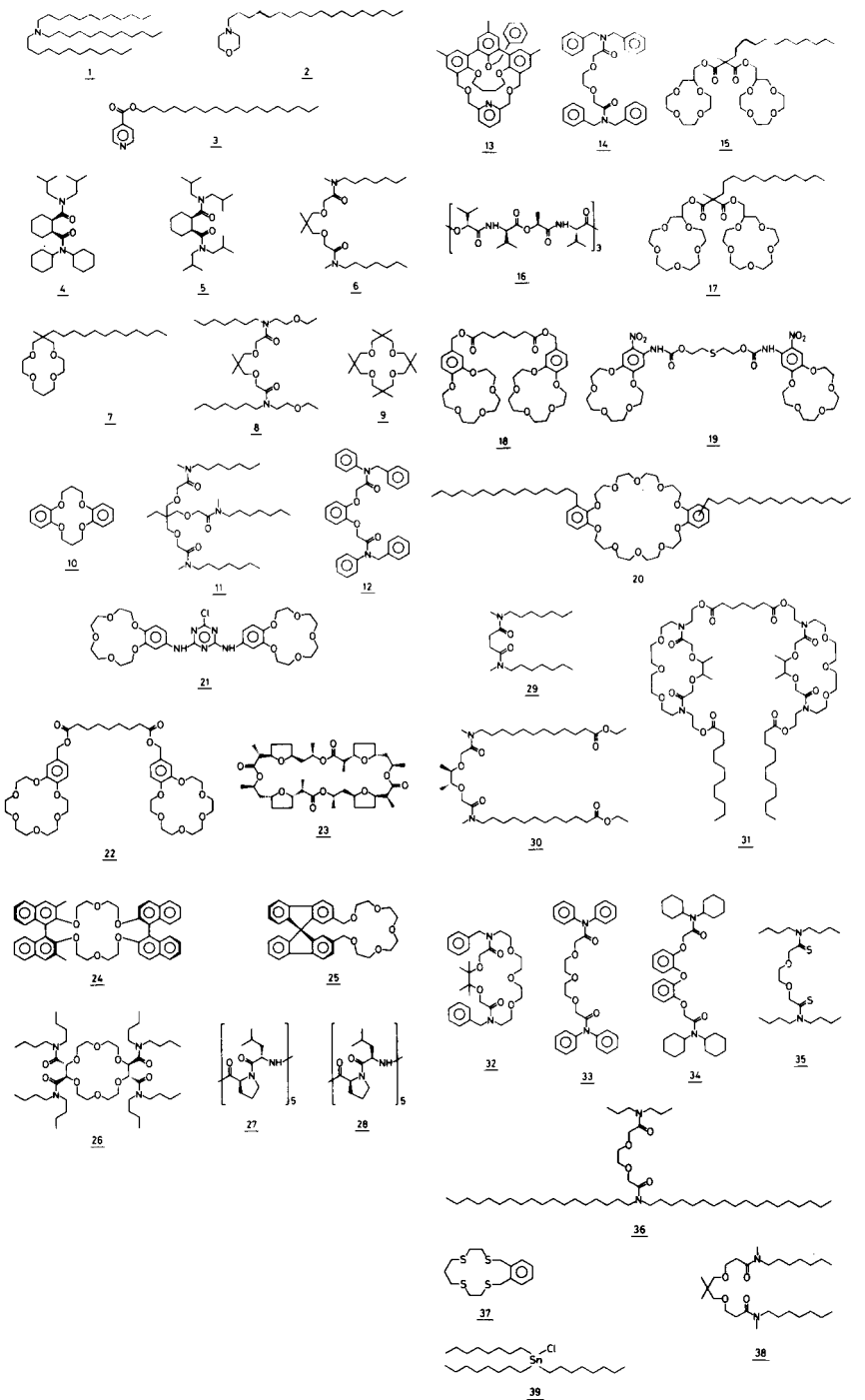


Fig. 2. Selection of proposed neutral carriers for ion-selective electrodes.

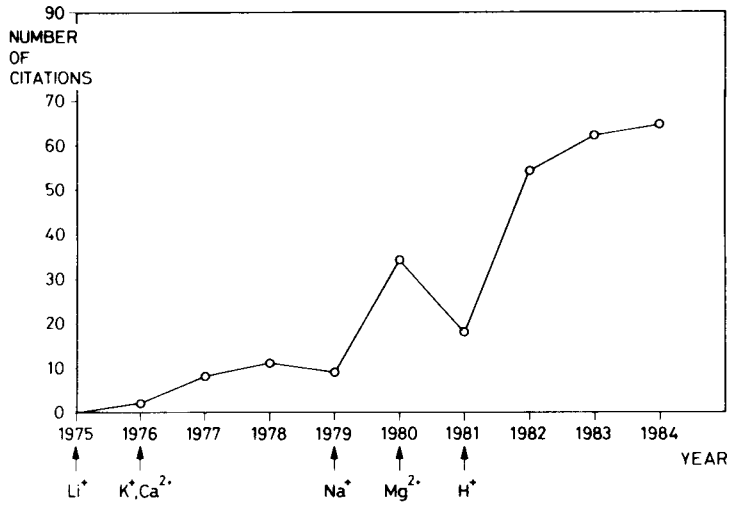


Fig. 3. Number of citations of the original publications from the authors' laboratory on various neutral-carrier-based microelectrodes as a function of the year of citation. The numbers of citations were evaluated by SCISEARCH, a computer-aided science citation search using a multidisciplinary index. Publications were considered only if experiments with neutral carrier microelectrodes were involved. The following literature was used for the search: H^+ [81]; Li^+ [53]; Na^+ [90]; K^+ [96]; Mg^{2+} [71]; Ca^{2+} [110, 111, 118, 119, 120]; reviews [1, 92, 121, 122].

is safe and free of sample preparation and sample destruction. Furthermore, they will eventually allow continuous in vivo monitoring of blood electrolytes on a bedside scale.

Neutral carriers for anions based on tin-organic compounds have been reported [78]. There is a prospect that such compounds will be suitable for sensors exhibiting different selectivity patterns.

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CRYSTALLOGRAPHIC STUDY ON METAL SULPHIDE-ACTIVE MATERIAL FOR CADMIUM ION-SELECTIVE ELECTRODES

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SUMMARY

Correlation between the electrochemical function of ion-selective electrodes made of differently prepared mixed sulphides and their crystalline structure was investigated. X-ray diffraction and x-ray photoelectron spectroscopy reveal recrystallization of CdS on Ag_2S grains during co-precipitation or co-milling. This study supports the idea that there is a strong correlation between the electrochemical function and crystalline properties of the electrode materials.

Different mixed metal sulphides are the active materials of ion-selective membranes essential for selective potentiometric determination of heavy metal ions, such as cadmium, lead and silver. These mixed sulphide-based active materials have to be prepared under rigorously controlled conditions. To date, precipitation or mixing of membrane components was followed by special high temperature treatment before, during, or after pressing (Table 1). The electrochemical functioning of membranes was attributed to material properties developed during this heat treatment. The authors however, are of different opinions concerning the properties of the materials necessary for proper electrode function.

Crystallographic structure seems to influence the electrode properties. Structural investigations were reported by several authors. For the CdS-Ag_2S system, van der Linden and Oostervink [1] detected the presence of acanthite (Ag_2S) and greenockite (CdS) both in the precipitate and pellet, using x-ray diffraction methods. The difference found between the diffraction patterns of these two samples was an increase in the intensity of CdS lines with respect to the Ag_2S lines as a result of hot pressing. They explained this phenomenon by orientation changes of CdS crystallites or by the decrease of crystallinity of Ag_2S during the phase transformation from acanthite to

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TABLE 1

Survey on the preparation conditions of CdS-Ag_2S-based cadmium ion selective electrodes

Before pressing		During pressing			After pressing		Ref.
Temp. (°C)	Time (h)	Press (MPa)	Time (h)	Temp. (°C)	Temp. (°C)	Time (h)	
—	—	980	2	150	—	—	[1]
600	3 (in H_2S)	—	—	—	—	—	[2]
—	—	750	15—20	150	—	—	[3]
—	—	800	3	150	—	—	[4]
—	—	300—700	1—3	200—500	—	—	[5]
—	—	500—1500	1—3	300—600	—	—	[6]
—	—	—	2—8	100—200	—	—	[7]
—	—	1500	3 min	—	150	72	[8]
—	—	—	—	—	300	3 (in N_2)	[9]
1200 (in vac.)	—	—	—	—	—	—	[10]
—	—	1500	2.5 min	—	—	—	[11]

argentite and vice versa, or both. This phenomenon, however, was not observed by Mascini and Liberti [2]. According to other investigations [4] Ag_2S has an acanthite structure, while CdS has a greenockite structure in the mixed precipitate powder as in the membrane.

Recently Ikeda et al. [12, 13] failed to find any response to Cd^{2+} from pure CdS or Ag_2S, while CdS activated by 1.6—90 mol% Ag_2S gave a good potential response. The best results were found when using a membrane with composition of 40 mol% Ag_2S and 60 mol% CdS. The surface state of the membrane and the structure of the membrane material were investigated and no evidence for the formation of a new phase in the CdS-Ag_2S system was found. Methods of preparing ion-selective electrode materials without the use of high temperature heat treatment are of great technical interest. Two such methods were developed recently and used for preparing CdS-Ag_2S mixtures [11].

The purpose of the present investigation was to study the correlation between the electrochemical properties of the electrodes pressed from co-precipitated or mixed-and-milled materials of different stoichiometries and their structures determined by x-ray diffraction methods.

EXPERIMENTAL

Precipitation of ion-selective membrane material

Two methods were used for the precipitation of the starting materials of the electrodes.

Procedure 1 (co-precipitated material). A 0.05 M Na_2S solution was titrated with a mixture of $Cd(NO_3)_2$ and $AgNO_3$ to the chemical equivalence point [11]. The precipitates were decanted, washed, filtered and dried under controlled conditions. The mole ratios of the starting nitrates were varied between 3:1 and 1:12.

Procedure 2 (mixed-and-milled samples). The membrane components were prepared individually as described in Procedure 1. Their mixtures of appropriate mole fractions were homogenized in a Fritsch-mill for 1—20 h. Pellets were prepared from each material by pressing 1 g at 1.5×10^3 M Pa for 2.5 min at room temperature.

Measuring techniques

Potentiometric measurements. Electrode potentials were measured in all cases in a potentiometric cell:

$$\underbrace{\text{Ag, AgCl}\,|\,0.1\text{ M KCl}\,\|\,0.1\text{ M KNO}_3}_{\text{Reference electrode}}\,\|\,\underbrace{\text{Standard-solution}\,|\,\text{Cd ion-selective membrane}\,|\,5\times10^{-3}\text{ M CdCl}_2\,|\,\text{AgCl, Ag}}_{\text{Cd}-\text{ISE}}$$

by precision digital pH-meter (Radelkis OP-208). The reference electrode was a double junction Ag, AgCl (0.1 M KCl) electrode (Radelkis OP-821) immersed in 0.1 M KNO_3. As standard solutions 10^{-2}—10^{-5} M $Cd(NO_3)_2$

were used; the ionic strength was adjusted to 0.1 by adding the appropriate amount of KNO_3.

Structural investigations. X-ray diffraction patterns of the precipitated and mixed-and-milled samples were taken in a Guinier-de-Wolff-type focussing camera, using CuK_α radiation. Exposures were taken on Geveart D7 or CEA Reflex 25 x-ray films.

Surface analytical investigations were done by x-ray photoelectron spectrometry (Kratos XSAM 800) and x-ray fluorescence spectrometry (Kevex system 700).

RESULTS

Structural properties of precipitates

CdS. All CdS samples precipitated by the method described above were quasi-amorphous according to the x-ray diffraction investigations. On the x-ray pattern shown in Fig. 1a very diffuse reflections of β-CdS (cubic) appear. No changes in the diffraction pattern were observed after pressing the material into pellets. On the contrary, milling caused a pronounced change of the x-ray pattern (Fig. 1b). The diffraction maxima remain diffuse, but the half-width is decreased, indicating an increase in the crystallite size. Together with the decrease in the half-width of the strongest CdS line, diffuse but well resolved maxima are visible at the angles of the (100) and (101) reflections of the hexagonal CdS crystal, shown for comparison in Fig. 1c. An estimation of the crystallite size, Λ, from all lines yields for Fig. 1a, $\Lambda \simeq 30$ Å, Fig. 1b $\Lambda \simeq 70$ Å and Fig. 1c $\Lambda \simeq 1000$ Å.

Ag_2S. Silver sulphide is crystalline in the precipitated state as seen on the x-ray pattern in Fig. 2a. The measured positions and intensities of the diffraction line system correspond to that of argentite. Milling did not significantly alter these data (Fig. 2b), but pellet pressing resulted in an increase of the widths of the lines (Fig. 2c). The small changes in the intensity distribution indicate a partial change in the structure from argentite to acanthite.

CdS-Ag_2S co-precipitated samples. Co-precipitated samples with mole fractions of CdS/Ag_2S of 6:1, 4:1, 2:1, 1.5:1, 1:1, 1:2 and 1:6 were prepared according to the method described above. On the x-ray pattern of the samples the diffraction line system of Ag_2S is present together with the lines

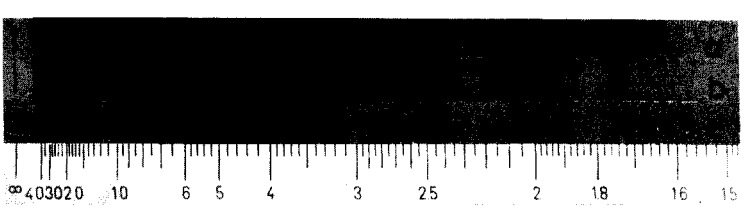


Fig. 1. X-ray patterns of CdS: (a) precipitated; (b) precipitated, milled for 24 h; (c) reference powder sample of hexagonal CdS.

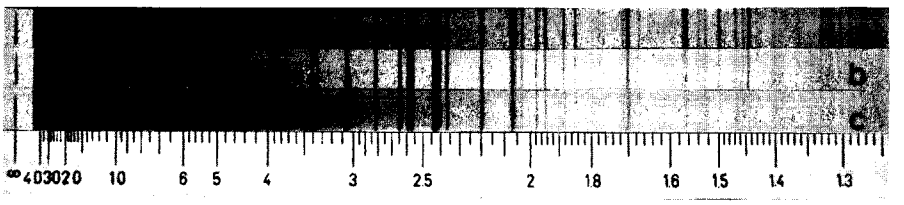


Fig. 2. X-ray patterns of Ag_2S: (a) precipitated; (b) milled for 12 h; (c) pressed for pellet.

of hexagonal CdS (Fig. 3). As can be seen from a comparison of Figs. 1, 2 and 3, co-precipitation results in much sharper CdS lines and slightly wider Ag_2S lines.

Effect of milling on structural properties

The various mixtures of CdS and Ag_2S samples precipitated separately show interesting changes when milled in a Fritsch mill. The precipitated pure CdS is quasi-amorphous, giving very diffuse diffraction lines; these lines were still diffuse even after 24 h of milling. Separately precipitated CdS when milled together with Ag_2S became more crystalline after a rather short period of milling.

The degree of crystallinity, indicated by the decrease in line-width corresponding to the increase in crystallite size, depends on the CdS/Ag_2S ratio. In the diffraction pattern from the sample mixed in mole ratio 6:1 of the separately precipitated CdS and Ag_2S and milled for 10 h, the diffraction lines of CdS were much more diffuse than in the pattern of the co-precipitated sample of the same composition (the half-widths of the CdS (100), (002) and (101) lines were considerably larger in the first instance). However, for samples of composition 1:2 mole ratio $CdS:Ag_2S$, the half-widths of the two constituents became comparable regardless of the origin of the

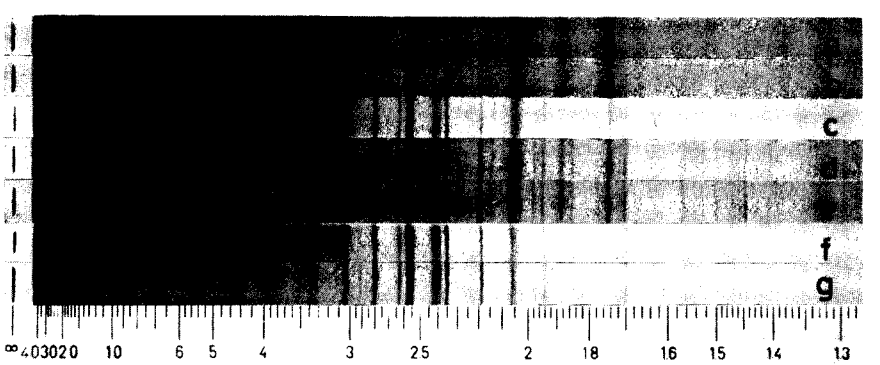


Fig. 3. X-ray patterns of co-precipitated CdS/Ag_2S of mole fractions: (a) 6:1; (b) 4:1; (c) 2:1; (d) 1.5:1; (e) 1:1; (f) 1:2; (g) 1:6.

sample. In all cases milling remarkably decreased the width of the CdS lines and at the same time slightly increased the widths of the originally very sharp Ag_2S lines.

For the sample with composition 1:2 CdS:Ag_2S (which is very important practically) the effect of milling time on the structure of the sample was also investigated. After milling for 1—9 h, the x-ray patterns of the samples showed sharp, well-defined diffraction lines characteristic of materials of crystallite size of a few μm. After $\geqslant$ 10 h of milling, however, the half-width of the lines began to increase, indicating a decreasing crystallite size (Fig. 4). This effect showed a correlation with the quality of the electrodes made from the corresponding material (Table 2).

The effect of initial crystallinity of the constituents on the final structure and electrochemical behaviour of the milled mixtures was also investigated. Mixtures of crystalline CdS and precipitated Ag_2S and of separately precipitated (quasi-amorphous) CdS and Ag_2S were milled for 10 min and 12 h, respectively. According to the x-ray patterns, the specimens did not differ after 12 h of milling.

Correlation between structure and electrochemical properties

The electrochemical properties of electrodes pressed from separately precipitated, co-precipitated and mixed-and-milled materials were measured in the electrochemical cell described above. According to the measurements, membranes prepared of separately precipitated Ag_2S or CdS did not show any response to cadmium ions. This is in accordance with earlier data for CdS or Ag_2S [12, 13] prepared by different methods. Electrochemical data of membranes made of a co-precipitated CdS-Ag_2S membrane of mole ratio 1:2 gave the greatest response and stability.

Table 2 gives electrochemical data for CdS-Ag_2S-based electrodes made from the appropriate mixture of separately precipitated or commercial substances after milling for 0.15—20 h, together with data for the best co-precipitated sample. It is evident from this Table that milling leads to an appropriate crystalline structure necessary for good electrochemical properties. Extended milling, however, spoils the good electrochemical properties,

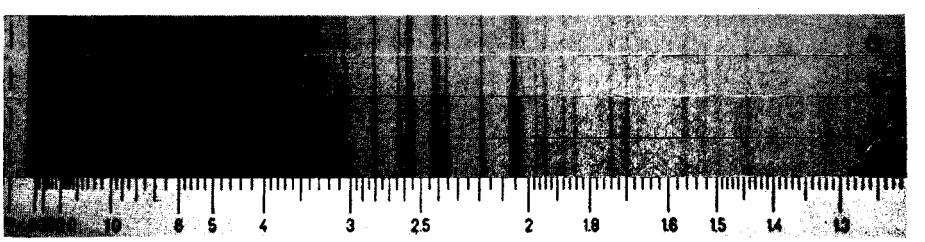


Fig. 4. Effect of milling time on the structure of samples mixed from separately precipitated CdS and Ag_2S in mole ratio 1:2. Diffraction patterns taken after milling times of: (1) 1 h; (b) 3 h; (c) 12 h; (d) 20 h.

TABLE 2

Electrochemical data for CdS-Ag_2S (1:2 mole ratio) based ion-selective electrodes developed in the authors' laboratory

No.[a]	Time of homogenization (h)	$S(^2E_{60} - {}^3E_{60})$[b] (mV/decade)	Stability ($^3E_{180} - {}^3E_{90}$)[c] (mV)	Remarks
1	0.15	4.0	4.0	
2	0.15	4.0	2.0	CdS crystalline
3	0.5	6.5	3.1	
4	1	11.1	2.3	
5	3	15.2	2.8	
6	5	21.0	1.5	
7	6	24.5	2.5	
8	12	28.5	0.4	
9	12	29.3	0.3	CdS crystalline
10	20	27.5	1.1	
11	—	29.5	0.2	

[a]CdS-Ag_2S samples 1—10 were prepared by mixing individually precipitated CdS and Ag_2S (1.156 g CdS and 3.9623 g Ag_2S). Sample 11 was co-precipitated CdS-Ag_2S, 1:2 mole ratio. [b]S is the slope of the calibration graph determined on the basis of the potential data measured in 10^{-2} — 10^{-3} M Cd^{2+} solutions. $^2E_{60}$ e.m.f. measured in 10^{-2} M $Cd(NO_3)_2$ (I = 0.1) 60 s after immersion; $^3E_{60}$ e.m.f. measured in 10^{-3} M $Cd(NO_3)_2$ (I = 0.1) 60 s after immersion. [c]Stability is defined as the potential difference measured in 10^{-3} M Cd^{2+} solution (I = 0.1) 1.5 and 3 min after immersion. $^3E_{90}$ e.m.f. measured in 10^{-3} M $Cd(NO_3)_2$ (I = 0.1) 90 s after immersion; $^3E_{180}$ e.m.f. measured in 10^{-3} M $Cd(NO_3)_2$ (I = 0.1) 180 s after immersion.

correlating with the increase of the half-width of the diffraction lines of the material.

X-ray photoelectron spectrometric investigations

A comparison of the x-ray diffraction patterns of precipitated (Fig. 1) and co-precipitated CdS (Fig. 3) indicates an improved growth of CdS crystals in the presence of Ag_2S. The Ag_2S crystals act as seeds for the crystallisation of CdS. A similar result has been found for milled mixtures of separately precipitated CdS and Ag_2S. An increasing Ag_2S content causes the formation of a greater amount of large CdS crystals. Thus, CdS grows epitactically on Ag_2S-crystals. When this model holds, parts of the surface of the Ag_2S crystals must be covered by CdS after co-precipitation as well as after milling.

Using a surface analytical technique like x-ray photoelectron spectroscopy (x.p.s.) these conditions can be proved. As Ag_2S-crystals are partially covered with CdS, x.p.s. can be used because of the extremely small depth (ca. 10 Å) from which information is obtained, by an increasing cadmium and a decreas-

ing silver signal. Figure 5 gives the results of x.p.s. investigations of a mixture of individually prepared CdS and Ag_2S (1:2 mole ratio) after different milling times. For the unmilled mixture, the spectrum shows a weaker cadmium signal and a stronger silver signal. Milling for 0.5 h did not change this Cd/Ag ratio, whereas, milling for 12 h caused a pronounced increase in the cadmium signal. When milling is continued for 20 h the silver signal again becomes higher than the cadmium signal.

The x.p.s. results are in good agreement with the x-ray diffraction patterns which show, for an increased milling time, first a decreasing line width (increasing CdS crystallite size) followed by an increase of the line width. Comparing the x.p.s. results to the corresponding electrode functions (Table 2) also shows good agreement. Besides, Table 2 gives for unmilled co-precipitated electrode material (sample 11) electrochemical results which are as good as the electrochemical properties of CdS-Ag_2S (1:2) mixed-and-milled for 12 h.

An x.p.s.-spectrum of co-precipitated CdS-Ag_2S (1:2) is also given in Fig. 5. It can be seen that the ratios of cadmium and silver signals of the co-precipitated sample and the 1:2 mixed sample milled for 12 h are similar.

To confirm the x.p.s. results, all specimens were analyzed by x-ray fluorescence (x r.f.). As this method obtains information from $\leqslant 50\ \mu m$ depth, the

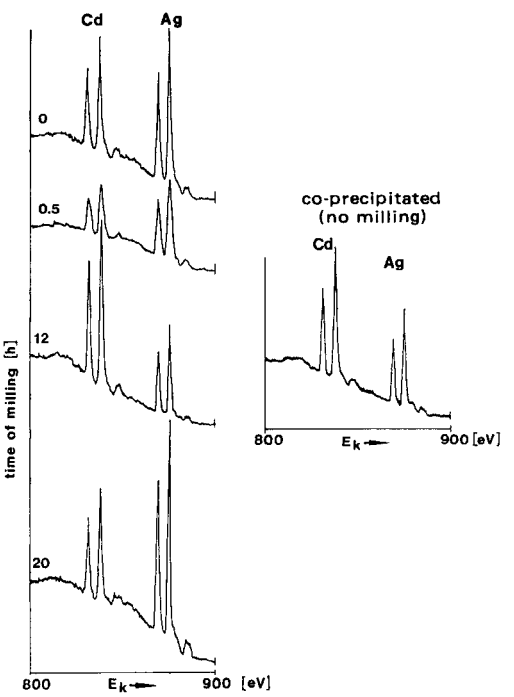


Fig. 5. Dependence of x.p.s. results (MgK_α radiation, Cd_3d- and Ag_3d-spectra) of a CdS/Ag_2S (1:2) mixture on the time of milling, and of co-precipitated CdS/Ag_2S (1:2).

results are obtained from the bulk material and therefore CdS coverage of the Ag_2S crystals has little influence on the bulk composition. All samples gave identical characteristic AgK_α and CdK_α signals which proves an identical bulk composition of the specimens investigated by x.p.s.

CONCLUSIONS

Correlated diffraction and electrochemical investigations on co-precipitated and mixed-and-milled CdS-Ag_2S electrode materials has shown that the optimal electrochemical function of the relevant electrodes can be attributed to a certain crystalline state of the samples. According to the electrochemical results, the samples with molar ratios CdS:Ag_2S = 1:2 were the best as electrode materials among the co-precipitated samples studied. CdS-Ag_2S electrode materials prepared by the mixing of separately precipitated or crystalline samples in the above ratio exhibited the same electrochemical response in solid membrane electrodes only after milling for an appropriate period of time. This suggests that the electrochemical properties of solid ion-selective electrodes made with the use of differently prepared mixed sulphide electrode materials strongly correlate with the crystalline structure of these materials as indicated by x-ray diffraction patterns. According to x.p.s. investigations, both co-precipitation or co-milling result in the coverage of Ag_2S with CdS crystallites.

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CHEMICALLY-SENSITIVE FIELD-EFFECT TRANSISTORS

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Development of integrated solid state electronic circuits has affected deeply all areas of analytical chemistry, mainly in data acquisition and data processing, resulting in faster, more flexible and more compact instrumentation. Chemically-sensitive field-effect transistors (CHEMFETs) are representative of future development in this direction, i.e., miniaturization. They are not the only devices in which chemistry and biology meets solid state electronics and they are closely related to another type of electroanalytical device, ion-selective electrodes, which are discussed elsewhere in this issue.

In direct sensing there are two steps: selective recognition, which is usually done by the chemical membrane, and amplification which in most cases is done electronically. Thus, in an ion-selective electrode, the membrane containing a highly specific ionophore produces a weak electrical signal in response to the change of activity of a specific ion. This signal is amplified by a remote voltmeter. In CHEMFETs these two functions are combined on one silicon chip (Fig. 1). Although some real advantages as well as real problems result from this integration, CHEMFETs are not fundamentally different from ion-selective electrodes [1]. The same parallel holds for potentiometric enzyme electrodes and enzyme-selective field-effect transistors (ENFETs).

The only CHEMFET which does not have its counterpart in a macroelectrode is a transistor in which the analytical signal is derived from the change of electron work function of a chemically sensitive layer. The oldest and most studied device of this type is a palladium MOSFET in which the work function of the palladium gate metal changes as the result of interaction with hydrogen [2]. In principle, other chemically sensitive layers could be used instead of palladium in a similar configuration. However, the problem is that examples of a gas penetrating inside the gate structure are very rare. A transistor structure in which the gate metal is suspended above the gate insulator, thus forming a gap, is a more general version of this type of sensor (Fig. 2). In this case the fluid sample can penetrate inside the gate gap and interact with the electric field [3]. The selectivity is achieved by chemical modification of the gap surfaces which can conveniently be done by electrochemical deposition of organic or inorganic layers [4]. This type of sensor cannot be

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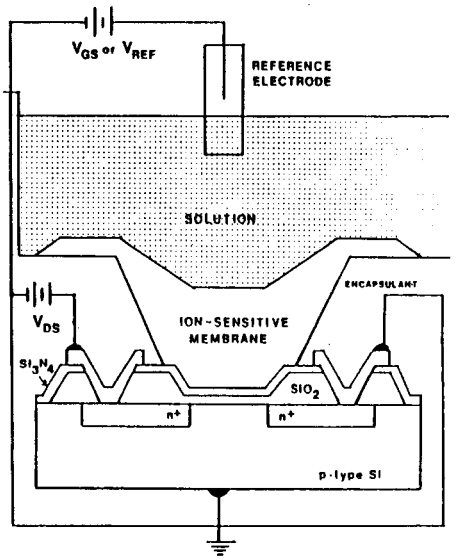


Fig. 1. Schematic diagram of ion-sensitive field-effect transistor.

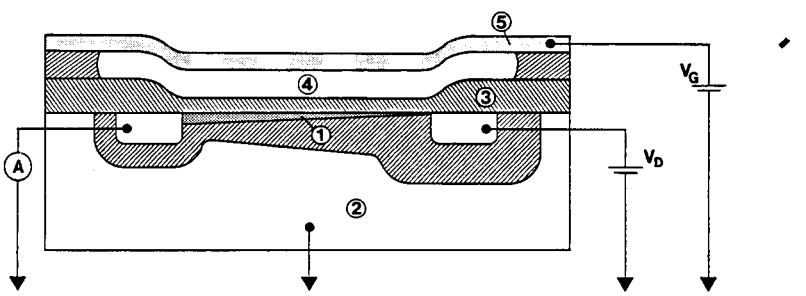


Fig. 2. Suspended-gate field-effect transistor: (1) channel; (2) substrate; (3) insulator; (4) gate gap; (5) suspended platinum gate.

constructed, in principle, in a macroelectrode configuration [1]. Thus, the work function transistors represent a truly new type of analytical probe.

Other advantages obtained from the integration of solid state electronics and electrochemistry are mainly of a practical nature. Perhaps the most significant is the possibility of building miniature multisensors. Several attempts along this line have already been attempted [5—7]. The main problems in realizing the full potential of multisensors center around encapsulation and membrane separation. Once these problems are solved the full power of statistical data processing will be available, possibly on the same chip. This blurring of the line between signal acquisition and data processing will signal a new approach to chemical sensing [8].

The main area of application of CHEMFETs seems to be in biomedical monitoring where the necessity usually dictates the use of small probes and where only small amounts of samples are usually available [6, 9].

The development of solid state chemical sensors is truly an interdisciplinary activity. These devices were introduced by electrical engineers and were further developed with the help of electroanalytical chemists. The most pressing problems at present seem to lie in the area of materials science.

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DEVELOPMENT AND APPLICATION OF COMPUTERIZED BATTERY-POWERED FIELD PROGRAMMABLE DATA ACQUISITION SYSTEMS

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SUMMARY

A computer-based, battery powered, data acquisition system is described, which forms part of a field-based chemical system for use with sensors generating a voltage. The equipment has been developed using complementary metal oxide semiconductor (CMOS) "low power" integrated circuit technology and is based on a multiplexed bus. One 2k-byte erasable programmable read only memory (EPROM) controls the data acquisition process. Another 2k-byte EPROM contains the monitor program, while a 2k-byte random access memory stores the data before and after manipulation. A microprocessor unit provides all the control signals used by the instrument and data are collected on a real-time basis. The electronics are housed in a self-contained box to which the sensors and an independent keyboard can be connected. The instrument can be linked with as many as eight sensors. With the use of battery-operated CMOS technology, the measurement noise level is low and analytical techniques can be used to determine low concentrations of variables in the field. The viability of the instrument has been demonstrated by obtaining extensive data on the measurement of temperature and pH. Field-based potentiometric titrations, ion-selective electrode applications and studies on battery life are also reported.

The increasing demand in chemical industry for in situ or on-line analytical data has accelerated research into the development of automated methods of chemical analysis. The requirement is to produce immediately useful information rather than a record of data of historical value only. In addition, the present emphasis on chemical monitoring of the environment has further stimulated investigations into stand-alone field-based instruments which include data storage, manipulation and transfer capabilities. Both these pressures have produced a demand for effective instruments which can measure variables in an isolated situation, be it in industry or in the environment [1—5].

An even more recent trend, requiring instantaneous results from isolated monitoring stations, has introduced a new concept into analytical chemistry, the stand-alone battery-powered chemical field laboratory. In oceanography and meteorology, microcomputer-controlled stand-alone field devices [6] are available for the measurement and recording of physical parameters such

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as temperature, wind velocity, tidal flow and barometric pressure. In contrast, there is very little computer-based chemical monitoring equipment which has a long autonomous period of operation without operator intervention and which can monitor a wide range of parameters. At present, instrumentation exists which can be used to monitor chemical species in the field but operator support is required. For example, a portable digital voltammeter [7] designed by the Commonwealth Scientific and Industrial Research Organisation, Division of Mineralogy and Geochemistry, Australia and manufactured by Chemtronics, has value as a field laboratory but requires extensive operator attendance. "The Squirrel" from Grant Instruments [8] has the capability of measuring a wide range of variables. However, these instruments are clearly not autonomous. In addition, neither has any computer-managed chemical control to annul the effects of interferences. The latter is an essential aspect for monitoring chemical species in a field situation as chemical sensors are rarely specific.

In order to meet the challenges peculiar to field-based instrumentation, new developments in suitable sensing devices and assemblies [9, 10], data processing systems [11] and chemical pretreatment procedures [12] need to be combined in a systematic fashion. For completely autonomous operation, the sensing and data acquisition systems available to the analytical chemist ideally should have the ability to perform on-line monitoring with the capability to warn users of dangerous or undesirable chemical levels or chemical events in the environment or industrial stream and an ability to collect, manipulate and store or relay data on a long term basis, with minimal operator intervention.

The main difficulty in the development of battery-operated, computer-based data acquisition systems which can be used in the field has been the relatively high power consumption of the available semiconductor devices. The latest step in this technological development has been the advent of CMOS (complementary metal oxide semiconductor) microprocessor and related technology. If these devices are used for data acquisition systems, truly low power consumption and operation via battery-based power supplies becomes a reality. For example, a conventional microprocessor-based data acquisition system, built from a Motorola MEK 6800 D2 "kit" of the kind described elsewhere [13] has a power requirement of ca. 40 W, whereas the field instrument discussed in this paper dissipates less than 250 mW.

In addition to energy saving, another advantage in using battery powered technology in the construction of monitoring equipment is the removal of noise originating from the mains frequency of a mains-based power supply. As a result of the improved signal-to-noise ratio, more sensitive analytical techniques can be developed. For example, the use of chromatography with battery-based electrochemical detection provides improved performance over conventional approaches based on generating a d.c. potential and detecting current via a mains operated device [14].

In the present paper an example of a suitable battery-powered computer-based data acquisition system is described which can be used with electrochemical sensors. Based on CMOS technology, this device offers scope for numerous applications of analytical procedures away from the confines of the chemical laboratory.

EXPERIMENTAL

Chemicals

All chemicals used were of analytical reagent grade. Standard pH solutions were prepared from pHydrion Buffers (Micro Essential Laboratory Inc., Brooklyn, NY).

Instrumentation

The sensing devices used in conjunction with the Field Data Acquisition System (FDAS) described in this article were readily available standard items. The pH probes were an Orion Research Ross Combination pH electrode Model 81-02 or a Sensorex (Westminster, California, U.S.A.) combination pH electrode, Model 120C. The ion-selective electrode was an Orion copper-(II) electrode, Model 94-29, used in conjunction with a Ag/AgCl (saturated KCl) Orion double-junction reference electrode, Model 90-02, in which the outer chamber contained 2 M sulphuric acid.

An Inter Ocean Systems Model 513A Probe used with a Model 514A Readout which can monitor conductivity and an Orion Research Model 701A Digital Ion Analyzer were both used in conjunction with the FDAS as primary sensors and to provide a basis for comparison.

The temperature sensors of the Field Instrument were constructed with either a semi-conductor type Analog Devices AD 590 device encapsulated in I.C.I. tensol-7-acrylic or an alumel-chromel IDA Type K thermocouple (S.I.R.S. Sales, Geelong, Australia). Reference temperature measurements were made with a Dobros precision thermometer No. 17261 (Australia).

Batteries were either standard alkaline D cells or an Exide RE 12-6, 12V, 6AH recombination electrolyte battery.

The field data acquisition system. The following design objectives were deemed to be essential for a battery powered field data acquisition system which would be used with electrochemical sensors.

(i) State of the art "low power" CMOS technology to conserve the power source.
(ii) Multiple input channels to give the user considerable flexibility in monitoring a variety of parameters with passive sensors.
(iii) A simple, cheap and, above all, reliable method of data recording.
(iv) A portable keyboard for field programming and a display unit to give the user meaningful visual information when inspecting the field installation.
(v) An hermetically sealed metal box for the instrument power source and data recorder to provide suitable weather protection.

(vi) A solar cell support system to maximise power efficiency and extend battery life.
(vii) Firmware monitoring software that prompts the operator regarding the state of the instrument and the batteries.

With the above objectives in mind the data acquisition system described below was developed.

General design features of the CMOS-based field data acquisition system

The FDAS constructed in this project uses CMOS integrated circuits exclusively and is based on a multiplexed (address then data) bus. Fig. 1 is a block diagram of the FDAS showing the major components which are:

(i) An 8-bit microprocessor unit (MPU) Motorola type MC146805E2P which has the primary function of providing all the control signals used throughout the instrument. A secondary function is to indirectly interface the "outside world" via 16 Input/Output (I/O) lines to the analog-to-digital converter (ADC), the input multiplexor (MUX) and the input modules from the sensors.
(ii) A real time clock (RTC) operates while battery power exists and allows data acquisition to occur on a real time basis. In addition, the clock can

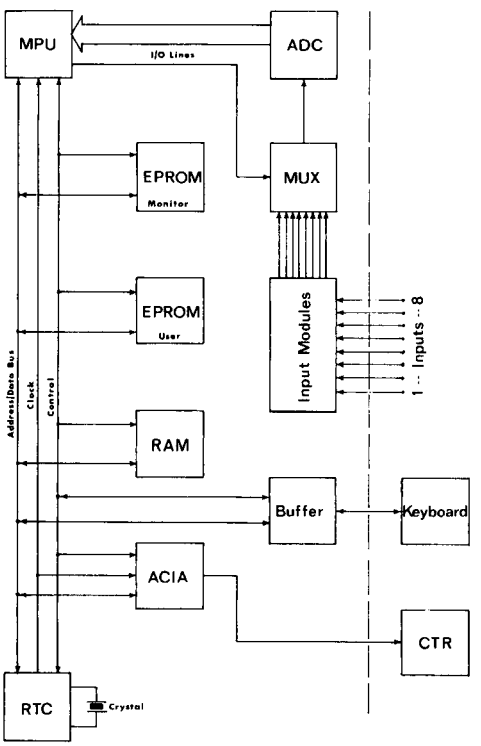


Fig. 1. Block diagram showing the major components of the FDAS.

be set or adjusted using the keyboard. Obviously, with this device, the data record can be precisely labelled with time, day and date.

(iii) A firmware monitor program is contained in a 2048 × 8 bit erasable programmable read-only memory (EPROM). This software controls the scanning of the keyboard and display and contains all permanent routines that may be regularly useful to the user.

(iv) User software in a second 2048 × 8 bit EPROM. This software is user-generated to control the data acquisition process for individual input modules and conditions. The FDAS collects data at user-specified intervals, averages a selected number of data values and processes the results according to the user's requirements.

(v) A 2048 × 8 bit random access memory (RAM) type 6116 for storage of data before manipulation, and for storage of data prior to transmission to the cassette tape recorder (CTR).

(vi) An asynchronous communications interface adapter (ACIA) type 65SC51 for use in controlling serial data communication with the CTR which is to be used to record data in the field.

(vii) An ADC Intersil type ADHC12B, which converts the input analog signal selected by the input multiplexor to a 12-bit parallel format for use by the system.

(viii) Various signal conditioning input modules which condition the analog signals from the sensors to voltage levels suitable for the multiplexor which selects inputs under software control for the ADC.

(ix) The keyboard and display combines a 20-key water-resistant key pad with a 32-character liquid crystal display (LCD). This type of display allows the firmware or user software to show meaningful prompts or data to the operator.

(x) The CTR is an inexpensive National model RQ2309 modified to employ a zero-crossing recording technique at 1200 baud (bits s^{-1}). This format allows approximately 48 kbytes of data to be recorded on a 60-min cassette tape.

Circuit diagram of the instrument

Figure 2 details the circuitry and components used in the instrument.

Hardware. The FDAS makes extensive use of modern CMOS technology in order to minimize power consumption. The first requirement was that the power supply consumed negligible standby power. Perusal of the circuit diagram in Fig. 2 shows that by using the base-emitter junction of a 2N3641 transistor [15] as a reference this requirement was achieved. The standby current drawn by the power supply is of the order of 20 μA and yet acceptable load and line regulation is achieved.

The heart of the system is the MPU, a Motorola type MC146805E2P [16] which utilizes a multiplexed address then data bus (BO-7) thus minimizing the number of pins allocated to these two functions. This allows allocation of pins to I/O functions, in this instance 16 lines PAO-7 and PBO-7. Twelve

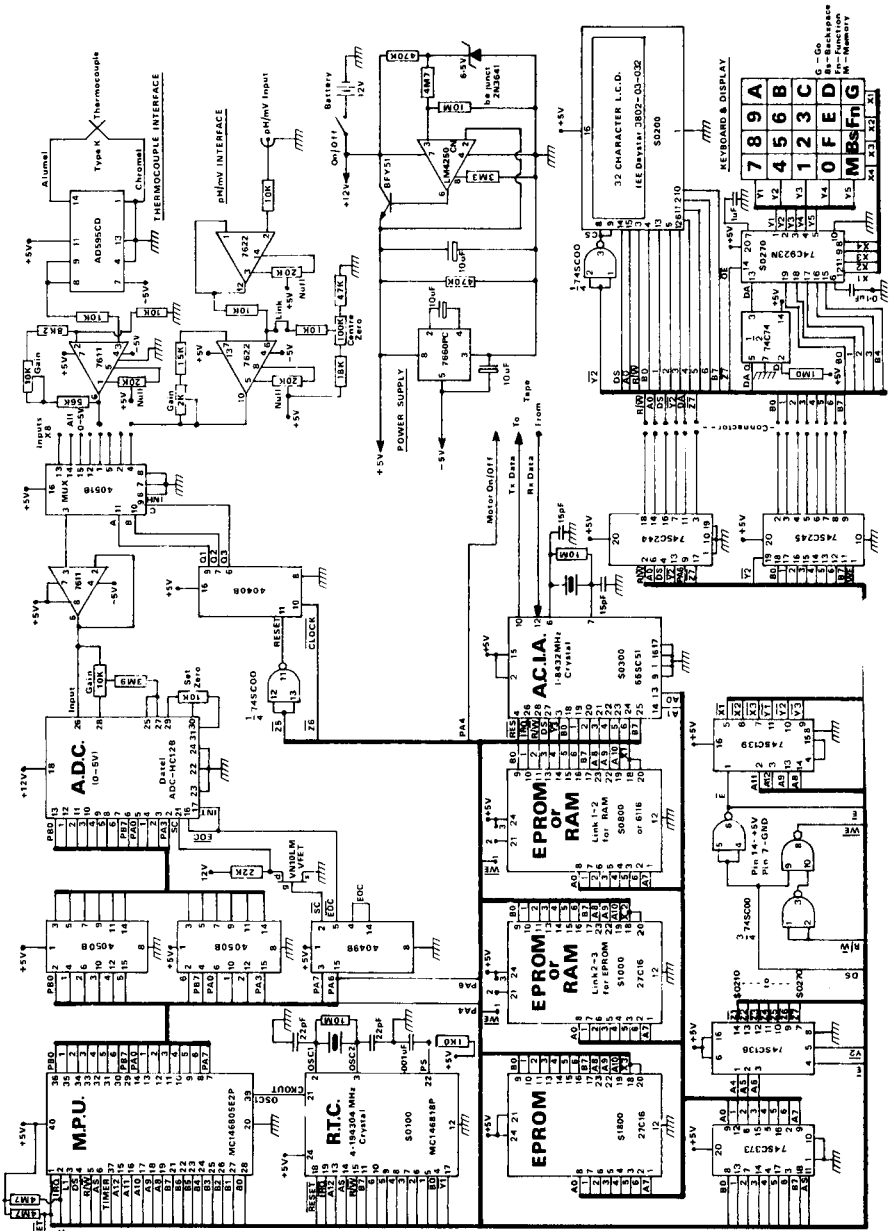


Fig. 2. Circuit diagram of the FDAS.

of these I/O lines connect via logic level translators (2 × 4050B) to the ADC type ADC-HC12B. This 12-bit device [17] is ideal in that it operates in the interrupt mode, hence it only consumes significant energy while actually doing a conversion. Between conversions, only standby power is used, which amounts to drawing ca. 100 μA from the supply.

Any one of eight unipolar 0–5 V inputs can be selected using the 4051B multiplexor (MUX) which drives the ADC via a high impedance buffer based on a 7611 CMOS operational amplifier. The input selection is controlled by the 4040B device which in turn is controlled by decoded chip select lines and appropriate software.

Timing for the whole system is provided by a MC146818P (16) real time clock (RTC) device operating at a clock frequency of 4.194304 MHz. As this device is a companion to the MPU it has the great advantages of being easy to implement into the system and being able to generate "real time" interrupts for the MPU to service, as required by the user. A much lower frequency crystal could be used thus decreasing power consumption, but clock accuracy was thought to be more important in this application.

Memory space, restricted to 8192 (8k) bytes, is allocated in the following way. The lower 2k page is taken up by the peripheral devices, viz. the MPU ports, keyboard, display and the ACIA. The remaining 6k is divided into three 2k pages for EPROM or RAM. The upper page will always be devoted to EPROM, as a monitor program of some sort will always be needed. The type of application will determine whether the other two 2k pages are allocated to EPROM or RAM, either of which can be selected via links on the printed circuit board. All remaining devices are devoted to keyboard buffering, latching and general address decoding.

Two interface modules are shown, namely, a thermocouple interface and a pH/mV interface. Both use CMOS devices and simple techniques to provide a unipolar 0 to 5 V signal as required by the MUX. The outputs of both interfaces are read and scaled by suitable software and the resultant data are stored either in RAM or on tape as required.

The keyboard and display provides the operator with meaningful messages via the 32-character LCD and the ability to control the whole process via hexadecimal (0 to F) keys and four control keys i.e., M (memory examine and change), BS (backspace), F(n) (function) and G (go and execute). Thus up to sixteen functions can be readily provided using the F(n) key and the hexadecimal keys in conjunction with appropriate software. For example, field programming of parameters, resetting the RTC or setting the time between samples becomes an easy task for an operator. One input channel could be devoted to periodical monitoring of the state of charge of the battery and warning the operator with a suitable message on the LCD.

Data storage has been accomplished by connecting a modified portable cassette tape recorder to the ACIA device, and appropriate software has been developed to store and read data at 1200 baud. This enables the storage of about 48kbytes on a standard C60 cassette. Internal modification of the tape

recorder enables the recording of the data in a zero-crossing format. The tape recorder motor and electronics are actuated and de-actuated at appropriate times by I/O line PA4 coming from the MPU.

Software. All software has been written at an assembler level using a cross assembler called CRASMB purchased from Frank Hogg Ltd. running under the FLEX 9 operating system in a South West Technical Products Corporation 6809 MPU system. The monitor program has been placed in the EPROM at address $1800 (hex.). This provides the usual monitor functions, which are best described by reference to the keyboard functions above and other useful functions such as the ability to set and read the RTC. Software for specific tasks has generally been programmed into a second EPROM at address $1000 (hex.). Examples of this software include sampling of the outputs of the interfaces described above and either storing or displaying the results provided by these modules.

Operation of the FDAS

The equipment can be used in a number of different operating modes.

Long term recording – time interval controlled. Large quantities of long-term data can be accumulated on the CTR. This information can be inspected later using manual or computerised methods. Although these data are of an historical nature, they can be used for the prediction of potentially harmful trends.

Immediate warning procedure in the field — event controlled. In the event of severe departures from normal levels of specific parameters, warnings can be issued via a suitable link from the remote sensing station to a central laboratory. Such links could be a data communication line or telemetry.

Immediate warning procedure in industry — event controlled. One analytical device which can be used in conjunction with the above monitoring system is a delta-shaped flow-through cell [12]. This is a high capacity device for use with any ion-selective electrode system and is particularly applicable if chemical treatment of the solution is necessary to eliminate interferences. When chemical interference is not a problem a simpler flow-through device can be used [4]. If these types of cells are incorporated with the FDAS and used in a process-variable measurement and control context, an operator warning can be given in the event of unacceptable levels of a given factor being sensed in the process circuit stream.

General use. In general, the capacity and versatility of the FDAS enables an operator to change any parameter being sensed by visiting the field station and using a separate keyboard to alter the software without interfering with the measurements. The inbuilt program of the system can be changed by simple replacement in the field.

RESULTS

Use of the FDAS with different sensors

Temperature. Comparisons were made between the semi-conductor type temperature sensor, the Inter-Ocean probe and a certificated precision thermometer. Some representative data obtained for the measurement of water temperature at intervals over an extended period of time are contained in Table 1. The average offset for the FDAS/precision thermometer comparison was 0.34° C and for the FDAS/Inter-Ocean probe was 0.09° C. The precision of the temperature measurement can be improved by using a K-type thermocouple and by powering the instrument with a battery which is less sensitive to variations in the ambient temperature than alkaline D cells. When the K-type thermocouple and a recombination electrolyte battery were used over a 45-h period, the 45 water bath temperature readings showed an average variation of 0.1° C lower than the set value of 28.0° C.

TABLE 1

Comparison of temperature readings with different devices obtained during a period of one week

Precision thermometer (° C)	Difference (° C)	F.D.A.S. semiconductor sensor (° C)	Difference (° C)	Inter-Ocean probe (° C)
8.6	0.2	8.4	0.2	8.2
8.6	0.3	8.3	0.1	8.2
8.9	0.7	8.2	−0.3	8.5
10.3	1.0	9.3	−0.5	9.8
10.4	0.7	9.7	−0.3	10.0
9.6	0.2	9.4	0.3	9.1
10.4	0.9	9.5	−0.4	9.9
12.1	0.6	11.5	−0.1	11.6
11.4	0.2	11.2	0.2	11.0
11.7	0.6	11.1	−0.2	11.3
11.9	0.7	11.2	−0.3	11.5
12.1	0.8	11.3	−0.4	11.7
12.1	0.7	11.4	−0.3	11.7
12.8	0.6	12.2	−0.2	12.4
13.0	0.6	12.4	−0.1	12.5
11.6	−0.2	11.8	0.7	11.1
11.7	0.7	11.0	−0.2	11.2
12.2	0.6	11.6	−0.2	11.8
11.8	−0.4	12.2	0.9	11.3
12.3	0.1	12.2	0.5	11.7
12.8	0.0	12.8	0.6	12.2
12.4	0.0	12.4	0.5	11.9
13.7	0.3	13.4	0.3	13.1
13.3	0.4	12.9	−0.1	13.0

pH. The mV readings obtained when a combination pH electrode was used with the instrument indicate sufficient accuracy and stability in comparison with an Orion 701A Digital Ionanalyzer system. Table 2 summarises data from the solutions of different pH values used in the comparison.

Potentiometric titrations. Figure 3 illustrates a potentiometric (pH) titration curve obtained with the FDAS system. The end-point is 18.0 ± 0.1 ml. The acid end-point was identical within the limit of experimental error to values obtained by using an Orion Research 701A Ionanalyzer.

Ion-selective electrodes. Calibration curves were obtained for the determination of copper(II) in a 2 M sulphuric acid solution typical of that obtained in a copper refinery [12], by using two completely different analytical systems, the FDAS and an Orion Research Model 701A Digital Ionanalyzer using different copper ion-selective electrode and reference electrodes. The Nernstian slope for the FDAS curve is 28.7 mV and for the Orion instrument is 29 mV. Each form of instrumentation gave the copper concentration of an unknown as 156 ± 1 μg ml^{-1} copper.

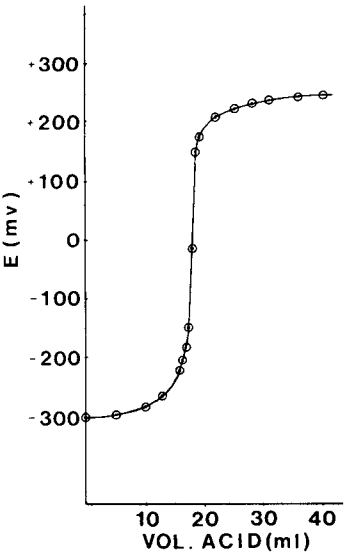
Simultaneous measurement of conductivity and temperature. The FDAS has the capability of measuring eight parameters simultaneously. To test the multiplexing capability of the instrument, the simultaneous measurement of conductivity and temperature of sea water was examined. The FDAS was coupled with existing commercially-available probes (Inter-Ocean systems equipment) for the measurement of conductivity. Temperature was measured as described above. The temperature and conductivity of a solution

TABLE 2

Comparison of the measured response at three different pH values obtained from the FDAS and an ORION 701A instrument interfaced to the FDAS

Time[a] (min)	Solution A pH 4.0, 20° C (mV)		Solution B pH 7.0, 22° C (mV)		Solution C pH 9.0, 24° C (mV)	
	FDAS[b]	ORION[c]	FDAS[b]	ORION[c]	FDAS[b]	ORION[c,d]
0	+162	+164	−12	−12	−129	−133
5	+162	+164	−12	−12	−129	−133
10	+162	+164	−12	−12	−129	−132
15	+161	+164	−12	−12	−129	−132
20	+162	+164	−12	−12	−129	−132
25	+162	+164	−13	−13	−129	−132
30	+163	+164	−12	−14	−128	−132
35	+163	+164	−13	−13	−128	−132
40	+163	+164	−12	−13	−128	−132
45	+163	+164	−12	−13	−128	−133
50	+163	+164	−12	−13	−128	−133

[a] Average of 10 readings taken at 1-ms intervals after stated time. [b] Sensorex electrode. [c] Ross electrode. [d] Offset of 4 mV compared with FDAS because of use of different electrodes.



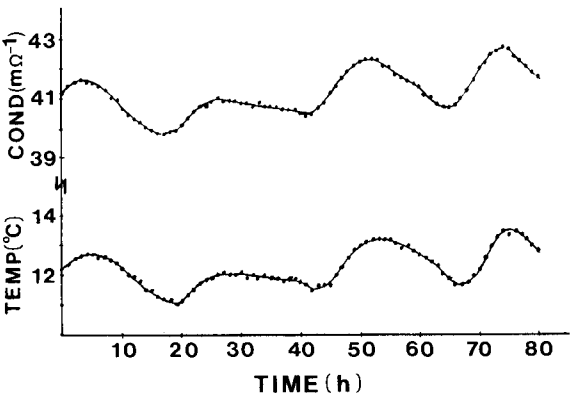


Fig. 3. Potentiometric titration curve obtained with the FDAS for neutralisation of a strong acid (HCl) with a strong base (NaOH) using a Ross combination pH electrode.

Fig. 4. Simultaneous monitoring of conductivity and temperature with the FDAS. Conductivity was measured using an Inter-Ocean probe with data collection on the FDAS. Temperature was measured directly on the FDAS using the semiconductor type sensor. The expected correlation between the two parameters can be observed.

were measured. Figure 4 shows the expected correlation of temperature and conductivity for sea water. The ability to simultaneously measure eight variables is a distinct advantage of the CMOS-based FDAS system.

Battery life. Alkaline cells were tested for suitability by continuously recording the temperature of the water in a thermostatted water bath set at 25.6° C by using the semiconductor temperature probe. This combination of probe and cell is the worst case encountered. When departures from the set value reached more than ±2° C, the batteries were described as unreliable. Under these limitations some alkaline batteries had a life of only one week. In addition this class of cells was clearly sensitive to the ambient temperature producing an apparent small lowering of temperature every 24 h when night temperatures were below 10° C. This fluctuation was within the ±2° C variation considered acceptable in these tests. Other sensors including the Type K thermocouple were far less susceptible to battery variability and could be used for periods well in excess of one week with far greater precision (a variation of <0.1° C). Other batteries have different power capabilities and lifetimes and a self-testing power routine can be incorporated into the FDAS to verify that batteries are operating adequately during a course of experiments. For example, the Exide battery provided substantially extended and improved performance at lower and higher temperatures than alkaline batteries.

CONCLUSIONS

Battery-powered analog instruments have been available for field applications for a considerable time. However, analog devices require the services of an operator to collect the data. In contrast, a CMOS-based FDAS of the kind described in this paper has the capability to apply microcomputer control to a data logging and treatment system at a field installation without any intervention from an operator. It has the advantage of being computer-based and is therefore a logical extension to the digital form of field-based instrumentation. This approach has been extremely successful in meteorology and oceanography and should be an equally powerful approach in chemical analysis.

The range of electrochemical and other sensors which could be connected to this type of system via suitable input modules is almost limitless. In fact any sensor, preferably passive, that produces a voltage could be attached, providing the user has the ingenuity to produce the necessary software routines. Examples of the parameters examined in the present work include pH, concentration of ions measured by ion-selective electrodes and temperature.

Recent developments in CMOS processors indicate that more powerful MPU devices and companion peripheral devices can now be implemented using this low power technology. Hence more powerful, smaller and better systems similar to the FDAS will be designed. These instruments will have supporting solar panels for recharging the battery and data transmission techniques incorporated.

The versatility of the system can be improved by linking the instrument to a chemical treatment procedure in an electrochemical cell with the valves and pumps under microcomputer control, by using supporting solar panels for recharging the 12-V battery, incorporating data transmission techniques at the field station, and extending the measuring capability to include current. Ultimately the equipment should be able to carry out not only potentiometric but also coulometric, amperometric and voltammetric measurements.

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ANALYSIS OF HIGH PURITY ARSENIC BY SPARK-SOURCE MASS SPECTROMETRY

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SUMMARY

Very low ($\mu g\ kg^{-1}$) concentrations of most elemental impurities are determined in an arsenic matrix by spark-source mass spectrometry when working with a cut ion-sensitive plate. The mass spectrum of arsenic is relatively simple and the lines for almost all analytically important ions are interference free, at the mass resolution used (ca. 4000). In order to have semi-quantitative results at least, one element should be accurately determined by an independent technique, such as atomic absorption spectrometry. The measurements are then semi-quantitative.

One of the main fields of the application of spark-source mass spectrometry (s.s.m.s.) is sensitive bulk multi-element analysis of industrial materials, especially semiconductors and products for semiconductor manufacture. Indeed, the presence of impurities in semiconductor materials influences the electronic properties, even when present in extremely low concentrations. The technology for manufacturing high-purity silicon and germanium is well developed, but there is still a need for characterization of a variety of new materials for the microelectronics industry based on high-purity Ga, As, Se, Te, In, etc. Several analytical techniques such as emission spectrography, atomic absorption spectrometry (a.a.s.) and neutron activation analysis have been used for this purpose [1]. Ideally, the technique should have multi-element capability with high sensitivity and maximum detection selectivity. The above techniques, except s.s.m.s. meet one or two but not all of these criteria, hence their application is restricted.

In the literature, references can be found to the analysis of various semiconductor materials by s.s.m.s. [1]. Very little attention, however, has been paid to the analysis of arsenic. In this article some aspects of the analysis of this matrix are discussed.

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EXPERIMENTAL

The measurements were made using a double-focusing spark-source mass spectrometer (JMS-O1, BM-2, JEOL, Tokyo) with Mattauch-Herzog geometry, equipped with an automatic spark control system.

The high purity polycrystalline samples (6N-grade) were cut in the form of bars, ca. 1 mm thick, 2 mm wide and 10 mm long. These bars were used without further preparation as electrodes for sparking. Before analysis, the sample electrodes were presparked for at least 1 h in order to remove surface layers and simultaneously to coat the surface of the source housing and the slits of the mass spectrometer with a layer of arsenic, thus removing the silicon background by cleaning the tantalum slits with quartz abrasive as well as decreasing memory effects from previous analyses. Table 1 shows the experimental spark parameters which ensure stable sparking between the arsenic electrodes as well as the slit settings and vacuum conditions. A 1.5-mm wide β-slit was chosen which eliminates, to a large extent, the discrimination effects on the sensitivity of ions with various mass, at the expense of a slight deterioration of the mass resolution to $\Delta m/m \simeq 4000$ [2]. Ilford Q2 ion sensitive plates were used as the detector.

The plates were developed at low temperature (8°C) according to a procedure described by Franzen and Schuy [3], because this results in less fogging at high exposures than the standard room-temperature development. The transmission profiles of the mass lines were stored on magnetic tape after automated densitometry and the data reduction was performed on a VAX 11/780 computer, using the program described by Pilate and Adams [4]. Fourteen exposures were recorded on each plate in the range 5×10^{-4}–6×10^{2} nC, increasing in steps of $\sqrt{10}$. For one sample, a highest exposure of 1200 nC was registered. The transmission profiles were integrated using the Hull function [5]. Two parameters in this function, γ and Ts, were determined by optimizing the isotopic abundance of several isotope pairs simultaneously.

TABLE 1

Experimental conditions

Spark voltage	32 kV
Pulse width	20 μs
Repetition frequency	3 kHz
Accelerating voltage	27 kV
Main slit-widths	25 μm
α Slit-width	0.8 mm
β Slit-width	1.5 mm
Source pressure while sparking	10^{-7} torr
Analyzer pressure	10^{-8} torr

All relative sensitivity factors were assumed to be unity, so that the accuracy of the results is limited for most elements to a factor of ca. 3. At least one element in each sample was accurately determined by a.a.s., and this element was used as an internal standard. Indeed, the singly charged arsenic-matrix line cannot be used for this purpose as it is too intense, and the multiply-charged arsenic lines appeared to be critically dependent on the experimental conditions.

RESULTS

A spectrum of a typical arsenic sample is shown in Fig. 1. It represents a plot of the consecutive data points of automatically obtained microdensitometer readings from a 600-nC exposure. Arsenic is monoisotopic and the spectrum of the matrix is, therefore, relatively simple; it is dominated by the matrix lines of the singly- and multiply-charged arsenic ions. In the mass range studied, even highly charged ions such as As^{9+} can be detected, as can be expected from the high energy available in the spark discharge. For the impurity elements, the singly-charged ion lines are the most intense and are the only ones normally used for analysis. Typical impurities detected in the spectrum of high purity arsenic are Na, Mg, Al, Si, P, S, Cl, K, Ca, V, Cr, Fe, Ni, Cu and Zn. The charge-exchange ions As^{4-3} and As^{3-2} were observed in each spectrum, whereas As^{5-4} could be detected in the 1200-nC exposure only, and no other such ions could be observed in the mass range (8—250) studied. The ion charge thus decreases by one unit at most, under the experimental conditions used (10^{-8} torr in electrostatic and magnetic analyzers). The As_2^+ and As_3^+ ions appear in relatively high apparent concentrations of the order of 10^4 and 10^3 $\mu g\ kg^{-1}$ respectively.

Background lines, due to the presence of residual gases, are an artefact of the mass-spectrometer source region which were observed consistently. Carbon, nitrogen, oxygen and a series of hydrocarbon lines were always observed, the former limiting the detectability for the determination of C, N and O. The hydrocarbon lines were exceptionally prominent compared to other high-purity materials, a fact also observed by Brown et al. [6], who explained this through the open-lattice structure of arsenic. Crysorption pumping decreased these background lines considerably and proved to be successful for the determination of C, N and O down to concentrations of 0.01, 0.005 and 0.15 $\mu g\ kg^{-1}$ respectively. The higher detection limit of oxygen may be attributed to the surface oxidation of the matrix when exposed to air.

A last class of ions which complicate the spectra are the heterogeneous compound ions, formed by association of the matrix with hydrogen, carbon and oxygen. A few ions of this type are indicated in Fig. 1. Doubly-charged molecular ions, e.g., As_3^{2+} and AsO^{2+}, were invariably detected. They appear with a low apparent concentration of the order of 0.1—10 $\mu g\ kg^{-1}$.

As a result of all the spectral features discussed above, only 20—30% of

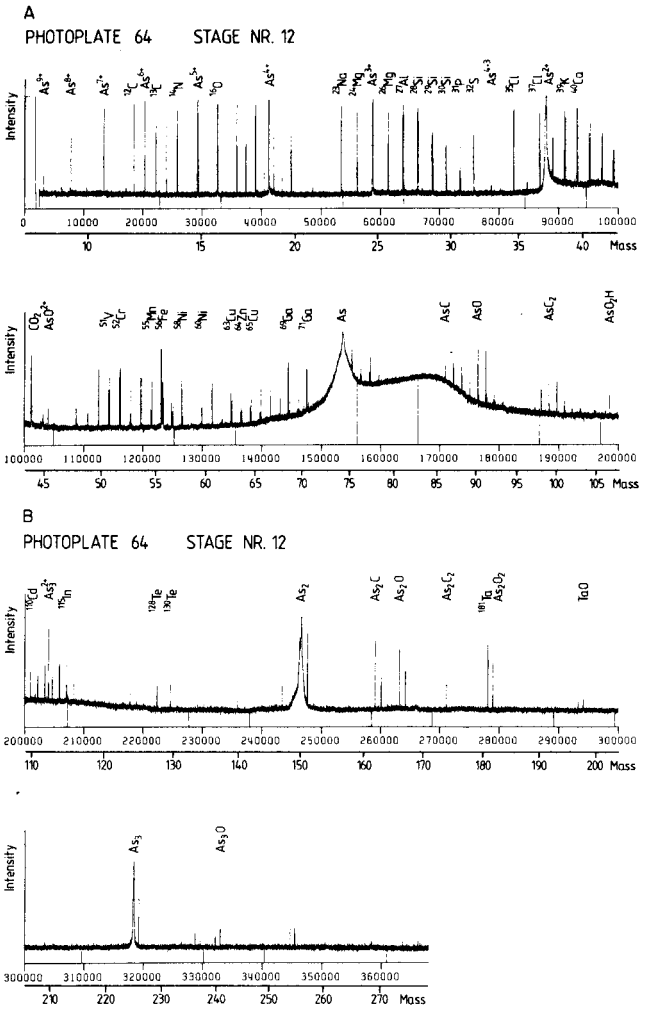


Fig. 1. Mass spectrum of typical arsenic sample obtained at 600 nC. Mass ranges 0—40, 40—110, 110—205 and 205—280.

the lines in the spectra of highest exposures are useful for quantitative analysis of the high-purity material. An important limitation of the sensitivity is the intense halo which extends on the high-mass side of the matrix lines (see Fig. 1). This halo results from secondary-emission processes; low energetic ions and electrons are sputtered away from the emulsion of the plate by high energetic ion bombardment. In addition, soft x-rays, and fluorescence radiation occur [7]. Different procedures to minimize the production of the halo were tried such as changing the composition of the developer and working at a low temperature (8°C instead of 20°C) for plate development, painting a small conducting strip of silver at the As^+ line

location, which was earthed to the photoplate, and finally cutting the plate to prevent ions from interacting in the m/z = 73—76 range. It was found that the cutting procedure was the most effective, with the halo effect decreasing to a negligibly low level, and this method was hence used for the actual determinations. A disadvantage of this technique is the more elaborate procedure required for data reduction and mass calibration.

A second limitation in sensitivity is a general, nearly uniform, blackening of the entire spectrum; it is due to non-focused particles and to the faint light emission of the ion beam itself [7]. It should be noted, however, that the blackening becomes apparent only at exposures of 600 nC and even at 1200-nC exposure it does not seriously affect detection limits. The latter exposure is a practical limit, however, as it requires ca. 8 h under the normal measurement conditions and 4 h when the main slit is opened to 75 μm.

Figure 2 shows the detection limits of various elements for a 1200-nC exposure and naked-eye observation of the elemental lines with a microdensitometer. Practical quantitative determination limits are a factor of 3—5 times higher. These detection limits correspond to an absolute sensitivity of the photographic plate of between 1500 and 4000 ions for the typical line area in the spectrometer (3 mm long; 0.03 mm wide) and 27 keV impinging ions. There has been controversy in the literature on this value with extremes between 2000 and 10000 ions [6].

Differences between detection limits from one element to another can be attributed mainly to their isotopic abundances, their mass (square root relationship) and their relative sensitivity. The values in Fig. 2 were determined assuming a relative sensitivity of unity for all elements relative to the internal standard element (Fe). Also, interferences of the mass lines may, in some cases, degrade the limit of detection. The only interferences observed

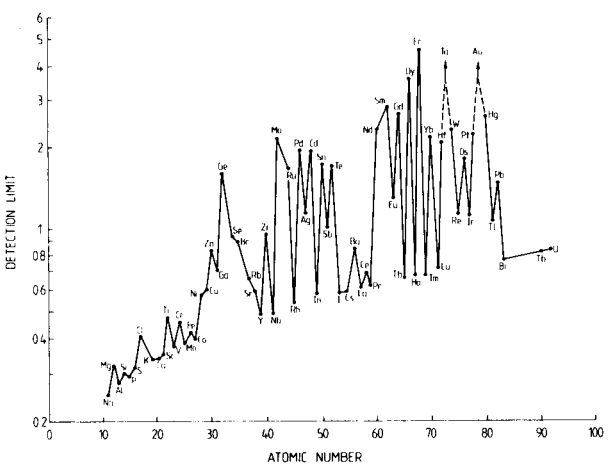


Fig. 2. Detection limits (parts per billion-atomic) as a function of atomic number for naked-eye observation of photoplates (1200-nC exposure).

were ^{28}Si through CO and AlH; ^{41}K through ^{40}CaH, and the ^{54}Fe—^{54}Cr isobars. Only the first interference is important for the present analysis, as $^{28}Si^{+}$ is the most abundant isotope of this element. Figure 3 shows the mass spectra near the Si isotopes when working at a mass resolution of ca. m/z = 4000. The carbon monoxide concentration can be significantly decreased by cryogenic pumping, thus allowing the use of the ^{28}Si line.

Table 2 shows the results of four analyses of one typical sample. It was analyzed four times with measurements up to 600 nC. The results give an idea of the RSD obtained, which is 38%. The average RSD of all results is ca. 30%. This is satisfactory in view of the low concentrations measured; it is partly due to the heterogeneity of the sample considering that only small amounts of material are analyzed. The uncertainty of the results mainly depends on the relative sensitivities and is estimated to be a factor of 3 from generally obtained variations in elemental sensitivity.

In order to estimate the accuracy, some arsenic samples, in which the concentration of some elements were determined by a.a.s., were also analysed by s.s.m.s. So far a comparison has been made for Al, Ca, Fe, Cu, Zn, Sb and Te. The average concentration ratio s.s.m.s./a.a.s. amounts to

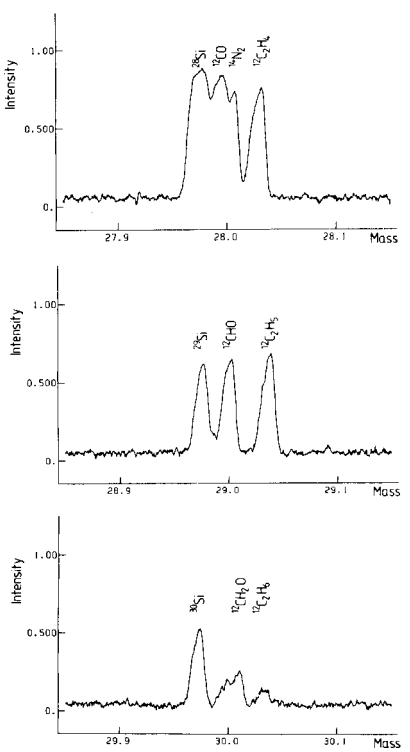


Fig. 3. Mass spectra near ^{28}Si, ^{29}Si and ^{30}Si with interfering mass lines.

TABLE 2

Comparison of results of s.s.m.s. with a.a.s.

Element	Concentration ($\mu g\ g^{-1}$)				
	S.s.m.s.				A.a.s.
Na	0.048	0.085	0.049	0.045	0.29
Mg	0.006	0.002	0.003	0.008	<0.002
Al	0.023	0.026	0.010	0.010	0.03
Si	0.17	0.20	0.11	0.13	0.6
P	0.009	0.015	0.009	0.010	—
S	0.011	0.018	0.011	0.010	—
Cl	0.11	0.10	0.21	0.18	—
K	0.16	0.055	0.070	0.062	0.07
Ca	0.028	0.060	0.042	0.048	0.26
Ti	<0.003	<0.007	<0.004	<0.002	<0.01
V	0.006	0.015	0.008	0.011	<0.03
Cr	0.018	0.015	0.008	0.017	<0.03
Mn	<0.002	<0.006	<0.003	<0.002	<0.01
Fe	0.20[a]	0.20[a]	0.20[a]	0.20[a]	—
Co	<0.003	<0.007	<0.004	<0.002	<0.03
Ni	0.043	0.075	0.032	0.063	<0.03
Cu	0.012	0.034	0.022	0.036	<0.02
Zn	0.013	0.020	0.024	0.039	0.07

[a]Internal standard determined by a.a.s.

0.41, 0.60, 1.43, 0.41, 0.24 and 0.24 for Ca, Al, Cu, Sb, Te and Zn, respectively. It should be taken into consideration that inhomogeneities of the samples and the errors of both methods contribute to this total error. The uncertainty for the elemental determinations can be estimated at a factor of 2.

The volume, v (μm^3), of arsenic consumed in making an exposure of n^+ ions ($n \simeq 3 \times 10^3$ for a barely detectable line with naked-eye observations [8]; $n \simeq 10^5$ for a readily measurable line) in order to determine an impurity of concentration, C (atoms per 10^6 matrix atoms), can be estimated by $v = An^+ \times 10^{20}/N_A \rho \Delta CI$, where A is the matrix atomic mass, I is the abundance (%) of the isotope line measured, N_A is Avogadro's number and ρ is the density of the matrix (g cm^{-3}). Δ is the useful yield, i.e., the number of atomized atoms necessary to deliver 1 ion to the ion sensitive detector. The value of Δ depends on the specimen material and on experimental parameters such as breakdown voltage and slit-widths [9].

For the present experimental parameters, from weight loss measurements and coulometer readings, a Δ-value of 4.7×10^{-8} was obtained. For the determination of a monoatomic element at 1 $\mu g\ kg^{-1}$ in arsenic under the given experimental conditions, about 1.4×10^6 μm^3 of material needs to be atomized to produce a barely visible line on the ion-sensitive plate. From material-consumption experiments, it was calculated that ca. 124 μm^3 of

arsenic is atomized per pulse train. Since it takes ca. 15 s to record 1 nC, an exposure of 0.25 nC is required to have a just visible line and 8.4 nC is necessary to have a measurable line for a monoisotopic element at 1 μg kg^{-1}. Calculations, such as those given above, are useful when a certain limit of detection is required or when dealing with small samples. For the latter, the transmission Δ can be increased when working at lower breakdown voltages (but analysis time seriously increases) or at wider main slit-widths (but mass resolution decreases).

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DETERMINATION OF WEAR METALS IN OIL USING ENERGY DISPERSIVE X-RAY SPECTROMETRY

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SUMMARY

Trace elements in oil can be determined by adsorption of the oil sample onto magnesium oxide followed by thermal degradation of the organic material. The resulting powder is easily pressed into a pellet suitable for x-ray spectrometric analysis. The lower limit of detection depends upon the trace impurities in the magnesium oxide and is a few $\mu g\ g^{-1}$ for most elements determined.

Determination of metals in oil is an analytical problem known world wide. Several investigations of trace element determinations have been reported [1—4]. There are a number of reasons for performing these determinations. One is to monitor engine performance by the determination of wear metals in lubricating oils taken from engines in use or under test. Elements such as As, Se and Pb may poison catalytic converters and these and other elements are potentially harmful to the environment. These determinations rely heavily on atomic absorption and inductively-coupled plasma atomic/emission spectrometry [5]. Atomic absorption methods require tedious digestion procedures and inductively coupled plasma emission methods are hampered by problems with viscosity of the oil samples.

X-ray spectrometry should be a useful method for the determination of metals in oil. For the most part, the elements of interest lie in the practical range of atomic number for determinations by x-ray spectrometry. Energy-dispersive x-ray spectrometry provides the capability for simultaneous determination of several elements. However, like other methods, x-ray spectrometry has limitations. In the case of the determination of metals in oil, the limitations are primarily lack of adequate detection limit and matrix effects due to the composition of the oil, including metal content and the content of carbon, hydrogen and sulfur. As a working figure, most transition elements can be determined from a few $\mu g\ g^{-1}$ upwards in a hydrocarbon matrix. Further improvement will require some type of preconcentration technique. The matrix-effect problem can be approached in two ways. The sample can be prepared as an "infinitely thin" specimen in which matrix effects are minimized, or some type of data treatment using computer software can be used.

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Kubo et al. [6] prepared specimens by pipeting 0.05—5.0 mg of oil sample spiked with a sulfuric acid solution of Rh, or Rh and Cr as an internal standard onto Formvar film. The two-element spike was required for viscous samples which did not flow into films sufficiently thin to ignore absorption by the sample matrix. The technique was tested on only two types of samples, only one of which was a standard reference material. The results that were obtained were good; however, the major deficiency in the approach is the long counting times of several hours required for acceptable x-ray statistics.

Giauque et al. [7] used the incoherent scatter of the source x-rays from a molybdenum x-ray tube as an "internal standard" following standardization of the instrumentation with solutions of dissolved metals. Specimen preparation probably resulted in a thickness intermediate between infinitely thin and infinitely thick specimens. Results reported for NBS SRM 1634 fuel oil were not acceptable for most applications.

Christensen and Agerbo [8] used a combination of secondary target excitation and quantification calculations based on the Fundamental Parameter approach [9] for the determination of sulfur and heavy metals in crude oil and petroleum products. Samples were analyzed as liquids placed in commercial specimen cups with liquid depths of ca. 2 cm. X-ray counting times ranged from 100—1000 s, and a variety of secondary targets were required. This work presented a methodical investigation of the effect of the carbon: hydrogen ratio in the matrix. However, results were presented for only S, V and Ni. The V and Ni results were compared to another x-ray spectrometric determination and instrumental neutron activation analysis.

Sanders et al. [10] used a combination of the measurement of the scatter of source radiation and a fundamental parameter approach [11] for the determination of heavy elements in unweighed oil samples. Specimen preparation consisted of pipeting sufficient sample into a commercial liquid specimen cup to assure that the bottom of the cup was covered. Titanium and zirconium secondary-target sources were used sequentially. The titanium-excited spectrum is collected in a helium atmosphere and is used for elements in the Al—Ca range. The zirconium source is used for elements in the Ti—Pb range. The two spectra are stripped of the unused portions and summed to obtain a composite spectrum. The zirconium radiation scatter is used in conjunction with first approximations of the heavy element content obtained from fluorescent intensities to obtain the mass of the specimen. A computer program, SAP3, was used to perform the computations [12]. Results for sulfur in NBS oils were reported and were good. The results of trace element determination were reported for only one NBS oil standard reference material. Although the results were good, the test did not prove the method applicable to a variety of oil types. The SAP3 program is not readily available to users in a well documented form and requires a specific and relatively expensive minicomputer.

An alternate approach to those given above is to prepare a sample which has an essentially constant matrix to minimize matrix effects, and to make

the sample infinitely thick so that sample mass is not an issue. Adsorption of oil onto an inert substrate with subsequent thermal degradation of the organic material is the approach reported in this paper.

EXPERIMENTAL

Apparatus

All x-ray measurements were made using a Tracor X-ray Inc. (Mountain View, CA) Spectrace 440 x-ray spectrometer equipped with a Tracor Northern (Madison WI) TN 2000 analyzer. A molybdenum x-ray tube operated at 25 kV and 0.30 mA with a 0.05-mm molybdenum primary filter was used as the x-ray source. The detector used was a 10-mm^2 Si(Li) semiconductor detector with a resolution of 155 eV FWHM at 5.9 keV. X-ray emission intensities were extracted from the spectra using the Tracor "XML" spectrum fitting software. Counting times were 100 s (livetime) for elemental concentrations above 100 $\mu g\ g^{-1}$, and 500—1000 s for concentrations below this.

Materials

Magnesium oxide used as the substrate was either J. T. Baker Analyzed, or Aldrich Chemical Co. Gold Label (99.999%). Standards were Conostan (Conoco Oil) organometallic standards. Test samples for the results shown in Table 2 were prepared analytically by addition of weighed amounts of Conostan standards to a base oil stabilizer also obtained from Conoco. These samples were prepared by a colleague and the values remained unknown to the analyst until after the test runs were completed.

Specimen preparation

Specimens were prepared by thoroughly mixing 1.1 g of magnesium oxide powder with 1.3 g (accurately weighed) of oil in a crucible. If necessary, the oil was preheated to 60—70°C to lower the viscosity. The mixture was heated to 260—270°C for 20 min with frequent stirring on a hotplate in a fume hood. The powder was removed and pressed into a 2.5-cm diameter pellet at 28,000 psi. The pellet was placed in a sample cup (Spex Industries) and supported using Kapton film (Spex Industries).

RESULTS AND DISCUSSION

An investigation of the loading of the magnesium oxide substrate showed that the weight of oil that could be adsorbed was approximately equal to the weight of substrate. Because the lower limit of detection was an issue, the maximum reliable ratio of oil to substrate was sought. An additional factor to consider is that the final specimen was to be "infinitely thick" for the most energetic x-ray measured. Tests performed using Zn as the analyte element showed that this criterion was met if more than 0.5 g of MgO was used. A compromise sample preparation procedure was to add 1.3 g of oil sample

to 1.1 g of MgO. If a greater ratio of oil to MgO was used, the oil separated from the substrate and adhered to the wall of the crucible.

It was possible to add oil to the substrate repeatedly after each heating. As many as five additions were performed in this way to improve the detection limit by preconcentration of the trace elements in the oil onto the substrate. The practical upper limit of repeated additions was not determined.

Heating time was found not to be a critical parameter. If the oil/substrate mixture was occasionally stirred, after ca. 15 min the mixture became an almost characteristic gray-beige color. After 20 min, an investigation showed that the mean percent weight loss of the organic material was 97.6 ± 0.2%. Further heating increased the weight loss to near 100% of the weight of the organic material with no measurable effect on the results. Furthermore, it was found that if the specimen was not heated beyond 20 min, the stability of the resulting pellet was improved. This may be a result of a small amount of residual organic material acting as a binding agent for the magnesium oxide.

Figure 1 shows a typical x-ray spectrum of an oil sample, in which identification of Ca, Ti, V, Cr, Mn, Fe, Ni, Cu, and Zn is clear. In other samples, additional elements may be observed. Concentration standards of 100, 500, 1000, 1500 and 2500 $\mu g\ g^{-1}$ were prepared for V, Cr, Mn, Fe, Ni, Cu and Zn using the Conostan standards and the sample preparation method described. Plots of x-ray intensity vs. elemental concentration resulted in excellent linear calibration graphs. Table 1 contains the statistical parameters for linear regression fits to these data. Replicate determinations made on all standards showed that relative standard deviations of less than 4.5% were obtained for all elements except vanadium which gave 8.5%.

To estimate the lower limit of detection for the elements investigated, a set of replicate blank specimens was prepared from the two types of magnesium oxide available. From the standard deviation of the x-ray intensities for the element peaks in this blank specimens and the slopes of the calibration graphs given in Table 1, the lower limit of detection was estimated as that concentration which would give a signal three times the standard

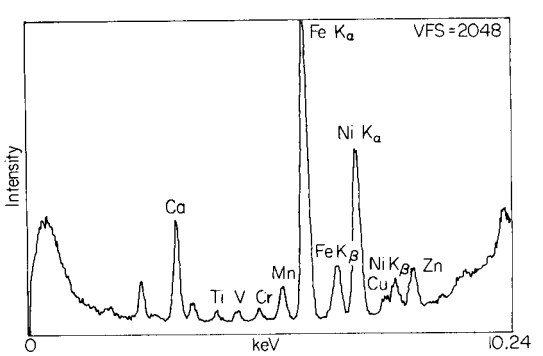


Fig. 1. X-ray emission spectrum of an oil sample.

TABLE 1

Statistical parameters for the linear regression fits of standards data

Element	Slope ± SD (Cts/200 s/μg g^{-1})	Intercept ± SD	Correlation coefficient
V	2.75 ± 0.02	72.6 ± 34.2	0.99988
Cr	5.45 ± 0.02	26.5 ± 21.5	0.99999
Mn	8.27 ± 0.05	98.3 ± 63.3	0.99995
Fe	14.32 ± 0.20	−59.5 ± 283	0.9997
Ni	33.15 ± 0.34	−366 ± 474	0.99984
Cu	41.93 ± 0.03	−599 ± 421	0.99992
Zn	65.91 ± 0.68	−1009 ± 958	0.99984

deviation of the intensities of the blank. The results are shown in Table 2. The major difference is due to the impurities in the magnesium oxide from J. T. Baker which give rise to a high background signal, especially for iron.

To be certain that the recovery was high for the trace elements, several samples were analyzed by standard additions and compared with the results from the calibration graph method. Four additions were used in the standard additions method and plots of the intensity vs. concentration added were linear. The largest difference between the results from the two methods was 6% relative. This was taken as evidence that the sample preparation method gave consistent recovery.

The first evaluation of the method was to determine V, Cr, Mn, Fe, Ni, Cu and Zn in test samples which were prepared by dissolving Conostan standards in a base oil. The results of these determinations are shown in Table 3. With the exception of vanadium, the RSD was better than 10% in all cases. The relative error between the value reported for the preparation and the results was also less than 10% in most cases. These results demonstrate the reliability of the calibration graph approach, and that the detection limit achieved is suitable for the determination of wear metals in oil.

No standard reference materials for oils were available. However, oil samples were obtained which had been previously analyzed by atomic absorption, wavelength-dispersive, and energy-dispersive x-ray spectrometry. No details of the analytical procedures were provided. For comparison

TABLE 2

Comparison of the lower limit of detection for two sources of MgO

	Limit of detection (μg g^{-1})						
	V	Cr	Mn	Fe	Ni	Cu	Zn
J. T. Baker	3.6	4.0	7.6	11.5	1.7	1.0	0.7
Aldrich Gold	2.8	2.0	1.5	3.8	0.6	0.9	0.7

TABLE 3

Results of analyses of test samples prepared from standards ($\mu g\ g^{-1}$)[a]

Sample no.	V	Cr	Mn	Fe	Ni	Cu	Zn
1	300 ± 15 (304)	310 ± 12 (309)	398 ± 9 (408)	620 ± 5 (612)	107 ± 3 (108)	1220 ± 12 (1220)	1048 ± 3 (1048)
2	400 ± 17 (393)	200 ± 15 (197)	950 ± 10 (981)	428 ± 11 (411)	1009 ± 3 (1013)	120 ± 6 (120)	515 ± 8 (515)
3	203 ± 15 (210)	1995 ± 14 (1981)	205 ± 12 (205)	200 ± 5 (199)	594 ± 7 (591)	395 ± 2 (398)	120 ± 3 (114)
4	1010 ± 29 (1010)	420 ± 13 (403)	110 ± 4 (125)	1200 ± 5 (1202)	295 ± 7 (294)	202 ± 5 (206)	403 ± 5 (410)
5	705 ± 14 (701)	580 ± 19 (568)	480 ± 9 (504)	105 ± 2 (102)	998 ± 12 (1001)	253 ± 5 (251)	530 ± 4 (529)
6	140 ± 7 (159)	63 ± 8 (64)	14 ± 1 (20)	164 ± 1 (185)	47 ± 2 (46)	30 ± 1 (33)	60 ± 1 (65)
7	28 ± 4 (34)	307 ± 8 (320)	33 ± 2 (33)	23 ± 1 (32)	95 ± 1 (96)	63 ± 1 (64)	16 ± 1 (18)
8	98 ± 6 (109)	83 ± 4 (89)	70 ± 2 (78)	ND (16)	156 ± 1 (154)	36 ± 1 (39)	80 ± 1 (82)

[a]Results reported as the mean ± SD for 5 replicates. The values in parentheses are the concentrations calculated from the preparation.

purposes, the results for V, Cr, Fe, Ni, Cu and Zn are shown in Table 4. No results were reported in these samples for manganese, and this element was not detected by the present method. These data cannot provide detailed information on the absolute accuracy of the proposed method. However, the general agreement between the results obtained and the range of results reported by the other analysts is good.

One of the factors which can effect results of trace element determinations

TABLE 4

Results of determinations of trace elements in oil ($\mu g\ g^{-1}$, mean ± SD)

Sample		V	Cr	Fe	Ni	Cu	Zn
F126	Reported	—	5—14	96—172	—	30—63	—
	Found	—	5 ± 1	94	—	67 ± 1	1650 ± 4
F127	Reported	—	5—22	244—405	—	0—7	—
	Found	20 ± 3	—	256 ± 2	—	4 ± 1	945 ± 4
6-Oil	Reported	15	—	20	20	0.5	—
	Found	15 ± 1	—	10 ± 1	20 ± 1	—	—
VTR	Reported	14	—	86	7.0	0.7	—
	Found	10 ± 1	—	45 ± 1	10 ± 1	—	—
#5	Reported	—	5.9	176	—	47	—
	Found	—	8 ± 1	320 ± 2	—	55 ± 1	1620 ± 23

by x-ray spectrometry carried out directly on oil specimens is the carbon-to-hydrogen ratio. Oil samples of different carbon-to-hydrogen ratios were identified and analyzed. These were a "gas oil," which is a high viscosity oil, an SAE 30W and an SAE 10W oil. A set of standards was prepared from mixtures of toluene and cyclohexane. A plot of the ratio of the elastic (Rayleigh) to inelastic (Compton) scatter intensity of the Mo*K* line from the x-ray tube vs. the carbon-to-hydrogen ratio gives a smoothly increasing curve. The curve can be used to estimate the carbon-to-hydrogen ratio in oil. The samples chosen ranged in carbon-to-hydrogen ratio from 9.3 to 8.0. No significant difference was observed between results obtained using the calibration graph method for samples of these oils which had been doped to 10, 30 and 50 $\mu g\ g^{-1}$ of the elements determined using the Conostan standards. These results suggest that the method is independent of the carbon-to-hydrogen ratio and viscosity of the oil.

CONCLUSIONS

Use of MgO as a substrate for the adsorption of oil provides a simple and rapid specimen preparation technique for the determination of trace elements such as wear metals in oil. Successive adsorption of oil, followed by heating, provides for preconcentration of the trace elements. Detection limits are a few $\mu g\ g^{-1}$ and the accuracy and precision are 5—10%. The method appears to be independent of the matrix composition of the oil sample.

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CONTINUUM-SOURCE ATOMIC ABSORPTION SPECTROMETRY — NEW LAMPS FOR OLD?

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SUMMARY

A short review is presented on the development of continuum-source atomic absorption spectrometry (CSAAS). The current status of the technique is assessed and the application of microcomputer controlled single channel CSAAS to the analysis of standard reference materials is described.

The field of atomic absorption spectrometry (AAS) has grown enormously in the last 25—30 years. The pioneering work of Walsh [1] and Alkemade and Milatz [2] has profoundly affected the way in which trace element determinations are carried out in laboratories throughout the world. Although there have been significant developments in AAS instrumentation in the intervening years, the principle of the measurement has remained essentially the same since its inception. The use of a narrow-line spectral light source for AAS measurement was hailed, rightly, as a major breakthrough in the development of analytical atomic spectrometry. The classic exposition of the technique by Walsh [1] has been reiterated in countless papers and textbooks. It is now almost axiomatic that a narrow-line light source, such as a hollow-cathode lamp, is required in order to make sensitive atomic absorption measurements. Yet the future development of the technique may well depend on questioning this rationale, since it imposes severe restrictions on the design of instrumentation for AAS.

HISTORICAL PERSPECTIVES

In offering at alternative basis for atomic absorption measurement, it is pertinent to consider the historical development of the subject. Lockyer [3] described an experimental system for studying absorption spectra as long ago as 1875. A pictorial representation of the apparatus used, taken from a text published around that time [4] is shown in Fig. 1. The experimental

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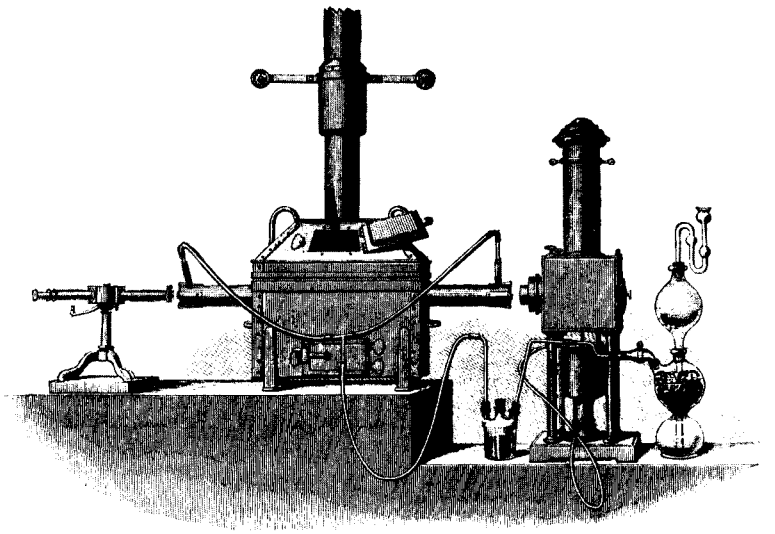


Fig. 1. Atomic absorption equipment of Lockyer reproduced from [4]. Note the electric lamp light source to the right and the spectroscope on the left hand side. The "atomizer" consisting of an iron tube was mounted in a coal fired furnace in the centre and a Kipp's apparatus was used to generate hydrogen to produce a non-oxidising atmosphere in the centre of the tube furnace.

arrangement consisted of a continuum source (electric lamp), a furnace and a spectroscope. This apparatus was used to study the absorption spectra of metal vapours at elevated temperatures. An iron tube, some 4 ft. in length and filled with hydrogen, was heated in a furnace supplied with coke or charcoal. Metals were inserted via a T-piece side tube, once the main iron tube had reached the desired temperature. It is remarkable that this furnace is in many respects similar to electrically heated "constant temperature" type atomisers devised almost a century later. For measurements at still
er temperatures, the iron tube was replaced by one of chalk, and an
ıydrogen blow-pipe was used as a source of heat.
ockyer was interested in observing atomic absorption phenomena under
ratory conditions, primarily as part of an investigation into solar
nistry [5]. He discovered helium in the atmosphere of the sun 27 years
re the element was found on earth [6], and in this sense his observations
: made from an astronomical viewpoint. Nevertheless, he made valuable
ributions to the development of quantitative analysis by emission
troscopy [7], perhaps best illustrated by his description of a procedure
he assay of gold ingots [4]. Lockyer also vigorously promoted the use
hotographic procedures for recording absorption and emission spectra,
icularly with regard to documenting the intensity and width of spectral
. His description of the observation of the atomic absorption of
um vapour contained in a heated glass combustion tube is quite

remarkable [4]. It is evident from an examination of the work of Lockyer that the origins of the technique of continuum source atomic absorption spectrometry (CSAAS) are contempory with the early development of emission spectroscopy.

In 1905 and 1908, A. S. King developed arc-heated and resistively-heated tubular graphite furnaces, respectively, for studying the emission spectra of elements [8, 9]. The King furnaces are acknowledged to be the precursors of the modern electrothermal atomisers used in AAS today. Indeed, the quantitative analytical technique of electrothermal atomic emission spectrometry [10] has its origins in this work. The King furnace, while used extensively as a purely thermal emission source in order to characterise spectra, was also used for absorption measurements. Paul reported the observation of absorption spectra of rare-earth elements [11, 12] and manganese and silver [13] using the King furnace as an atomizer. Several types of continuum source, including a 500-W tungsten filament lamp, the positive crater of a carbon arc and a hydrogen lamp were employed in these measurements. Garton and Bovey [14—17] also used the King furnace in assigning the absorption spectra of copper, gallium, lutetium and thulium. Again, a variety of light sources were utilised, including a hydrogen lamp, a Lyman continuum, a xenon high-pressure arc and an over-run 12-V 48-W car bulb. At this time, with the exception of the special case of the determination of mercury [18], AAS was employed primarily as a tool in fundamental spectroscopic investigations, e.g. in the measurement of oscillator strengths, or in astronomical observations.

ANALYTICAL DEVELOPMENT OF ATOMIC ABSORPTION SPECTROMETRY

The application of AAS to chemical analysis per se was first considered by Walsh [1] in an influential paper published in 1955. Although Walsh introduced the hollow-cathode lamp as a light source for AAS, thereby providing the basis of modern instrumentation, it is interesting to note that his first experiments, carried out in 1952, were made using a continuum source [19]. The description given of the measurement of sodium by AAS is reminiscent of a similar experiment performed by Lockyer and Frankland [4] in 1869. However, sensitivity limitations attributed to the low resolution of the spectrograph employed caused Walsh to abandon the use of continuum light sources and, subsequently, a functional line-source atomic absorption instrument was developed. The rejection of continuum sources was justified on the basis of theoretical considerations. The resolution required accurately to measure an absorption line profile was estimated to be about 500 000. It was considered unlikely also that a continuum source could provide sufficiently high energy over the small spectral slit width to yield an analytically useful signal-to-noise ratio. The use of the total absorption method, which offered a means of overcoming this problem, would give rise to non-linear growth curves. In view of the

considerable expense of high resolution spectrometers at that time, it appeared more practical to pursue the relatively low-cost and less complex alternative of line source-based instrumentation.

Despite this rather powerful dismissal of the utility of continuum sources for AAS, occasional reports appeared in the literature describing their application [20—27]. Gibson et al. [20] were the first to reassess the potential of the continuum source for qualitative and quantitative analysis. An evaluation of a CSAAS instrument incorporating a multi-pass optical system, described by Fassel et al. [24] demonstrated that detection limits in general comparable to those reported for line-source AAS could be obtained for a number of elements, at wavelengths >300 nm. Although this work demonstrated the utility of a continuum source-based instrument for the determination of elements for which hollow-cathode lamps were not available, it was clear that detection limits for the elements most often determined on a routine basis were substantially inferior to those of line-source AAS.

McGee and Winefordner [25] reported the development of a CSAAS system with a long path flame tube which provided detection limits comparable to the literature values of the time for line-source flame AAS. Unfortunately, the use of non-standard experimental arrangements in these studies must qualify the validity of direct comparisons. The use of primary continuum light sources with conventional atomic absorption equipment was less successful, giving rise to poorer sensitivity than that obtained using the established procedure [27]. A relatively poor linear dynamic range was also a particularly disadvantageous characteristic of CSAAS at that time. However, advocates of the technique pointed out that a wide range of elements could be determined using the same lamp, unlike conventional AAS where a separate lamp was required for the determination of each element. In this respect, the continuum-source approach could compete favourably with line sources, both in terms of cost and performance, since line sources of adequate intensity, lifetime and stability were not available for all elements. Unlike line-source AAS, continuum source-based instrumentation provided the means for simple and rapid qualitative analysis. In addition, since the same lamp was used for the determination of all elements, the set-up time for analysis could be considerably decreased. It is interesting to note, however, that the possibility of simultaneous multi-element analysis offered by CSAAS was not cited in discussion of the potential of the technique. A theoretical comparison of line and continuum sources for AAS [26] indicated that, with a monochromator of spectral bandwidth <0.02 nm, the absorption signal measured with a continuum source was not much smaller than that obtained with a line source. The detection limits afforded by either technique were, therefore, dependent on the relative magnitude of noise sources. Subsequent developments in CSAAS were devoted to improving signal-to-noise ratios by examination of the characteristics of the spectrometer, the light source and the type of detection system employed.

Spectral resolution

It was apparent that improvements in sensitivity in CSAAS could be achieved by increasing spectral resolution, thus better isolating the absorption line from the spectral continuum. Increased effective spectral resolution was achieved by using a Fabry-Perot interferometer in conjunction with a medium-resolution monochromator [28, 29]. These studies were successful in that sensitivities and calibration graphs comparable to those of line-source AAS were obtained, thus demonstrating the utility of CSAAS given conditions of adequate spectral resolution. However, such systems are complicated and could not be recommended for application in routine analytical situations.

A more direct approach to the problem, implied by Walsh's rejection of continuum sources, is to use a high resolution spectrometer. The introduction of commercially available echelle spectrometers provided a convenient means of achieving the resolution required for CSAAS, whilst maintaining light throughput. Keliher and Wohlers [30, 31], in two significant papers, reported the application of an echelle spectrometer to CSAAS measurement. CSAAS sensitivities achieved using air/acetylene and nitrous oxide/acetylene flames were reported which were only slightly poorer than line-source AAS sensitivities measured on the same instrument. An investigation of calibration graph linearity in CSAAS using the echelle spectrometer and a conventional atomic absorption monochromator, demonstrated clearly that the absorbance/concentration relationship was a function of resolution [30]. As a consequence of the relatively low cost (compared to spectrometers of similar resolving power) of the echelle spectrometer, its compact nature and ease of use, it has been used predominantly in subsequent CSAAS studies. A useful introduction to the application of echelle gratings in analytical spectrometry is provided in a review by Keliher and Wohlers [32].

Wavelength modulation

Whilst CSAAS characteristic concentrations were established to be almost equivalent to line-source AAS values, detection limits were acknowledged by most authors to be less comparable, due to inherently poorer signal-to-noise ratios. Modulation techniques are often used in atomic spectrometry to discriminate against unwanted components of the measured signal. In the initial experiments in CSAAS, Walsh [19] used mechanical modulation of the light source in conjunction with a synchronously-tuned detection system. This experimental arrangement results in amplitude modulation of the source intensity and is useful in discriminating against radiation emitted by the atomiser. A special form of amplitude modulation, known as wavelength modulation, is used commonly in CSAAS. The application of wavelength modulation to CSAAS was originally described by Snelleman [33].

Essentially, an oscillating optical component, such as a mirror or quartz refractor plate, is mounted on a scanning motor located at the entrance or exit slit in the optical path of the monochromator. This mechanism is used to scan rapidly and repetitively the immediate wavelength region close to

the absorption line. The interval of this modulation is usually 2–2.5 times the spectral slit-width [34, 37]. The extent of the modulation interval may be controlled by constraining the degree of oscillation of the scanning motor. One of the main benefits of this technique in CSAAS is that it discriminates against low frequency noise such as source flicker noise [33] and consequently gives rise to signal-to-noise ratio enhancements [34–37]. In addition, wavelength modulation may be used to correct for the effects of broadband background emission, non-specific absorption and scatter of source radiation, and other spectral interferences which exhibit a linear change with respect to the short modulation interval, thus effectively providing a method of automatic background correction. It has also been shown that wavelength modulation is capable of correction for direct spectral overlap in CSAAS by use of the zero-crossing technique [38].

The combination of a high resolution echelle spectrometer, a wavelength modulation system [36, 38] and a high intensity xenon arc continuum source has produced the most effective CSAAS configuration, and detection limits obtained have been shown to be comparable with background-corrected line-source AAS values. Harnly and O'Haver [39] subsequently described the construction of a direct absorbance read-out circuit for this instrument which gave a real-time absorbance display, continuously corrected for source attenuation, and which allowed the system to be used with graphite furnace atomization. It was reported that this system could compensate for a non-specific source absorbance attenuation of 3 and that in a 3% sodium chloride matrix, the detection limits obtained were equivalent to or better than conventional line-source AAS or Zeeman-AAS values. However, it is undoubtedly true that detection limits in CSAAS are poorer than those of conventional AAS for elements with analytical wavelengths in the u.v. region, where the intensity of the continuum source falls off markedly. A comparison was made [40] of the radiant power of an Eimac (now Cermax) 300-W xenon short-arc continuum source and hollow-cathode lamps of the appropriate elements in the 170–600 nm region, using the echelle spectrometer operated at its highest resolution. It was found that although there was a severe decrease in radiance in the u.v., the continuum source was brighter than the corresponding hollow-cathode lamp, particularly at wavelengths above 300 nm. Thus, it would appear that state-of-the-art continuum sources are sufficiently intense to overcome the criticism of Walsh [1] that such lamps would provide insufficient radiant power within the confines of a narrow slit to yield adequate signal-to-noise ratios. It is evident, however, that an increase in intensity in the far u.v. region is required to improve detection limits for elements such as Cd and Zn, which are not competitive, at present, with line-source AAS values. Such an improvement would also allow full advantage to be taken of the multi-element capability of CSAAS.

When the wavelength modulation unit is placed behind the entrance slit of the echelle spectrometer, the entire spectrum is modulated at the focal plane of the instrument and consequently it is possible to achieve automatic

background correction at all wavelengths simultaneously. The echelle spectrometer can be utilised as a single-channel sequential instrument, or as a multi-channel direct reading polychromator. In the latter mode, it is possible to carry out background-corrected simultaneous multi-element analysis by CSAAS. A 16-channel multi-element atomic absorption spectrometer, SIMAAC, has been devised based on this principle [41]. A PDP 11/34 minicomputer is used to control the movement of the wavelength modulation unit, aquire data from the echelle polychromator and perform subsequent data processing functions such as background correction, calculation of height and area absorbances, analytical calibration, report generation and storage of results, for each of the 16 channels. The instrument may be used with either flame [42] or furnace [43] atomization. Software control [44] of the analytical procedure results in a considerable increase in the flexibility of operation of the instrument.

Wavelength-modulated CSAAS, unlike conventional AAS, provides a means of observing the whole of the absorption line profile. Consequently, raw intensity data are collected with respect to both wavelength and time. Such a system is ideal for the study of spectral interference effects [45], particularly when electrothermal atomisation is employed [46]. It is also possible to select the most appropriate wavelengths for absorbance measurements [47]. At low concentrations, the absorbance measurement is made at the centre of the line profile, since maximum sensitivity is usually required. Reference intensity levels are usually made at wavelengths close to the analyticaly line, usually at the extreme ends of the modulation interval. At higher concentrations, where the calibration graph is strongly curved, this sensitivity is no longer advantageous and in this case a less sensitive absorption measurement can be made in the 'wings' of the absorption line profile. This will give rise to a second, less sensitive but more linear calibration graph. During the initial development of the SIMAAC system software, six background corrected absorbances were computed for different positions across the line profile, producing a family of calibration curves covering in total some 4—6 orders of magnitude of concentration [47]. More recent studies [48] have shown that a similar linear dynamic range can be achieved by computing absorbances at only two wavelengths, by utilising a staircase (or 5-step square wave) modulation waveform. This development has substantially decreased the number of calculations required per cycle and consequently the computer power required to operate a multi-channel system is also significantly less. A survey of the applications of the SIMAAC instrument has been reported by O'Haver [49].

SINGLE-CHANNEL MICROCOMPUTER-CONTROLLED CSAAS

A microcomputer-controlled single-channel version of the SIMAAC instrument has recently been constructed and its performance characteristics described [50, 51]. The system, shown in Fig. 2, is based on a single-channel

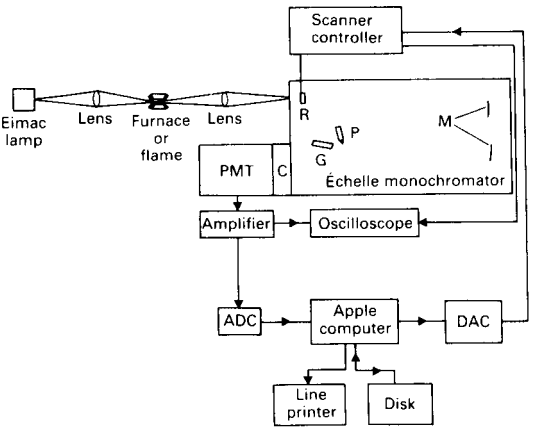


Fig. 2. Schematic diagram of the single-channel computer-controlled continuum source atomic absorption and atomic emission system: R, quartz refractor plate; A, echelle grating; P, order-sorting prism; M, mirrors; C, exit slit cassette assembly.

version of the echelle spectrometer modified for wavelength modulation, and employs a Cermax 300-W short-arc lamp as a continuum source. A variety of atomizers, including flames and furnaces (and plasmas, under a different software regime [52]) may be operated with the instrument. An Apple IIe microcomputer, fitted with appropriate interfaces, is employed to control the wavelength modulation unit (via a scanner controller which accepts externally-generated waveforms), and to perform all data acquisition and data processing functions, under software control. Programs are written in BASIC (compiled versions), incorporating a machine code subroutine for rapid data acquisition.

Since wavelength modulation can be used for background correction in flame [34] and furnace [53, 54] emission spectrometry, the instrument offers the unique capacity for accomplishing automatic background correction in absorption or emission measurements using essentially the same technique. The operating software automatically provides the option of sine, 3-step square wave or staircase modulation waveforms. The staircase waveform is used primarily to extend the linear calibration range in both absorption and emission modes, in a similar manner to that described for the SIMAAC instrument [47, 48, 55]. However, it is possible to tailor the modulation waveform for specific requirements, e.g., the use of a triangular waveform for line profile measurement [45, 46, 51, 56], and consequently the system is extremely flexible.

The computer stores the data acquired during the period of modulation in memory (either in total or as ensemble averaged values for each wavelength), and carries out background correction, calculation of peak height and area absorbances or intensities and may be used to plot signals on a visual display unit (VDU) and to a printer in real time, compatible with the

cycle time of graphite furnace operation. As the raw data is retained until the next atomisation, it is possible to reprocess the results using different integration limits, scale expansions or software smoothing filters, if desired. Indeed, all of the acquired data may be saved on disk for future examination. Usually, however, it is sufficient to save characteristic height and area data, the temporal location of the peak signal and an identification number. Other recent additions to the operating software include a subroutine to allow checking of spectrometer wavelength alignment directly on the computer VDU during a run and the development of a post-run data manipulation program. The data processing program has been developed to allow automatic calibration of peak height or area measurements for standards and unknown samples after the completion of an experiment. The calibration routine has been designed specifically for application to growth curves which deviate from linearity at high concentrations, and was originally developed as a data handling facility for the measurements from commercial atomic absorption instruments [57]. The completion of the program has allowed the system to be applied to the analysis of real samples on a routine basis.

In contrast to the SIMAAC system [49], the single-channel microcomputer-controlled system developed at the University of Strathclyde [51] is used mainly for research in electrothermal atomisation involving continuum-source atomic absorption and thermal or non-thermal atomic emission measurements. Recent participation in standard reference material certification campaigns organised by the E.E.C. Community Bureau of Reference, however, has involved the use of this system for CSAAS with flame atomisation. Since this remains by far the most important form of atomisation in use in AAS, the results make a significant contribution to the increasing evidence which demonstrates the viability of CSAAS compared to line-source AAS. The use of standard reference materials has allowed a further examination of the accuracy of this measurement system and a comparison with the performance of line-source AAS and the more complex SIMAAC instrument. The final section of this paper contains a report on the procedures developed and the results obtained for the analysis of E.E.C. BCR single-cell protein and milk powders.

EXPERIMENTAL

Instrumentation

The instrument used for these studies has been described elsewhere [51]. The system (see Table 1) consists of an echelle monochromator incorporating a wavelength modulation unit (a 5-mm thick quartz refractor plate mounted on a G300PD optical scanning motor supplied by General Scanning Inc., Watertown, Mass.). A compatible CCX-101 scanner controller was used to drive the refractor plate motor according to a waveform supplied by an Apple IIe microcomputer via an Interactive Structures A003 8-bit digital-to-

TABLE 1

Instrumentation

Light source	Cermax 300-W xenon short-arc continuum source lamp with internal parabolic reflector, operated at 20 A.
Spectrometer	Spectrametrics SM1 III echelle monochromator. Slit width 50 μm. Slit height 300 μm. Matched entrance and exit slits. Reciprocal linear dispersion 0.062 nm mm^{-1} at 200 nm
Power supply	+ 28 V d.c. supply to EHT unit (laboratory constructed).
Detector	Hamamatsu R713, R292 photomultipliers.
Wavelength modulation	Frequency 50 Hz (detection). General Scanning Inc. G300PD optical scanning motor fitted with 5-mm thick refractor plate. CCX101 scanner controller.
Amplification	Laboratory constructed preamplifier in current-to-voltage configuration.
Interfaces	Interactive Structures Inc. 12-bit 16-channel A113 ADC with 0-5 V input range. Output via an AØØ3 8-bit 2-channel DAC. Grappler + printer interface.
Computer	Apple IIe 64K RAM microcomputer, 2 disk drives, monochrome monitor and dot matrix printer.
Atomizer	Perkin-Elmer air/acetylene flame unit, 10-cm burner. Perkin-Elmer HGA 500 graphite furnace.

analog converter. Signals from the photomultiplier were amplified and passed to an Interactive Structures AI 13 12-bit analog-to-digital converter and thence to the computer. The computer is equipped with twin Apple disk drives, a monochrome monitor and dot matrix printer fitted with a Grappler + interface which is capable of handling Apple graphics screen dumps. A Cermax 300-W xenon short-arc lamp was used as the continuum source and was operated at 20 A. The spectrometer was used with matched entrance and exit slit dimensions of 300 μm height and 50 μm width. A standard Perkin-Elmer air/acetylene flame unit with a 10-cm burner was employed for all measurements and was operated under the conditions specified for each element by the Manufacturers (see Table 2). All results were processed using the CALGRAF-EXP program [57] on the Apple IIe computer. A Perkin-Elmer 5000 flame atomic absorption spectrometer was used to obtain comparative values in the analysis of single-cell protein samples.

TABLE 2

Conditions for analysis by CSAAS

Element	Wavelength (nm)	Atomizer	Sample dilutions	
			Milk powder	Single-cell protein
Ca	422.7	air/C_2H_2 flame, stoichiometric	x 20	x 10
Fe	248.3	HGA 500	—	x 10
K	766.5	air/C_2H_2 flame, lean	x 100	x 20
Mg	285.2	air/C_2H_2 flame, stoichiometric	x 10	x 20
Na	589.0	air/C_2H_2 flame, lean	x 50	—

Reagents

Dissolution procedure. Sample (ca. 1 g, accurately weighed), as received, was placed in a 150-ml beaker and 20 ml of 50% (v/v) nitric acid added. The solution was heated gently from ambient temperature to 170°C on a hot plate, taking care to avoid excessive frothing. After 1 h, once the original volume of the solution had decreased to ca. 5 ml, a mixture of (1 + 5) perchloric/nitric acid (10 ml) was added, and the decomposition was continued until fumes of perchloric acid were observed. The solution was cooled, 30 ml of water added and boiled for 5 min to complete the dissolution. This solution was cooled, filtered and diluted with distilled water to 100 ml in a volumetric flask. This final solution was diluted as required, to bring the analyte concentration into the most precise range for instrumental measurement. For the analysis of the milk powder sample, two separate dissolutions were performed. In the analysis of the single-cell protein sample, results are reported for 5 separate dissolutions. All figures are retrospectively corrected for a separately performed moisture content determination.

Calibration procedure. All results reported are the mean of five 5-s integrations. Calibration standards were matched in perchloric acid content to that of the diluted sample solution. For the determination of magnesium and calcium, 1% lanthanum chloride was added to all solutions as a releasing agent. For the determination of potassium and sodium, 1% caesium was added to all solutions as an ionisation buffer. All acids used were of AnalaR grade. Standard solutions of Mg, Ca, K and Fe were prepared freshly as required on a daily basis from 1000 μg ml^{-1} spectrosol solutions (BDH Chemicals). A 1000 μg ml^{-1} sodium solution was prepared from AnalaR sodium chloride. Lanthanum chloride additions were made from a Spectrosol standard solution (BDH). Blank solutions of all reagents were prepared in an identical procedure and put through the dissolution procedure where appropriate. Blanks established were subtracted from the results in the usual way.

ANALYSIS OF REFERENCE MATERIALS BY CSAAS

The E.E.C. Community Bureau of Reference (BCR) has, in recent years, established a range of reference materials. The CSAAS instrument was used for the determination of four major elemental components in milk powder (BCR RM63) [58] and in single-cell protein, a candidate reference material (RM 274) [59]. The results obtained for milk powder are shown in Table 3. The values reported were obtained for two separate dissolutions and are corrected for moisture content. It is clear that these results are in excellent agreement with the certificate values for all four elements. The results obtained for the analysis of single-cell protein, carried out as part of the certification campaign for this material, are shown in Table 4. The results presented represent individual determinations on 5 separate dissolutions. The agreement between these determinations is very good, except in the case of iron, where graphite furnace rather than flame atomization was used. Precision in this case was poorer as a result of a degradation in the intensity of the continuum source lamp in the u.v. which produced a deterioration in signal-to-noise ratio for this element. The use of furnace rather than flame

TABLE 3

Determination of elements in E.E.C. RM63 (milk powder)

Element	CSAAS value found (%)	Certificate value (%)
Mg	0.110 0.108	0.112 ± 0.003
Ca	1.263 1.279	1.26 ± 0.03
K	1.70 1.76	1.78 ± 0.07
Na	0.446 0.446	0.457 ± 0.016

TABLE 4

Determination of elements in E.E.C. RM274 (single-cell protein)

Element	CSAAS values (%)	Mean r.s.d. (%)	Certificate value (%)	Line-sou value (PE (%)
Mg	0.261, 0.260, 0.261, 0.251, 0.253	0.257 ± 0.005	0.268 ± 0.018[a]	0.268
Ca	1.21, 1.23, 1.23, 1.22, 1.20	1.22 ± 0.013	1.197 ± 0.140	1.192
K	0.218, 0.220, 0.217, 0.223, 0.217	0.219 ± 0.0025	0.222 ± 0.005	0.211
Fe	0.0163, 0.0156, 0.0148, 0.0128, 0.0164	0.0152 ± 0.0015	0.0156 ± 0.0004	0.0165

[a]Not certified. Mean + r.s.d. of survey results.

atomization is also likely to have contributed to a loss of precision in this case. However, the mean values correspond closely to the certificate value and to the results obtained by line-source AAS.

CONCLUSIONS

In 1974, Walsh [19] concluded that atomic absorption was a technique which was "not stagnant but merely pregnant". Continuum-source atomic absorption spectrometry could indeed, in one sense, be considered as part of a new generation. Equally, it could be considered to be a technique reborn. The original objections to the application of continuum sources in analytical AAS have now been largely overcome. It is evident that CSAAS has matured to the stage at which it must be considered a viable alternative to conventional line-source AAS. Theoretical considerations [60] indicate that whilst the use of echelle spectrometers has dramatically improved the prospects for the technique, the optimum spectrometer configuration may not yet have been realised. Also, the development of more intense and more stable light sources, particularly for far u.v. wavelengths, will continue to be a priority. It is probable that spectral sources designed specifically for application in CSAAS would provide significant improvements in performance over those currently available commercially, which have been developed primarily for visible illumination purposes.

Intrinsically, CSAAS offers the realistic prospect of carrying out routine simultaneous multi-element analysis by AAS. This will require the introduction of atomizers which provide compromise conditions for multi-element determinations. The development of 'constant temperature'-type electrothermal atomizers, therefore, is likely to be closely associated with the exploitation of CSAAS in this respect [61, 62]. The cost of such a multichannel instrument, produced commercially, utilising microcomputer control and data handling facilities is likely to be relatively low in comparison to existing instrumental techniques with similar capabilities, such as ICP/AES and ICP/MS. The single-channel system described in this paper represents the first step in this direction. The unique ability of this type of detection system to provide background corrected absorption and emission measurements in both steady state and transient atomization modes provides much greater flexibility than is currently available to users of conventional line-source atomic absorption equipment. This symbiosis perhaps reflects, in some ways, the origins of continuum source atomic absorption in the emission spectroscopy studies of Kirchoff and Bunsen, and later Lockyer. The pregnancy is over. The result may well be twins!

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INVESTIGATION OF REACTIONS INVOLVED IN GRAPHITE FURNACE ATOMIC ABSORPTION PROCEDURES

Part 12. A Study of Some Factors Influencing the Determination of Selenium

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SUMMARY

An experimental and theoretical study of various factors influencing the determination of selenium by graphite furnace atomic absorption spectrometry (g.f.a.a.s.) is reported. It is shown that the atomization efficiency can be increased as compared to the L'vov platform technique by means of a constant temperature furnace as a consequence of the possibility of choosing a higher atomization temperature. This is explained by means of high temperature equilibrium calculations, which include the formation of the thermodynamically relatively stable gaseous diselenium, hydrogen selenide and selenium sulphide. The extent of losses of selenium during thermal pretreatment was established by measurements with ^{75}Se for different types of selenium compounds, Se(-II)-methionine, selenite and selenate, in aqueous solutions as well as in chloride and sulphate matrices. It is shown that the addition of 20 μg of nickel is effective in stabilizing Se(IV) and Se(VI) in the presence of sodium chloride, sodium sulphate and pure water. However, in the presence of both an organic matrix (glucose) and sodium chloride, nickel is shown to lose its stabilizing effect.

Much interest has been focussed in recent years on selenium determination, particularly because of the biological significance of this element. In living systems selenium is found either in low molecular weight compounds, for example dimethylselenide, or bound to proteins. A number of independent methods are readily available for the determination of selenium, such as neutron activation analysis [1], molecular fluorescence [2] and hydride generation a.a.s. [3]. In consequence, the reliability of methods for selenium determination in biological materials can easily be checked so that it becomes possible to identify the problems involved in the direct determination of selenium in body fluids by graphite furnace a.a.s. (g.f.a.a.s.). These can be attributed to the volatility of organoselenium compounds and decomposition products such as hydrogen selenide and selenium monoxide formed during ashing and atomization. Moreover, methods that use conventional background correction with continuum sources are subject to spectral interferences from

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phosphorus molecules [4, 5] which restrict the applicability of the direct methods. Norheim et al. [6] have shown that a nickel-silver modifier decreases problems with both spectral interferences and volatilization losses from biological samples. Nickel and silver are the most commonly used stabilizers for selenium [7], having found application in sea water [8], biological samples [9], blood and serum [10, 11] analyses. Copper [12, 13], molybdenum [14] and potassium iodide [15] have also been used as additives. Comprehensive reviews covering the determination of selenium have been given by Verlinden et al. [16] and Welz et al. [17]. Recently Slavin and Manning [18] proposed a modifier consisting of a mixture of nickel, nitric acid and magnesium nitrate for the determination of selenium in biological materials.

Further problems associated with the determination of selenium by g.f.a.a.s. originate from the susceptibility of selenium to interact with carbon and the possibility that selenium(IV) and selenium(VI) may not be stabilized to the same extent by nickel or copper [17]. Substantial improvements with respect to the conditions for selenium determination have been realized during recent years through the introduction of the L'vov platform technique, and Zeeman or Smith-Hieftje background correction. Recently Chung et al. [19] discussed the properties of the graphite surface in relation to the sensitivity and interference effects observed in the graphite furnace determination of selenium. In this study, the authors deposited samples on a graphite ribbon and found that selenium was stabilized by the relatively large ribbon surface area in contact with the sample.

The aim of this work was to investigate selected condensed-phase as well as gas-phase reactions which are of importance for the determination of selenium by g.f.a.a.s. The extent of analyte losses during thermal pretreatment was established for different types of selenium compounds in chloride and sulphate matrices. In order to study gas-phase reactions a constant temperature atomizer was used which permits the selection of atomization temperatures independently of the volatility of the analyte. As in the previous parts of this Series most experimental results are correlated to high temperature equilibrium calculations.

EXPERIMENTAL

Instrumentation

For the platform measurements, a Perkin-Elmer AA-3030 atomic absorption spectrometer was used equipped with an HGA 500 graphite furnace, an AS-40 autosampler and a PR-100 printer. Facilities for temperature registration of the graphite tubes were installed. A fibre-optic cable was directed towards the outer surface of the central part of the tube and the light emitted from the tube was monitored as a function of time with i.r.-sensitive photodiodes connected to a data acquisition system [20]. The emission intensities from the outer graphite surface were calibrated against the temperatures

inside the tube (not containing the platform) with an optical pyrometer (Keller Spezialtechnik Pyro Werk GmbH, Model PB0 6AF3). The variation of the tube temperature with time is included in the Figs. The constant temperature atomizer used for comparative experiments is shown in Fig. 1. The entire construction is home-made, with several design improvements compared to previously described constant temperature furnaces from this laboratory [21]. The terminal blocks are made of brass and fitted with silica rubber gaskets to provide electrical insulation and to prevent air from entering the furnace. The housing is covered with a quartz window.

Purge gas from beneath the furnace passes through a laminar flow block mounted on the base plate. A secondary gas flow is directed from both cup terminal blocks through the tube. The cup and tube are heated with separate power supplies. This allows independent selection of final temperature and heating rate of the cup and tube. A fibre optic cable is directed through a 1-mm bore in the left hand cup electrode to permit temperature control of the cup. Tube emission is monitored by another fibre optic cable mounted between the contact electrode pairs in one of the tube terminal blocks, through a 2-mm bore in a graphite rod positioned close to, but not touching, the tube. The principle of this feed-back control has been described earlier [22]. Temperature settings referring to the inner surface of the cup and the outer surface of the tube were calibrated with an optical pyrometer. The constant temperature furnace was installed in a Varian AA-6 spectrophotometer. The signal damping of the AA-6 readout module was modified to obtain a fast response time from the electronics. The value of the DAMP A time

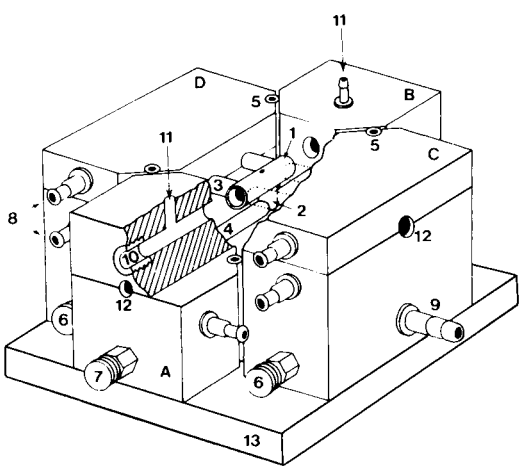


Fig. 1. Constant temperature atomizer. (1) graphite tube; (2) graphite cup; (3) tube contact electrode, spring-loaded; (4) cup contact electrode; (5) silicon gasket; (6) connection for power lead to tube; (7) connection for power lead to cup; (8) connection for cooling water supply; (9) connection for outer gas flow supply; (10) removable window assembly; (11) connection for inner gas flow supply; (12) fibre optic cable inlet; (13) baseplate with electrical insulation. (A), (B) cup terminal blocks; (C), (D) tube terminal blocks. (B) and (D) are spring-loaded.

constant was thus altered from the original 260 ms to 10 ms as described earlier [23]. In order to study in detail the time dependence of the analytical signals as well as the temperature, a fast on-line data acquisition system was connected to the spectrometer [20].

For the radioactivity measurements, a Perkin-Elmer HGA-74 furnace was used. γ-Radiation was measured with a 45 × 50 mm NaI(Tl) well-type scintillation detector (Berthold, SZ 44/50-W-N) connected to a high voltage power supply with integral discriminator (Berthold, LB 2220) and a scalar timer unit (Berthold, BF 2270-1). In these experiments temperature settings refer to the platform temperature and were calibrated with a NiCr-Ni thermocouple (below 1000°C) and with a PtRh-Pt thermocouple above that temperature. All temperatures pertaining to the constant temperature atomizer and the associated calculations were measured spectroscopically [24] and so directly reflect the conditions existing in the vapour phase.

Reagents

A 1000 μg ml^{-1} stock solution of selenium(IV) in water was prepared from solid selenium dioxide. The radioactive sodium selenite stock solution contained 0.11 mg Se ml^{-1} and had an activity of 1.19 mCi ml^{-1} (Radiochemical Centre, Amersham). From this solution sodium selenate was prepared by refluxing overnight in 30% hydrogen peroxide. The oxidation of selenium(IV) to selenium(VI) was verified by hydride generation a.a.s. No signal was observed from the selenium solution indicating complete conversion to selenium(VI). The radioactive Se-methionine stock solution contained 0.12 mg Se ml^{-1} and had an activity of 8.6 mCi ml^{-1} (Radiochemical Centre, Amersham). A 2% w/w nickel solution was made from nickel nitrate. Standard and "matrix modifier" solutions were prepared in 0.05 M nitric acid and stored in polyethylene bottles. All gases used were of SR-grade purity.

Materials

Pyrolytically-coated graphite tubes with pyrolytic platforms were used in the Perkin-Elmer HGA-500 and the HGA-74. In the experiments with the HGA-74 totally pyrolytic carbon platforms (Varian) were used. Before use, pyrolytically coated tubes were conditioned by heating four times to 2920 K using a ramp time of 60 s and a hold time of 0 s followed by twice heating to 2920 K using a ramp time of 1 s and a hold time of 5 s. The graphite cups for the constant-temperature furnace were manufactured from RW01 high-density graphite (Ringsdorff-Werke GmbH, Bonn-Bad Godesberg, F.R.G.) and subsequently pyrolytically coated by Ringsdorff-Werke. The coating was removed where the electrodes were in contact with the cup. The tube was made of solid pyrolytic graphite (Pye Unicam Ltd., Cambridge, Great Britain).

Determinations with the HGA-500

The temperature programme and the spectroscopic conditions are given in Table 1. The temperatures given in the Figs. refer to the tube wall. Volumes

TABLE 1

Furnace conditions for the Perkin-Elmer HGA 500. After ashing a cool-down step of 20 s was included

	Ramp (s)	Hold (s)	Temp. (K)	Gas flow (ml min^{-1})
Drying	15	20	440	300
Ashing	20	25	varied	300
Atomization	0	7	2275	0
Cleaning	1	6	2875	300
Wavelength (nm)		196.0		
Spectral bandwidth (nm)		2.0		
Lamp source		EDL (8 W)		
Selenium amount		2 ng		
Modifier amount		20 μg		

used were 20 and 5 μl for the solutions containing the analyte and modifier, respectively. Each result given in the Tables and Figs. represents the mean of at least three determinations. All values have been corrected for blanks. Typically, the results were obtained by running each of the solutions three times in the following sequence; aqueous blank, matrix blank, aqueous standard, standard with matrix, aqueous standard.

Determinations with the constant temperature atomizer

Sample aliquots (2—10 μl) were pipetted manually into the graphite cup through the injection port of the tube. After drying and ashing as described in Table 2, the tube was heated to a preselected atomization temperature. Cup heating was initiated after a selected delay time such that the vapour phase temperature had become constant [21].

Radioactive measurements

Standard ^{75}Se solution (20 μl) and matrix modifier (5 μl) were manually pipetted onto the platform. After drying and ashing as described in Table 3,

TABLE 2

Instrumental parameters for the constant temperature graphite furnace

	Cup		Tube	
	Temp. (K)	Time (s)	Temp. (K)	Time (s)
Drying	390	60	470	60
Ashing	600	25	600	25
Atomization	as tube	8	varied	11
Atomization delay	—	3	—	—
Purge gas	argon, 1000 ml min^{-1} unless otherwise stated			

TABLE 3

Furnace conditions for the Perkin-Elmer HGA 74 used for the radioactivity measurements

	Ramp time (s)	Hold time (s)	Temp. (K)
Drying	15	30	420
Ashing	20	40	varied

the platform was removed and the amount of ^{75}Se left was measured. Care was taken in positioning the graphite parts reproducibly in the detector in order to ensure a similar geometry for all counts. Relative losses of ^{75}Se were compared with the amount of ^{75}Se left after the drying step.

The platforms were cleaned three times at 2650 K for 15 s between each injection. Temperatures given in the Figs. were measured by a thermocouple in direct contact with the platform.

High-temperature equilibrium calculations

The calculations were done as described earlier [25, 26]. Table 4 lists the basic suite of species which was considered in all calculations. Thermodynamic data were extracted from [27—29]. Except where otherwise stated, the following input amounts (μmol) were used for the calculations: hydrogen 0.056, argon 4, carbon 1, analyte 10^{-5}. The amount of oxygen was varied with temperature to obtain the partial pressures given in Table 5. The relevance of using this oxygen scale has been discussed elsewhere [30].

TABLE 4

Species considered in all calculations

Gaseous	Ar, C, CH, CH_2, CH_3, CH_4, CO, CO_2, COCl, $COCl_2$, COS, CCl, CCl_2, CS, CS_2, C_2, C_2H, C_2H_2, C_2H_4, C_2H_6, C_3H_4, C_3H_6, Cl, Cl_2, ClO, ClO_2, Cl_2O, H, H_2, HCl, HCO, H_2O, H_2S, H_2S_2, O, O_2, O_3, OH, S, S_2, SCl, SO, SO_2, SH, Se, Se_2, Se_3, $SeCl_2$, Se_2Cl_2, SeO, SeO_2, CSe, CSe_2, H_2Se, SeS
Condensed phase	C, S, Se, SeO_2, $SeCl_4$, Se_2Cl_2, CSe_2

TABLE 5

Partial pressures of oxygen used in the equilibrium calculations

T(K)	1000—1300	1400	1500	1600	1700	1800	1900	2000
$p(O_2)$(bar)	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}	10^{-13}	10^{-14}	10^{-15}
T(K)	2100	2200	2300	2400	2500	2600	2700—3000	
$p(O_2)$(bar)	10^{-16}	10^{-17}	10^{-18}	10^{-19}	10^{-20}	10^{-21}	10^{-22}	

Determination of carbon monoxide

Carbon monoxide was determined by using a modified Varian CRA 90 carbon furnace assembly connected to the detector system for carbon monoxide as described by Lindberg [31]. A detailed description of this system is in preparation.

RESULTS AND DISCUSSION

A.a.s. experiments with platform and constant temperature atomizer

Figure 2 shows the calculated distribution of selenium species as a function of temperature. The validity of these results is very uncertain in the lower temperature region because of the slow reactions involved. It should be mentioned that a theoretical description of the chemical reactions taking place at low temperatures is also obstructed by the difficulties in characterizing the physicochemical properties of the graphite surface. Experimental results obtained without the use of a platform showed that selenium was stable up to 670 K and that signals from selenium could not be detected until 1000 K. According to the equilibrium calculations shown in Fig. 2, selenium atoms should be formed at 1200 K. From this it is evident that the use of a platform is beneficial for obtaining higher atomization efficiency, since the sample is vaporized at higher gas phase temperatures [32]. By combining the platform technique with a suitable stabilizing agent like nickel or copper the efficiency of selenium atom formation will still be increased as a result of decreased losses of molecular selenium species in the lower temperature region. This is shown in Fig. 3 where the sensitivity for selenium in the absence of a modifier (without ashing) is about five times lower than in the presence of a modifier. The interference from 0.1% sodium chloride can almost completely be eliminated at optimum ashing temperature in the presence of nickel. However, a depression is still observed for higher concentrations of matrix. The observed interference effect from 1% sodium chloride was even greater when glucose was added. However, only a small signal depression was obtained if only glucose was present. Figure 4 shows transient signals for selenium in water and in the presence of 1% of sodium chloride with and without modifier. The complex shape of the peaks seen for selenium

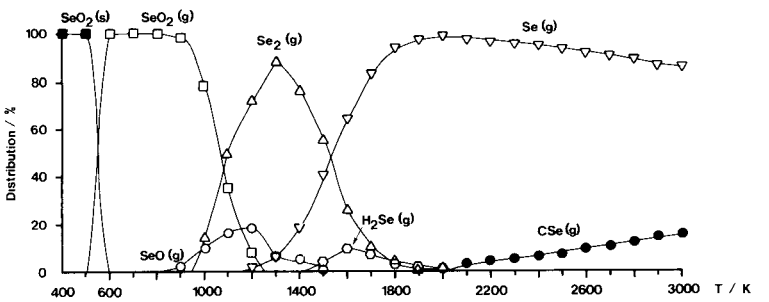


Fig. 2. Distribution of selenium species as a function of temperature.

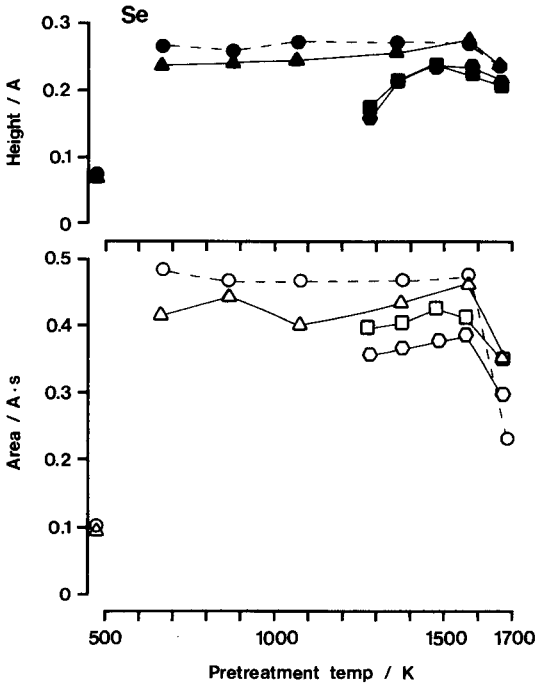


Fig. 3. Thermal pretreatment curves for selenium in: (○●) water; in the presence of (△▲) 0.1%, (□■) 1% sodium chloride and (◇◆) in 1% sodium chloride and 2% glucose. The data points on the left axis were obtained without modifier.

in the absence of a modifier, indicate that several reactions are involved in the atom formation process at lower temperatures.

Susceptibility to gas phase interference effects from chlorine is indicated by the equilibrium calculations in Fig. 5. In order to cause a decrease in the fraction of free selenium atoms it would be necessary for the partial pressure of chlorine to exceed 10^{-2} bar. In a separate investigation [33] the partial pressure of chlorine in a platform-equipped graphite tube was found to be significantly lower than this value in the temperature interval 1600 K to 2600 K. This means that the signal depression seen in Fig. 3 in the presence of sodium chloride is not caused by the formation of selenium chloride during atomization. Losses of selenium during thermal pretreatment will be discussed below.

In the presence of sodium sulphate and modifier, the selenium signal was significantly depressed as is shown in Table 6. It should be mentioned that the sodium sulphate matrix gave rise to a background which was overcompensated by the continuum light source so that negative absorbance values were obtained. This fact was taken into consideration when the results were evaluated. Table 7 shows the results obtained with the constant-temperature furnace in the presence of the same amount of sodium sulphate as that used

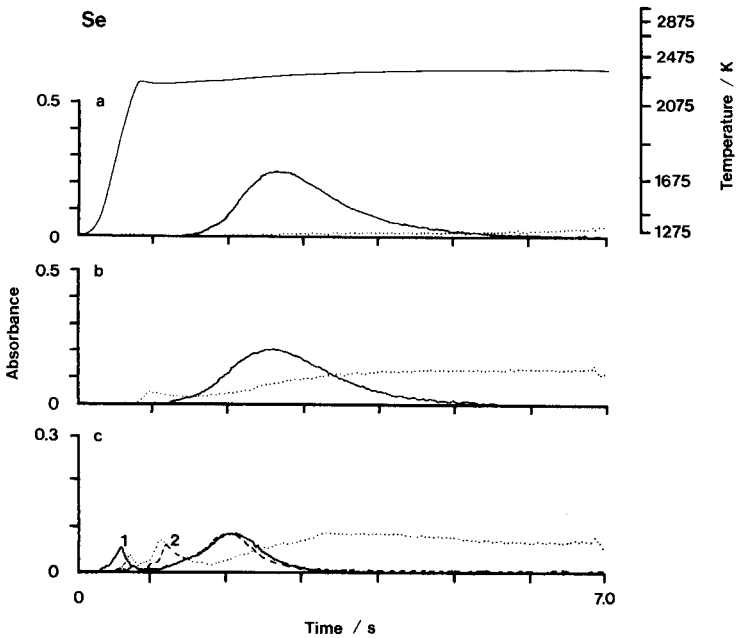


Fig. 4. Absorbance signals for 2 ng of selenium: (a) aqueous solution with modifier; (b) 1% sodium chloride with modifier; (c1) aqueous solution without modifier; (c2) 0.1% sodium chloride without modifier. Ashing temperatures were 1370 K (a, b) and 470 K (c). Background signals are shown by the dotted lines.

in the experiments with the HGA-500. The results demonstrate that the signal depression caused by sulphur can be eliminated simply by increasing the effective atomization temperature, which shows that the interference is caused by the formation of selenium sulphide during atomization. Under such high, constant temperature conditions, the large background signal observed with the HGA-500 was also completely removed. The results obtained with the constant temperature furnace are compared with those calculated and, in order to cope with the difficulties in estimating the true partial pressure of sulphur within the graphite tube [34], two levels are given. Adams and Kirkbright [35] showed that gaseous sulphur was generated during the atomization of potassium sulphate in a graphite furnace; 0.1 μmol is estimated to give rise to a partial pressure of sulphur of 5×10^{-4} atm (calculated value at 2170 K).

Selenium belongs to those elements which form stable dimers even at very high temperatures. Figure 6 shows the calculated ratios of monatomic to mono plus diatomic selenium as a function of temperature for two different input amounts of selenium. Clearly, much higher temperatures are required for complete dissociation of the larger amount. Since the average gas-phase temperature in the L'vov platform-equipped furnace is several hundred K lower than the wall temperature (see Fig. 4) [24] conditions for complete

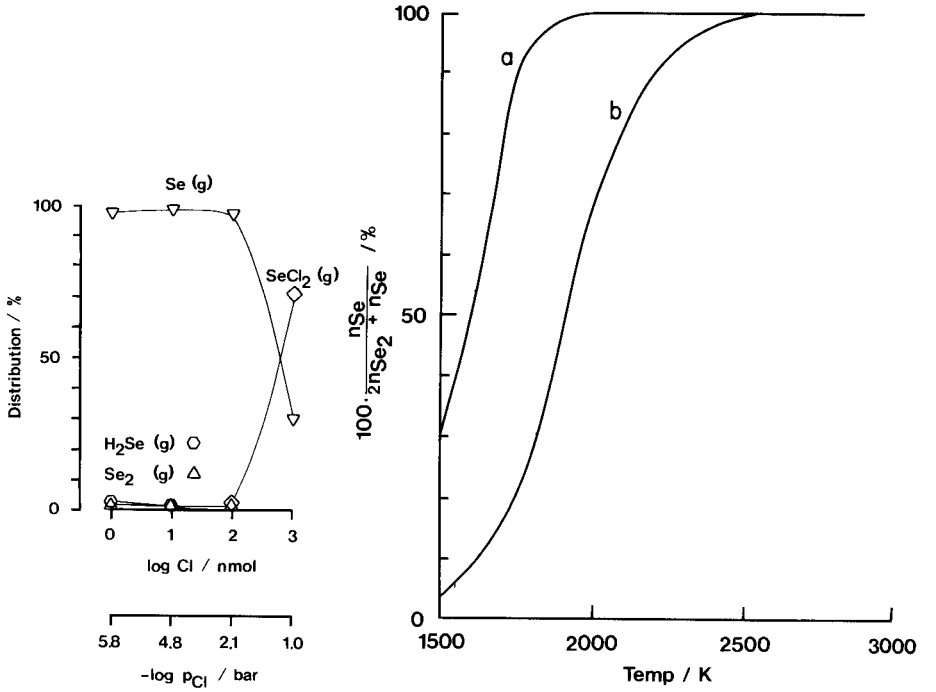


Fig. 5. Distribution of selenium species at equilibrium at 1900 K as a function of the partial pressure of chlorine.

Fig. 6. Calculated equilibrium distribution of Se(g) and Se_2(g) as a function of temperature for different input amounts of selenium. (a) 10^{-11} mol Se; (b) 10^{-9} mol Se.

atomization of selenium are not achieved for larger amounts. This is verified by the results given in Fig. 7. As can be seen, the bending of the calibration curves disappears at the highest temperature. It should be mentioned that the results of the experiments carried out at 1840 K are somewhat too low

TABLE 6

Peak height and peak area absorbance for 2 ng of selenium in aqueous solution and in the presence of sodium sulphate
(Ashing temp. = 1370 K. 5 μl of the nickel nitrate modifier was added. HGA 500 with platform was used.)

Na_2SO_4 (μg)	Se + Na_2SO_4		Se + H_2O		Recovery[a] (%)	
	Peak height	Peak area	Peak height	Peak area	Peak height	Peak area
2	0.325	0.393	0.318	0.425	102	92.5

[a]From Na_2SO_4 relative to H_2O.

TABLE 7

Comparison between experimental (constant temperature atomizer) and theoretical results for 3 ng of selenium in 2 μg of sodium sulphate
(No ashing step; 4.8 μg of nickel nitrate was used as modifier.)

Temp. (K)	Recovery[a] (%)	Calculated recovery (%)[b]	
		$p(S) = 10^{-4}$ bar	$p(S) = 10^{-5}$ bar
2000	75	70	96
2400	98	97	99.7

[a]Peak area measurements; recovery from matrix relative to water. [b]100 $p(Se)/[p(Se) + p(SeS)]$.

since the analyte was not completely released from the surface during the 10 s atomization time (see Fig. 8).

The relatively high stability of hydrogen selenide constitutes another factor of importance for the atomization efficiency. Figure 9 shows that the effect of hydrogen is less pronounced at higher temperatures. This is verified experimentally by the results given in Table 8. These values were corrected for the change in diffusion with temperature [36]. No correction was made, however, for the effect of the different gas compositions on the diffusion coefficient.

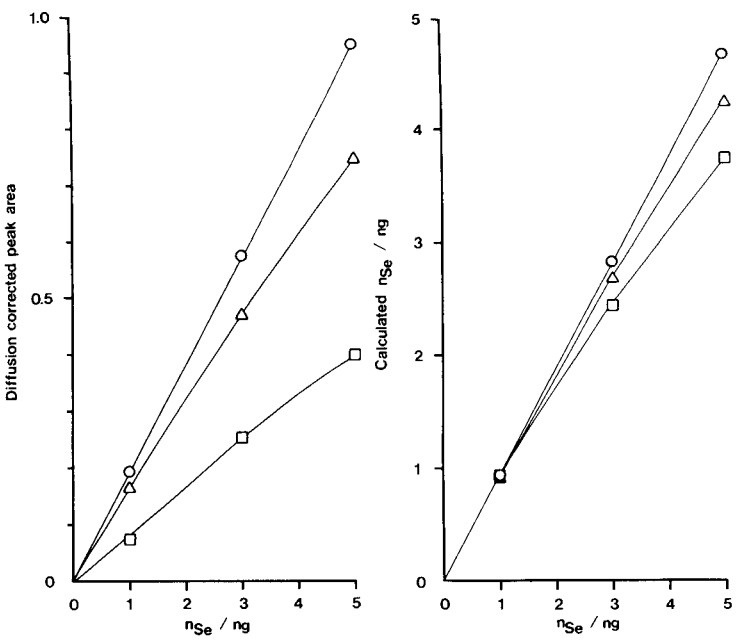


Fig. 7. Comparison between experimentally obtained (corrected for the change in diffusion with temperature) and calculated calibration curves using the constant temperature atomizer. (○) 2210 K; (△) 1910 K; (□) 1840 K.

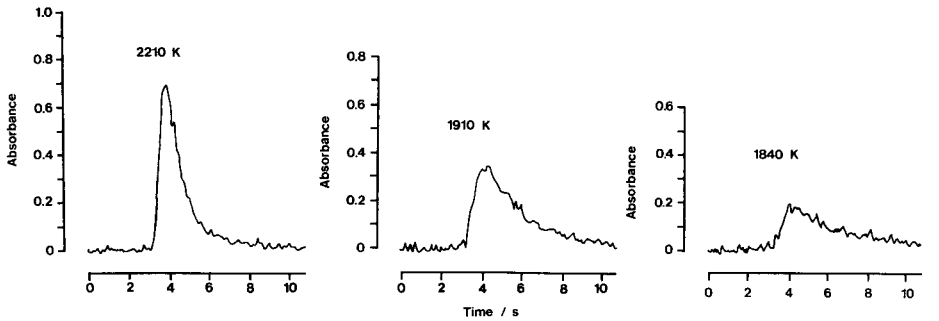


Fig. 8. Absorbance/time profiles for 5 ng of selenium for the constant temperature atomizer.

TABLE 8

Comparison between experimental (constant temperature atomizer) and theoretical results for 1 ng of selenium in pure argon and in a 20/80% (v/v) hydrogen/argon mixture (No ashing step; 4.8 μg of nickel nitrate was used as modifier.)

Temp. (K)	Recovery[a] (%)	Calculated recovery of Se(g) (%)
1840	46	58
1910	60	71
2210	83	93

[a]Peak area measurements; recovery in 20% H_2 relative to pure argon.

As discussed earlier [37] significant amounts of hydrogen are formed in a graphite tube during ashing and atomization as a result of the reaction between adsorbed water and carbon: $C(s) + H_2O(l) \rightarrow CO(g) + H_2(g)$. Figure 10 shows the amounts of carbon monoxide generated during atomization from ordinary as well as pyrocoated graphite as a function of the ashing temperature. With reference to Fig. 9, it is evident that the amount of hydrogen generated (assumed to be formed in equal amount to carbon monoxide) is sufficiently large to affect the atomization efficiency. As exemplified by the sample containing 1% nitric acid (see Fig. 10) the total amount of reaction products may vary considerably.

Radioactive measurements

In order to study the conditions for losses of selenium during thermal pretreatment in a graphite tube, three different types of selenium compound were selected; Se(IV), Se(VI) and Se(II)-methionine. Figure 11a shows that loss of selenium from the methionine begins at very low temperatures. For Se(IV) and Se(VI) significant losses are obtained above 600 K. By comparing the results for Se(IV) in Figs. 11a and 11c it can be seen that for the larger

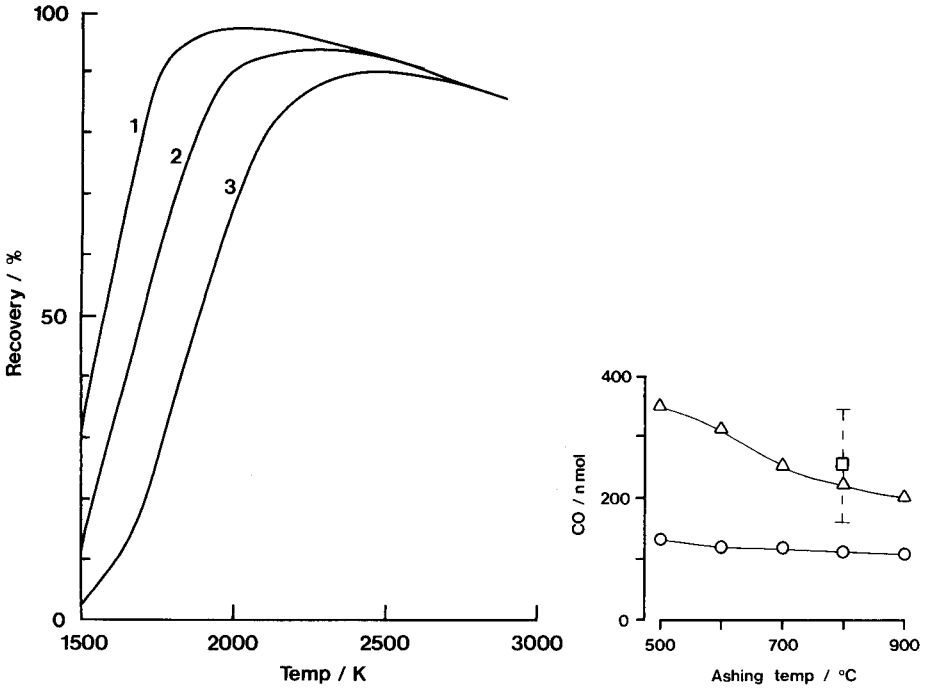


Fig. 9. Theoretical recovery of Se(g) at different temperatures and for different amounts of hydrogen added. (1) 0 μmol H_2; (2) 0.1 μmol H_2; (3) 1 μmol H_2; (1—3) 10^{-11} mol Se.

Fig. 10. Amounts of carbon monoxide liberated during atomisation at 1700 K from a Varian CRA 90 graphite tube: (○) pyrocoated graphite, 1 μl water; (△) normal graphite, 1 μl water; (□) normal graphite, 1 μl of 1% nitric acid.

amount (50 ng) the relative losses of selenium are more pronounced. This means that the ability of the graphite surface to provide sites for stabilizing Se(IV) is limited. From mass spectrometric measurements it can be concluded that the losses arise from the formation of compounds like selenium monoxide, selenium dioxide, and diselenium [38]. As is evident from the Figs., nickel is efficient in stabilizing these selenium species, except for Se(VI) at 1000 K. It should be pointed out that the decrease in recovery seen in Fig. 11b for Se(VI) at 1000 K is significant. It is interesting to note, however, that this loss can be avoided simply by using a higher ashing temperature. Welz et al. [17] found that in the presence of nickel (20 μg), Se(IV), in contrast to Se(VI) and Se(-II), gave decreased integrated absorbance signals after ashing between 500 K and 1400 K. These results cannot be explained by losses of selenium according to the measurements reported in Fig. 11b.

The stability of the same types of selenium compounds was also studied in 1% sodium chloride. The results are given in Fig. 12. As can be seen, whilst

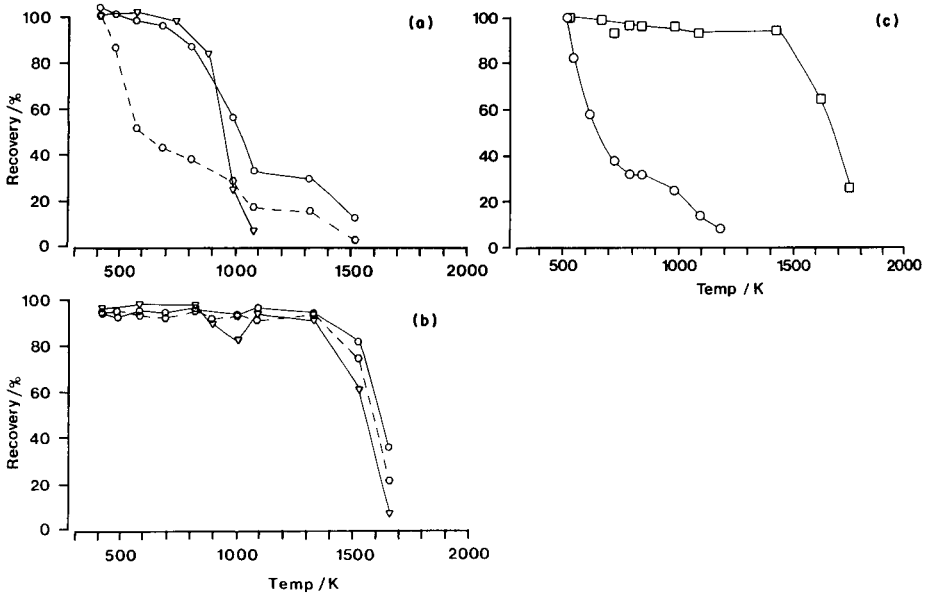


Fig. 11. Recovery of different ^{75}Se compounds as a function of the ashing temperature. (a) 2.4 ng Se in aqueous solutions, (▽) Se(VI), (⟨⟩) Se(-II) (Se-methionine), (○) Se(IV). (b) As (a) with 20 μg of nickel added; (c) 50 ng of Se(IV) in: (○) aqueous solution and (□) with 20 μg of nickel added.

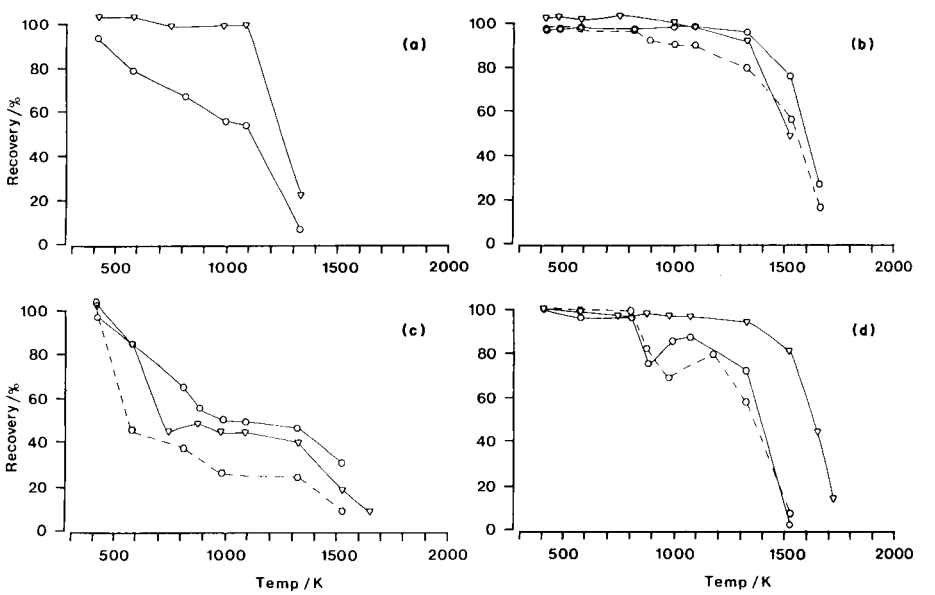


Fig. 12. Recovery of different selenium compounds as a function of the ashing temperature. (a) 2.4 ng of ^{75}Se in 20 μg of 1% sodium chloride; (b) as (a) with 20 μg of nickel added. (c) As (b) with 40 μg of 2% glucose added; (d) 2.4 ng of ^{75}Se and 2 μg of 0.1% sodium sulphate with 20 μg of nickel added. Symbols as in Fig. 11.

sodium chloride has a stabilizing effect on Se(VI), it promotes losses for Se(IV). Kilov et al. [39] found that Se(IV) can react with sodium chloride according to the reactions:

$$SeO_2 + 4NaCl \rightarrow SeCl_4 + 2Na_2O$$

$$SeO_2 + C + 4NaCl + O_2 \rightarrow SeCl_4 + 2Na_2O + CO_2$$

$$4SeO_2 + 4C + 4NaCl + O_2 \rightarrow 4SeCl + 2Na_2O + 4CO_2$$

These reactions can be used to explain the results obtained for Se(IV). According to Kilov et al. [39] the reactions leading to the formation of selenium chlorides are more probable with chlorides of zinc, iron, lead and calcium than with sodium chloride. The results for Se(VI) can be explained by the formation of the relatively stable $Na_2SeO_4(s)$.

As illustrated in Figs. 12b and 12c the stabilizing effect of nickel disappears when glucose is present. Slight losses of Se(-II) are observed at temperatures as low as 900 K even in the absence of glucose. These findings agree with the results reported by Saeed and Thomassen [4], who found that metabolized selenium was not efficiently stabilized by nickel. The results given in Figs. 12b and 12c agree with the findings represented in Fig. 3, although for the latter experiments a different furnace was used. In the presence of sodium sulphate (see Fig. 12d) and nickel the different selenium compounds are stable up to 800 K. For higher temperatures the stability is strongly dependent on the oxidation state of the selenium.

CONCLUSIONS

The atomization efficiency for selenium is increased as a result of the higher temperatures which can be achieved with the constant temperature furnace. The major source of uncertainty, however, is due to losses during thermal pretreatment steps. Thus, a better control of the reactions taking place during drying and ashing is required in order to improve the reliability of selenium determination, particularly when organic components are present in the sample.

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VERGLEICHENDE ATOMABSORPTIONSSPEKTROMETRISCHE UNTERSUCHUNGEN ZUR ELEMENTSPURENBESTIMMUNG IN URIN

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(Eingegangen den 20. Mai 1985)

SUMMARY

When using a direct determination procedure with graphite-furnace a.a.s. (e.t.a.a.s.), it is sufficient to make an addition of nitric acid in order to arrive at the optimal reduction of the spectral background. A "matrix modifier" (NH_4NO_3, $(NH_4)_2HPO_4$) produces a background which often cannot be compensated completely. Detection limits of the direct determination technique are: Cd 0.1, Co 8, Cu 4, Ni 5, Pb 2 and Tl 3 ($\mu g\ l^{-1}$). A similar power of detection can be achieved as with flame-a.a.s. due to a preceding preconcentration step (trace adsorption on highly dispersed silicic acid). After preconcentration and determination with e.t.a.a.s., the detection limits are: Cd 0.002, Co 0.1, Cu 0.05, Ni 0.09, Pb 0.09 and Tl 0.06 ($\mu g\ l^{-1}$). The trace concentrations in urine of healthy persons were found to be: Cd 0.2—0.8, Co $\leq$0.1, Cu 4—10, Ni 1—3, Pb 6—10 and Tl 0.7—1.3 ($\mu g\ l^{-1}$). Direct e.t.-a.a.s. is, therefore, found to be suitable for the determination of Cd, Cu and Pb. For the determination of Co, Ni and Tl concentrations, a preconcentration is required. Cobalt was not found in any of the urine samples at the limit of detection of 0.1 $\mu g\ l^{-1}$.

ZUSAMMENFASSUNG

Bei der direkten Bestimmung mit der Graphitrohr-a.a.s. (e.t.-a.a.s.) genügt ein einfacher Zusatz von Salpetersäure zur günstigsten Reduzierung des spektralen Untergrundes. "Matrixmodifier" (NH_4NO_3, $(NH_4)_2HPO_4$) erzeugen einen z.T. nicht mehr kompensierbaren Untergrund. Die Nachweisgrenzen der Direktbestimmung betragen für Cd 0,1, Co 8, Cu 4, Ni 5, Pb 2 and Tl 3 ($\mu g\ l^{-1}$). Durch einen vorgeschalteten Anreicherungsschritt (Spurenadsorption an Kieselsäure) kann für die Flammen-a.a.s. ein ähnliches Nachweisvermögen erreicht werden. Nach Anreicherung und Bestimmung mit der e.t.-a.a.s. betragen die Nachweisgrenzen: Cd 0,002, Co 0,1, Cu 0,05, Ni 0,09, Pb 0,09 und Tl 0,06 ($\mu g\ l^{-1}$). Als Eigengehalte in Urinen nicht belasteter Personen wurden gefunden: Cd 0,2 bis 0,8, Co <0,1, Cu 4 bis 10, Ni 1 bis 3, Pb 6 bis 10 und Tl 0,7 bis 1,3 ($\mu g\ l^{-1}$). Die direkte e.t.-a.a.s. ist somit geeignet, um Eigengehalte der Elemente Cd, Cu und Pb zu bestimmen. Für die Bestimmung der natürlichen Co-, Ni- und Tl-Konzentration ist eine Voranreicherung erforderlich. Kobalt wurde bei einer Nachweisgrenze von 0,1 $\mu g\ l^{-1}$ in keinen der Urine gefunden.

Die Elementspurenbestimmung in Urin wird in zunehmendem Maße aus arbeitsmedizinischen Gesichtspunkten gefordert. Insbesondere in den metallerzeugenden und -verarbeitenden Betrieben werden zur optimalen Überwachung der Schwermetallbelastung Verfahren angestrebt, die bei einer

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hohen Probenzahl einen möglichst geringen Personal- und Zeitaufwand bei vertretbarer analytischer Sicherheit gewähren. In der vorliegenden Arbeit werden nur solche Elemente berücksichtigt, die sich z.T. nur relativ schwierig auf einfache Weise bestimmen lassen und die voraussichtlich weiter an analytischer Bedeutung gewinnen werden. So wurde etwa auf die Untersuchungen des Zinkgehaltes verzichtet, da er durch die relativ hohe Konzentration (100—800 $\mu g\ l^{-1}$ [1, 2]) bereits direkt mit der Flammen-a.a.s. bestimmbar ist.

Der Konzentrationsbereich der anderen Elementspuren (Cd, Co, Cu, Ni, Pb und Tl) liegt im unteren Mikrogramm/Liter-Bereich; bei nicht belasteten Personen elementabhängig noch unter 1 $\mu g\ l^{-1}$. Aus rein wäßrigen Lösungen ist dieser Konzentrationsbereich gerade noch direkt mit der e.t.-a.a.s. (Elektrothermisches Atomisieren, Graphitrohr-a.a.s.) bestimmbar; Cd, Cu, Pb und Tl können auch mit elektrochemischen Methoden (z.B. Inversvoltammetrie) erfaßt werden. Durch die organischen Bestandteile des Urins ist jedoch bei den elektrochemischen Verfahren in jedem Falle eine Probenvorbereitung zur Zerstörung des organischen Anteils der Matrix erforderlich. Im Gegensatz dazu erübrigt sich bei der Bestimmung mit der e.t.-a.a.s. unter bestimmten Voraussetzungen eine größere Probenvorbehandlung. Wie Maßmann [3] gezeigt hat liefert Kochsalz, die anorganische Hauptkomponente des Urins, in dem gesamten für die a.a.s.-Messungen interessanten Spektralbereich einen kontinuierlichen Molekülbandenuntergrund; lediglich bei der Cu-Bestimmung (324,8 nm) wirkt sich diese Störung nicht mehr aus. Hinzu kommt, daß auch die organischen Bestandteile des Urins im Hauptwellenlängenbereich der a.a.s. (190 bis 325 nm) einen erheblichen Anteil der Hohlkathodenstrahlung absorbieren, sodaß sie den Untergrund zusätzlich stark erhöhen. Da die zu bestimmenden Konzentrationen bereits bei rein wäßrigen Lösungen teilweise an der Nachweisgrenze liegen, müssen bei der direkten Elementspurenbestimmung in Urin mit der e.t.-a.a.s. sehr kleine Atomabsorptionssignale auf einem hohen spektralen Untergrund gemessen werden. Da das Atomabsorptionssignal durch eine Differenzbildung aus Gesamtsignal und Untergrund erhalten wird, führt bereits ein kleiner systematischer oder statistischer Fehler in der Untergrundkompensation zu einem relativ großen systematischen bzw. statistischen Fehler in der Gehaltsbestimmung, wobei zudem ein systematischer Fehler bei der automatisch arbeitenden Untergrundkorrektur der meisten modernen Spektrometer nicht erkannt wird.

Eine weitere Schwierigkeit ist die Kalibrierung in der e.t.-a.a.s. Durch die starken Matrixeinflüsse und die von Probe zu Probe variierende Zusammensetzung des Urins erfolgt die Verdampfung der Elementspuren aus jeder Probe anders, so daß für jede Probe eine individuelle Kalibrierung erforderlich ist. Da es sich hierbei um eine generelle Problematik der Elementspurenbestimmung in Urin mit der e.t.-a.a.s. handelt, wird nur auf die Sekundärliteratur verwiesen [4]. Mit in die Untersuchungen einbezogen wurde ein bereits früher beschriebener e.t.-a.a.s. Versuchsaufbau, der einen Probeneintrag in einen bereits im zeitlichen Temperaturgleichgewicht befindlichen Graphi-

trohrofen gestattet; hierbei ist im Gegensatz zu den kommerziellen Plattformprinzipien ein echter isothermer Probeneintrag möglich [5]. Die im Vergleich zur e.t.-a.a.s. wesentlich interferenzärmere Flammen-a.a.s. kann nur im Verbund mit einem Anreicherungsverfahren eingesetzt werden, da ihr Nachweisvermögen im allgemeinen bei der Spurenbestimmung in Urin (Ausnahme: Zn, s.o.) nicht ausreichend ist. In dieser Arbeit wurde die adsorptive Anreicherung der Spuren an Aerosil nach einem totalen Säureaufschluß des Urins eingesetzt. Aerosil, eine hochdisperse Kieselsäure, zeichnet sich durch sehr kleine Eigengehalte an Spurenverunreinigungen aus [6]. Sehr tiefe Nachweisgrenzen sollten sich durch die Kombination von Spurenanreicherung und Ofen-a.a.s. erzielen lassen; diese Kombination wird jedoch durch die Blindwertprobleme des Gesamtverfahrens begrenzt.

Es wurden die Grenzen der Direktbestimmung mit der e.t.-a.a.s. ermittelt und mit dem Verbundverfahren verglichen; bei der Direktbestimmung wurde auch mit einer externen, einfachen Probenvorbereitung und mit Isoformierungsreagenzien ("Matrixmodifiern") gearbeitet. Für die Multielementanreicherung wurde ein vollständiges Verbundverfahren, bestehend aus Probeaufschluß, Spurenanreicherung und -bestimmung erstellt.

EXPERIMENTELLER TEIL

Der experimentelle Teil gliedert sich in die Direktbestimmung mit der e.t.-a.a.s., wobei ein handelsübliches Ofensystem sowie ein isothermes Probeneintragssystem eingesetzt wurden. Die Verbesserung des Nachweisvermögens durch ein Verbundverfahren (Aufschluß/Anreicherung/Bestimmung) wird unter anderem beschrieben, wobei als Bestimmungsmethoden die Flammen-a.a.s. und die e.t.-a.a.s. eingesetzt wurden.

DIREKTE OFENMETHODEN (e.t.-a.a.s.)

Bestimmung mit der herkömmlichen e.t.-a.a.s.

Die Schwierigkeiten und Grenzen der direkten Bestimmung mit der e.t.-a.a.s. sind vorweigend durch Untergrundprobleme (s.o.) sowie durch chemische Interferenzen gegeben. So können Matrixeinflüsse dazu führen, daß ionogen zugesetzte Elementspuren u.U. ein anderes Verdampfungsverhalten aufweisen, als die in der Probe als Eigengehalt enthaltenen Elementspuren; auch das Eichzusatzverfahren ergibt in diesem Fall falsche Ergebnisse. Zur Erzielung einer einheitlichen Verdampfung, aber auch zur Untergrundunterdrückung wird häufig die Zugabe von Isoformierungsreagenzien (NH_4NO_3 oder $(NH_4)_2HPO_4$) vorgeschlagen [4]. Um die Wirksamkeit der verschiedenen Vorbehandlungen vergleichen zu können, wurde der spektrale Untergrund einer Urinprobe in Abhängigkeit von der Wellenlänge bei einer einfachen Säurebehandlung und bei Zusatz von NH_4NO_3 bzw. $(NH_4)_2HPO_4$ gemessen. Dazu wurde ein "mittleres" Ofenprogramm gewählt, daß die Bestimmung der hier untersuchten Elemente erlauben sollte. Urinproben, die außerhalb des Graphitrohres zweimal mit hochreiner Salpetersäure in Quarzgefäßen

eingedampft wurden, zeigen nach Abb. 1 im gesamten Wellenlängenbereich den geringsten spektralen Untergrund. Proben, die lediglich vor der Eingabe in das Graphitrohr mit 3,5 M HNO_3 im Verhältnis 1 + 1 versetzt wurden, ergeben einen nur unwesentlich höheren Untergrund. Offensichtlich reicht das Eindampfen mit Salpetersäure unmittelbar im Graphitrohr aus, um den größten Teil des Chlorides zu verflüchtigen und die organische Matrix weitgehend abzubauen. Mit Ammoniumnitrat wird ein wesentlich höherer spektraler Untergrund gemessen; das Spektrum ähnelt dem des reinen Urins (eigene, frühere Messungen). $(NH_4)_2HPO_4$ verursacht einen z.T. nicht mehr kompensierbaren Untergrund. Da dieses Isoformierungsreagenz vorwiegend für die Bleibestimmung vorgeschlagen wird, dürften Bestimmungen auf der Hauptresonanzlinie (217,0 nm) stets durch einen nicht mehr zu kompensierenden Untergrund zu Fehlergebnissen führen.

Die Abbildungen 2 und 3 enthalten die zeitaufgelösten Signale von Gesamtabsorption, spektralen Untergrund und Atomabsorption eines unbelasteten und eines bleidotierten Urins (NH_4NO_3-Zusatz; λ = 283,3 nm). Diese Messungen erfolgten mit einem eigenentwickelten Datenübernahme-Interface und einer externen Datenverarbeitung [7]. Der von einer nicht belasteten Person stammende Urin (Abb. 2; Morgenurin, hoher Feststoffanteil) zeigt, daß das Gesamtsignal (Gesamtabsorption) lediglich durch den Untergrund bedingt ist. Das korrigierte Signal (Pb-Signal, Differenzsignal) erzeugt zwar an der höchsten Stelle des Gesamtsignals ein stärkeres Rauschen, jedoch keine gesicherte Abweichung von der Basislinie. Abbildung 3 enthält die Signale des gleichen Urins mit einer Dotierung von 100 $\mu g\ l^{-1}$ Blei. Das korrigierte Signal ergibt

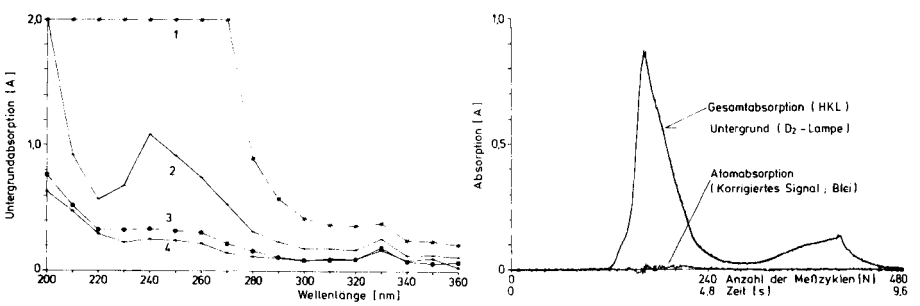
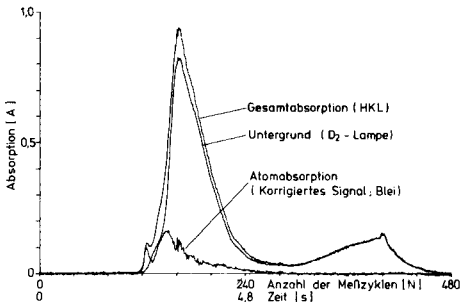


Abb. 1. Spektraler Untergrund des Urins in Abhängigkeit von der Probenvorbehandlung. Probenvorbehandlung: (1) $(NH_4)_2HPO_4$ (2 g l^{-1}) + HNO_3 (0,2%); (2) NH_4NO_3 (5 g l^{-1}) + HNO_3 (1%); (3) HNO_3 (1,75 M); (4) HNO_3 (14 M/1,75 M; zweimal eigedampft). (1, 2) Zusatz von Isoformierungsreagenzien; (3) nur Salpetersäurezusatz; (4) zweimaliges Eindampfen mit konz. HNO_3 außerhalb des Rohres. (Ofenprogramm: 10 μl: 110°C/Ramp 15/10"; 500°C/Ramp 15/10"; 2500°C/Ramp 1/6" (intern. flow 10 ml min^{-1}); 2700/ Ramp 1/3").

Abb. 2. Pb-Bestimmung: atomisierungs- und Ausheizschritt in zeitaufgelöster Messung; undotierter Urin; Modifier: NH_4NO_3.



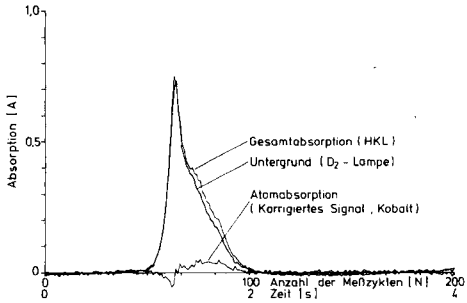
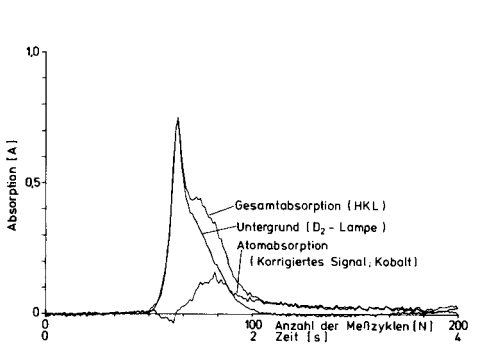


Abb. 3. Pb-Bestimmung: Atomisierungs- und Ausheizschritt in zeitaufgelöster Messung; Urin mit 100 $\mu g\ l^{-1}$-Pb-Dotierung; Modifier: NH_4NO_3.

Abb. 4. Co-Bestimmung: Atomisierungsschritt in zeitaufgelöster Messung; undotierter Urin; Modifier NH_4NO_3.

jetzt den für die Bleiverdampfung aus Urin typischen Doppelpeak, der beim Arbeiten mit einem Schreiber am Analogausgang des Spektrometers oder mit einer elektronischen Signalhöhenerfassung nicht erkannt würde. Wir deuten diesen Doppelpeak als Verdampfung verschiedener Bleiverbindungen, bedingt durch die chemisch stark heterogene Zusammensetzung der Matrix. Der erneute Anstieg des Gesamtsignals bzw. des Untergrundes nach dem Hauptsignal erklärt sich durch die mit weiter ansteigender Rohrtemperatur beginnende Verflüchtigung der schwerer verdampfbaren Matrixkomponenten; der nach etwa 8 s beginnende Signalabfall ist durch die Umschaltung des internen Gasstroms der Küvette (0 ml min^{-1} (Gasstop) auf 300 ml min^{-1}) bedingt. Charakteristisch für die Bleiverdampfung ist neben dem Doppelsignal die Lage des Bleimaximums vor dem Maximum des Untergrundsignals. Durch eine Optimierung des Ofenprogramms kann die Messung des elementspezifischen Signals zu einem relativ untergrundarmen Zeitpunkt erfolgen [7].

Bei der Kobaltbestimmung (Abb. 4) liegt das elementspezifische Signal direkt unter dem Untergrundsignal. Es zeigt stets (Wiederholungsmessungen an verschiedenen Urinen nichtbelasteter Personen) zuerst einen negativen, dann erst einen positiven Extinktionswert. Dieser negative Anteil kann durch einen strukturierten Untergrund oder (und) durch einen sich sehr schnell verändernden großen spektralen Untergrund hervorgerufen werden, da ein fein strukturierter Untergrund mit einer Kontinuumquelle und große, sich schnell verändernde Signale durch die alternierende Messung von HKL- bzw. D_2-Strahlungen vom Prinzip her nie völlig kompensierbar sind. Der nachfolgende positive Signalanteil sollte durch Kobalt verursacht sein; die analytische Aussagekraft ist jedoch geschwächt, da das Signal auch hier fehlkompensiert sein kann. Auf einen systematischen Fehler weist auch der Vergleich mit den zeitaufgelösten Signalen des gleichen Urins mit einer Dotierung von 40 $\mu g\ l^{-1}$ Co hin (Abb. 5). Bei dem undotierten Urin erreicht das Co-Signal bereits nach 2 s (100 Meßzyklen) wieder die Basislinie (Abb. 4); bei dem dotierten



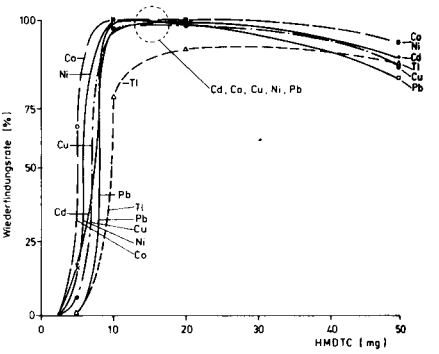


Abb. 5. Co-Bestimmung: Atomisierungsschritt in zeitaufgelöster Messung; Urin mit 40 µg l^{-1} Co-Dotierung; Modifier: NH_4NO_3.

Abb. 6. Wiederfindungsrate der Elementspuren in Abhängigkeit von der HMDTC-Menge. (+) Cd, 1 µg/50 ml; (○) Co, 1 µg/50 ml; (●) Cu, 10 µg/50 ml; (×) Ni, 1 µg/50 ml; (□) Pb, 10 µg/50 ml; (△) Tl, 1 µg/50 ml.

Urin wird das Co-Signal in diesem Bereich zwar verstärkt, doch klingt es jetzt erst nach etwa 4 s ab; wobei die Gesamtabsorption oberhalb von etwa 2 s (bis 4 s) ausschließlich durch Kobalt verursacht wird (Abb. 5). Das Untergrundsignal erreicht bei beiden Urinen nach 2 s wieder die Basislinie, dabei ist die zeitliche Reproduzierbarkeit dieses Vorganges erstaunlich hoch (Vergleich Abb. 4 und 5). Auf Folgerungen aus diesen Ergebnissen wird erst in der Gesamtdiskussion eingegangen.

Die statistischen Daten der Elementspuren-Direktbestimmung wurden an Proben ermittelt, die vor der Bestimmung lediglich mit Salpetersäure (3,5 M; 1 + 1) versetzt wurden. Auf eine Angabe von einzelnen Ofenprogrammen wird verzichtet, da diese zu stark geräteabhängig sind und individuell erarbeitet werden müssen. Die Kalibrierung erfolgte stets nach dem Eichzusatzverfahren. Die relativen Standardabweichungen wurden an einem nicht belasteten Urin ermittelt. Tabelle 1 enthält die Nachweisgrenzen (Signal aufzeichnung und Auswertung über einen Laborschreiber) sowie die ermittelten Eigengehalte des Urins. Für die Richtigkeit des Kobaltgehaltes gilt dabei die bereits angeführte Einschränkung. Thallium konnte in keinem der Urine nachgewiesen werden. Deshalb wurde hier zur Bestimmug der Standardabweichung ein synthetischer Kontrollurin (Lanonorm II) herangezogen.

Bestimmung mit isothermen Probeneintrag

Bei einer im Ofen angeordneten Plattform ist nur ein angenähert isothermes Arbeiten möglich, da die Plattform mit der Probe von Anfang an, wenn auch verzögert, mit aufgeheizt wird. In einer früheren Arbeit beschrieben wir einen Aufbau, bei der die zu analysierende Probe außerhalb des Graphitrohrofens mit einer Mikroliterpipette auf eine elektrisch beheizbare Wolframschlaufe aufgebracht, das Lösungsmittel verdampft und die Schlaufe

TABELLE 1

Statistische Daten für die direkte Elementspurenbestimmung in Urin mit der e.t.-a.a.s.

Element	Nachweisgrenze (3 s-Werte) ($\mu g\ l^{-1}$)	Relative s_r (%)	Standardabweichung bei c ($\mu g\ l^{-1}$)[a]
Cd	0,1	8,7	0,6
Co	8	12	59[b]
Cu	4	6,6	20
Ni	5	11	7,4
Pb	2	7,2	19
Tl	3	—	nicht nachweisbar
(Tl)		4,7	90[c]

[a]Eigengehalte. [b]Wert kann systematischen Fehler enthalten. [c]Synthetischer Kontrollurin (Lanonorm).

dann pneumatisch in das Graphitrohr eingeschossen wird. Diese Technik erlaubt eine mikrochemische Probenvorbehandlung (Matrixmodifizierung) unmittelbar auf der Schlaufe, wodurch bei der nachfolgenden Bestimmung die Interferenzen, und zwar der durch NaCl und der durch die organischen Substanzen verursachte spektrale Untergrund, erheblich gesenkt werden. Die Probe kann in den bereits im Temperaturgleichgewicht befindlichen Ofen eingebracht werden, so daß die Probenverdampfung unter wirklich isothermen Bedingunge erfolgt (Minderung von chemischen Interferenzen; "Plattformeffekt") [5].

Die mit Salpetersäure versetzten Urinproben (20 μl; 1,75 M HNO_3) wurden außerhalb des Graphitrohres auf der Schlaufe eingedampft und die Schlaufe dann in den jeweils vorgeheizten Ofen eingeschossen; die Ofentemperaturen betrugen dabei elementabhängig 1900° C bis 2600° C. Die bei der Cd-Bestimmung erreichten Verbesserungen sind bereits detailliert publiziert worden [5]. Bei der Bestimmung der Elementspuren Ni, Cu and Tl wurde mit dieser Technik nur noch ein spektraler Untergrund von $\leqslant 0{,}02$, bei Co und Pb wurde praktisch kein vom allgemeinen Rauschen zu unterscheidendes Untergrundsignal gemessen ($A \leqslant 0{,}01$). Somit konnte von einer fehlerfreien Untergrundkorrektur mit der D_2-Kompensation ausgegangen werden.

Untersucht wurden wiederum Urine von nicht belasteten Personen. Im Gegensatz zur üblichen e.t.-a.a.s., bei der stets Kobaltgehalte gefunden wurden, konnte mit dieser im Nachweisvermögen vergleichbaren, aber wesentlich interferenzärmeren Technik in keinem der Harne Kobalt nachgewiesen werden. Dieses Ergebnis unterstützt die Vermutung, daß die mit der einfachen e.t.-a.a.s. ermittelten Co-Gehalte durch Fehlkompensationen vorgetäuscht wurden.

ABTRENNUNG UND ANREICHERUNG DER ELEMENTSPUREN

Angestrebt wurde ein Gesamtverfahren, daß die Abtrennung und Anreicherung der untersuchten Elemente in nur einem gemeinsamen Arbeitsgang erlaubt. Nach einem Säuretotalaufschluß des Urins wurden Trägerfällungen mit den Gruppenreagenzien Dithizon, Tetramethylendithiocarbonsäure, Ammoniumsalz (APDTC) sowie Hexamethylammonium-hexamethylendithiocarbamidat (HMDTC) untersucht. Als Trägermaterialien wurden Aktivkohle sowie eine hochdispersive, sehr elementspurenarme Kieselsäure (Aerosil [6] eingesetzt. Diese Trägermaterialien haben durch ihre Säureunlöslichkeit den Vorteil, daß nach dem Wiederauflösen der Spuren in einem sehr kleinen Endvolumen vom Trägermaterial selbst her keine neue, die spektroskopischen Bestimmungsmethoden störende Sekundärmatrix gebildet wird. Optimale Anreicherungsergebnisse wurden zwar mit Aktivkohle erreicht, doch liegen hierbei die Blindwerte, verursacht durch Spurenverunreinigungen der Kohle, zu hoch. Reines Aerosil ergab zwar keine Blindwertprobleme, doch wurden hierbei nur unbefriedigende Wiederfindungsraten erzielt. Eine Mitfällung an Manganoxidhydrat (2 mg Mn(II)) ergibt in der Graphitrohr-a.a.s. bei einem Endvolumen von nur 0,5 bis 1,0 ml bereits wieder Störungen, so daß auch diese Methode ungeachtet der besseren Wiederfindungsrate verworfen werden mußte. Als optimale Kombination erwies sich die Komplexierung mit HMDTC als Gruppenreagenz, Aerosil als Trägermaterial und eine Fällungsverstärkung mit 2 mg Gold (Au(III)). Die Mitfällung mit einem Goldsalz hat den Vorteil, daß nach Gesamtabtrennung das Gold ebenfalls unlöslich zurückbleibt, so daß die nachfolgende Bestimmung der Elementspuren mit der e.t.-a.a.s. nicht gestört wird.

Zum Aufschluß des Urins wurde H_2SO_4/H_2O_2 verwendet [8]. Sämtliche benutzten Quarzgefäße wurden vor jedem Arbeitsgang längere Zeit mit Salpetersäure ausgedämpft; Reagenzien wurden ausschließlich in der höchsten handelsüblichen Reinheitsklasse (z.B. Suprapur(R)) eingesetzt bzw. selbst aufgereinigt (z.B. Subboilingdestillation der Salpetersäure). Die Wiederfindungsraten wurden durch Dotierungen an natürlichen Urinen im unteren $\mu g\ l^{-1}$-Bereich bestimmt. Parallel dazu wurden Untersuchungen mit synthetischen Kontrollurinen (Lanonorm(R)) sowie gefriergetrockneten, elementspurendotierten Kontrollurinen ausgeführt. Bei den rein synthetischen Kontrollurinen verläuft der Aufschluß einfacher und schneller. Da allein aus diesem Grunde eine echte Vergleichbarkeit mit natürlichen Urinen nicht gegeben ist, wurde das gesamte Verbundverfahren ausschließlich mit natürlichen Urinen ausgearbeitet.

Die Wiederfindungsrate der Elementspuren ist erwartungsgemäß von der Komplexbildnermenge abhängig (Abb. 6); ein großer Reagenzüberschuß verschlechtert jedoch wieder die Anreicherungsausbeute. Im Gegensatz dazu besitzt eine Veränderung der Aerosilmenge im Bereich von 10 bis 50 mg keinen Einfluß auf die Anreicherungsausbeute; erst bei Mengen <10 mg sinkt die Wiederfindungsrate ab.

Arbeitsvorschrift

25 ml Urin werden in einem 200 ml Quarzbecherglas mit 5 ml 36 M H_2SO_4 und 2 ml H_2O_2 (35%) versetzt; die Lösung wird langsam in dem teilweise abgedeckten Becherglas eingedampft, wobei in Schritten vom ca. 1 ml insgesamt 15 bis 20 ml H_2O_2 zugefügt werden. Das Einengen wird bis zum beginnenden Rauchen der Schwefelsäure fortgesetzt; die Lösung muß dabei farblos und klar werden. Nach dem Erkalten wird der Rückstand mit 50 ml Wasser aufgenommen, 2 mg Au(III) (100 μl: 20 mg ml^{-1}; 1 M HCl) zugefügt und dann der pH-Wert mit Ammoniaklösung (25%) auf etwa 2,5 eingestellt. Unter ständigem Rühren (Magnetrührer) werden 15 mg Aerosilmischung (1,5 ml: 250 mg Aerosil 380/750 mg Aerosil R 972/Degussa)/100 ml CH_3OH) als Suspension zugefügt, der pH-Wert mit 2,5 M CH_3CO_2Na auf 5,0 bis 5,3 eingestellt (Verbrauch ca. 2 bis 3 ml) und dann 20 mg Komplexbildner (2,0 ml: 500 mg HMDTC (Merck Nr. 11971)/50 ml Methanol) zugefügt. Bei einem Absinken des pH-Wertes unter 4,7 wird mit der 2,5 M CH_3CO_2Na Lösung eine Korrektur des Wertes auf 4,8 bis 5,0 vorgenommen.

Nach einer Rührzeit von 15 Min wird der Niederschlag über ein Membranfilter (z.B. Sartorius SM 113 25, 25 mm diam., Porendurchmesser 0,3 μm) abgetrennt; das Filter wird in einem 10 ml Quarzbecherglas bei 110°C getrocknet, wonach sich der Rückstand leicht vom Filter trennen läßt. Der mit den Spuren beladene Rückstand wird mit 0,5 ml 14 M HNO_3 zur Trockne eingedampft und dann mit 1 ml 3,5 M HNO_3 aufgenommen. Die Suspension wird in ein Miniaturzentrifugengefäß (z.B. Eppendorf-Reaktionsgefäß) überführt, zentrifugiert und die Elementspuren in der überstehenden Lösung mit der a.a.s. bestimmt.

Bestimmungen mit der Flammen-a.a.s.

Die Kalibrierung konnte gegen rein Salpetersäure Elementspurenlösungen erfolgen, da das Spurenkonzentrat hierbei keine Interferenzen verursacht. Die statistischen Daten des Gesamtverfahrens wurden aus der völligen Verfahrensdurchführung mit Urinen von unbelasteten Personen (Bestimmung der Nachweisgrenzen) sowie dotierten Urinen (Bestimmung der Standardabweichung) ermittelt. Die Daten sind in Tabelle 2 zusammengefaßt.

Bestimmungen mit der e.t.-a.a.s. (Graphitrohr-a.a.s.)

Zur weiteren Senkung der Nachweisgrenzen wurden die angereicherten Elementspuren mit der e.t.-a.a.s. einschließlich dem modifizierten Ofen für den isothermen Probeneintrag durchgeführt. Obwohl ein praktisch matrixfreies Spurenkonzentrat erhalten wird, mußte bei der herkömmlichen Graphitrohrtechnik mit dem Eichzusatzverfahren gearbeitet werden (Empfindlichkeitsunterschiede bis zu 20% gegenüber reinen Spurenlösungen), während beim isothermen Probeneintrag kein Unterschied zwischen Spurenkonzentrat und wäßrigen Lösungen festgestellt werden konnte. Mit Ausnahme von Kobalt wurden in allen untersuchten Harnen Elementspurengehalte gefunden, die weit oberhalb der Nachweisgrenze der Methode liegen. Die

TABELLE 2

Statistische Daten und Anreicherungsausbeuten des Gesamtverfahrens. Elementspurenbestimmung mit der Flammen-a.a.s.

Element	Anreicherungsausbeute	Nachweisgrenzen (3 s-Werte) ($\mu g\ l^{-1}$)	Relative s_r (%)	Standardabweichung bei c ($\mu g\ l^{-1}$)[a]
Cd	>95	0,3	3,6	10
Co	90—95	2	4,3	40
Cu	>95	0,4	3,7	48
Ni	>95	1	1,8	41
Pb	>95	2	3,6	47
Tl	90—95	1	3,9	40

[a]Dotierte Gehalte + Eigengehalte.

Nachweisgrenzen und die gefundenen Bereiche für die Eigengehalte sind in Tabelle 3 angegeben.

Benutzte Geräte

Für die e.t.-a.a.s. Messungen wurde ein Zweistrahlatomabsorptionspektrometer mit D_2-Kompensation und relativ hoher spektraler Auflösung (PE 4000), eine digital-geregelte e.t.-a.a.s. (PE/HGA 500), ein Eigenbauzusatz für den isothermen Probeneintrag sowie eine Kleinrechnereinheit mit eigenentwickeltem Interface und eigener Software eingesetzt. Die Flammen-a.a.s.-Messungen erfolgen an einem Einstrahlspektrometer mit D_2-Kompensation (SP9).

DISKUSSION

Schwerpunkt der Arbeit war die Feststellung der Grenzen der direkten Elementspurenbestimmung in Urinen mit der e.t.-a.a.s. (Graphitrohr-a.a.s.) sowie der Verbesserung des Nachweisvermögens durch die Entwicklung eines blindwertarmen Trenn- und Anreicherungsverfahrens in Verbund mit der Flammen-a.a.s. und der Graphitrohr-a.a.s. als Bestimmungsmethode. Für das jeweilige Element wurde ein eigenes Ofenprogramm ausgearbeitet, um möglichst günstige Bestimmungsbedingungen zu haben. Dabei wurde von Literaturvorschlägen und eigener Erfahrung ausgegangen. Widerlegt wird nach unseren Ergebnissen, daß mit den "Matrixmodifiern" NH_4NO_3 bzw. $(NH_4)_2HPO_4$ — insbesondere für die Bleibestimmung — in der Graphitrohr-a.a.s. bessere Ergebniss erhalten werden sollen. Insbesondere Phosphatzusätze erhöhen den spektralen Untergrund so stark, daß bei einer unzureichenden Untergrundkompensation mit systematischen Fehlern gerechnet werden muß. Die in Abb. 1 zur Messung des spektralen Untergrundes benutzten Modifier-Konzentrationen entsprechen den Mittelwerten aus verschiedenen Literaturstellen. Die beste Senkung des Untergrundes wird durch eine externe

TABELLE 3

Nachweisgrenzen des Gesamtverfahrens bei der Elementspurenbestimmung mit der e.t.-a.a.s. sowie Eigengehalte der Urine

Element	Nachweisgrenzen kommerzielle e.t.-a.a.s.	(3 s-Werte) ($\mu g\ l^{-1}$) isotherme e.t.-a.a.s.	Eigengehalte $\mu g\ l^{-1}$ (10 Urine)
Cd	0,002	0,004	0,2 bis 0,8
Co	0,1	0,1	<0,1
Cu	0,05	0,03	4 bis 10
Ni	0,09	0,08	1 bis 3
Pb	0,09	0,1	6 bis 10
Tl	0,06	0,04	0,7 bis 1,3

Salpetersäurevorbehandlung des Urins erreicht; fast vergleichbar günstig ist die unmittelbare Behandlung mit Salpetersäure nur im Graphitrohr. Obwohl die direkte Behandlung im Graphitrohr gegenüber der externen Behandlung einen etwas höheren spektralen Untergrund liefert, sollte ihr der Vorzug gegeben werden, da hierbei Kontaminationsprobleme durch eine externe Probenvorbehandlung entfallen.

Die Nachweisgrenzen für die direkte Bestimmung wurden aus 30 Messungen an einem nicht belasteten Urin ermittelt. (Auswertung der Schreiberaufzeichnungen). Durch die relativ hohen Eigengehalte konnten die Werte für die Nachweisgrenze z.T. nur aus dem Rauschen des Untergrundes abgeschätzt werden. Mit Ausnahme des sehr nachweisstarken Cadmiums (0,1 $\mu g\ l^{-1}$) liegen die Nachweisgrenzen zwischen 2 und 8 $\mu g\ l^{-1}$. Mit der direkten e.t.-a.a.s. konnten für alle untersuchten Elemente — ausgenommen Thallium — bereits Eigengehalte bestimmt werden (Ni an der Nachweisgrenze); auffallend ist dabei der sehr hohe Co-Gehalt (Tabelle 1). Vergleichsmessungen mit einem bereits früher betriebenen Probeneintragssystem in einem bereits im Temperaturgleichgewicht befindlichen Ofen bestätigten die Richtigkeit der gefundenen Eigengehalte, ausgenommen bei der Co-Bestimmung. Mit dieser speziellen Technik konnte in keinem der untersuchten Urine ($n > 20$) Kobalt nachgewiesen werden.

Die Zeitauflösung der e.t.-a.a.s.-Signale mit einem externen Kleinrechner weist bei der Co-Bestimmung auf einen systematischen Fehler hin (Abb. 4 und 5). Das dotierte Kobalt (40 $\mu g\ l^{-1}$) (Abb. 5) erscheint wesentlich später als der Kobalteigengehalt (Abb. 4). Dies bedeutet, daß entweder die Messung des Eigengehaltes im wesentlichen durch einen systematischen Fehler (z.B. durch eine falsche Untergrundkorrektur) bedingt ist oder aber sich Eigengehalte und Dotierung tatsächlich unterschiedlich verhalten, so daß bei der Kalibrierung ein systematischer Fehler entsteht. Zur Aufklärung und Beseitigung dieses Fehlers sind weitere Untersuchungen erforderlich.

Durch die Spurenabtrennung und -anreicherung aus 25 ml Urin auf 1 ml Endvolumen kann auch mit der Flammen-a.a.s. (Injektionsmethode, Mikroprobendosierung) das gleiche Nachweisvermögen wie mit der direkten

Graphitrohr-a.a.s. erreicht werden. Durch den erhöhten Arbeitsaufwand und den damit außerdem verbundenen Kontaminationsproblemen erscheint die direkte Bestimmung mit der e.t.-a.a.s. günstiger. Ebenso wie bei der e.t.-a.a.s. mit isothermem Probeneintrag konnte auch mit der Anreicherung und der Flammen-a.a.s. kein Kobalt nachgewiesen werden; alle anderen untersuchten Elemente ergaben Eigengehalte. Wird nach der Anreicherung die e.t.-a.a.s. als Bestimmungsmethode gewählt, so werden im Vergleich zur direkten Bestimmung ein bis zwei Größenordnungen tiefere Nachweisgrenzen erhalten (Vergleich Tabelle 3/Tabelle 1). Dieser Gewinn an Nachweisvermögen resultiert vorwiegend aus der Aufkonzentrierung (25/1 ml) und aus dem verbesserten Signal-Untergrundverhältnis (Urin/wäßrige Lösungen). Bei allen Urinen wurden mit dem Verbundverfahren aus Anreicherung und e.t.-a.a.s. Eigengehalte gefunden, die weit oberhalb der Nachweisgrenze des Gesamtverfahrens liegen. Lediglich beim Kobalt (NWG: 0,1 μg ml^{-1}) reicht auch hier das Nachweisvermögen nicht aus, um einen Konzentrationswert angeben zu können.

Insgesamt zeigt der Vergleich von direkter Bestimmung der Elementspuren und dem Verbundverfahren aus Anreicherung und e.t.-a.a.s., daß das Nachweisvermögen der D_2-kompensierten e.t.-a.a.s. ausreichend ist, um Eigengehalte der Elemente Cd, Cu und Pb zu bestimmen. Thallium (0,7 bis 1,3 μg l^{-1}) und Nickel (1 bis 3 μg l^{-1}) liegen an der Nachweisgrenze der direkten e.t.-a.a.s. oder darunter und erfordern deshalb zur Bestimmung in diesen Konzentrationsbereichen einen vorgeschalteten Anreicherungsschritt. Die Kobaltkonzentration in den Urinen konnte selbst bei einer Verfahrensnachweisgrenze von 0,1 μg l^{-1} nicht mehr bestimmt werden. Konzentrationen im arbeitsmedizinischen Bereich, z.B. im Urin von Personen aus der metallverarbeitenden Industrie (Pb 40 bis 100 μg l^{-1}), können mit der direkten e.t.-a.a.s. richtig bestimmt werden. Voraussetzung ist dabei die Kalibrierung nach dem Eichzusatzverfahren und eine optimal justierte und ständig überprüfte D_2-Untergrundkompensation. Durch eine zusätzliche Zeitauflösung der Signale können möglichst interferenzarme Temperatur/Zeitprogramme entwickelt sowie systematische Fehler besser erkannt werden.

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EMPIRICAL AND MOLECULAR FORMULA DETERMINATION BY GAS CHROMATOGRAPHY/MICROWAVE-INDUCED PLASMA ATOMIC EMISSION SPECTROMETRY

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SUMMARY

Following a review of gas chromatographic element-specific detection by microwave-induced plasma atomic emission spectrometry for the purposes of empirical-formula determination, current results for an atmospheric-pressure helium plasma are presented. Hydrocarbons and chlorinated organic compounds are used as models and compounds resulting from chlorination of fulvic acid are characterized. Accuracy and precision for carbon, hydrogen and chlorine determinations are reported. The former usually being better than ±5% and the latter in the range 1—3%. Manual and computer methods for empirical-formulae determination are presented and compared.

Since stoichiometry was first investigated by Dalton early in the nineteenth century, elemental analysis has been central to qualitative and quantitative identification of organic compounds. An empirical formula, defined as the simplest whole number ratio for the atoms of different elements in a compound, is typically obtained by various combustion methods [1]. The molecular formula can then be determined from knowledge of the molecular weight, which often can be obtained by vapor-phase osmometry or mass spectrometry. Isotope-abundance ratios from unit-resolution mass spectrometry or exact masses obtained from high-resolution mass spectrometry can sometimes establish a molecular formula, but accuracies and reliability are always dependent on purity. A net empirical formula will be obtained for an unresolved mixture of compounds. In order to determine the empirical formulae of components of a multicomponent sample, a prior separation scheme is necessary.

The ability to determine the empirical and molecular formulae of gas chromatographic eluents routinely has a significant impact on microanalysis and identification of unknown compounds. Ideally, a detector which responds

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with high specificity to each element in a compound is desirable. Atomic absorption and atomic emission techniques have been developed for element-specific detection of gas chromatographic eluates. Atomic emission affords the ability to detect several elements simultaneously by employing a multichannel or rapid-scanning spectrometer. Plasma excitation sources, for atomic emission, include the atmospheric pressure and reduced pressure microwave-induced helium plasma (m.i.p.) as well as the inductively-coupled plasma (i.c.p.) and direct current (d.c.p.) argon plasmas.

Dagnall et al. [2] reported the feasibility of obtaining inter-element ratios with an argon m.i.p. at atmospheric pressure. They showed that phosphorus-, iodine- and sulfur-to-carbon element ratios deviated from linearity by 4 to 8%. Chlorine- and bromine-to-carbon ratios were not obtainable because molecular and not atomic emissions were monitored. Most element ratioing investigations have utilized the reduced pressure helium m.i.p. Hydrogen-to-carbon ratios were obtained to within 3% relative error by McLean et al. [3]. In that study, the column elements were split for detection by both a flame ionization detector (f.i.d.) and a single-channel spectrometer. Element ratios were obtained by comparing the sequential carbon and hydrogen responses with those from the f.i.d.

Later, Van Dalen et al. [4] questioned the determination of empirical formulae because of an observed dependency of element response upon chemical structure. Dingjan and deJong [5, 6] used a multichannel spectrometer to compare hydrogen-to-carbon element ratios obtained with a helium and argon reduced pressure m.i.p. and a helium atmospheric pressure m.i.p., but the results were insufficiently accurate for the identification of unknown compounds. Poor hydrogen detection limits, however, were reported (130 pg s^{-1}) for the helium atmospheric pressure m.i.p., in comparison with other values obtained (7 pg s^{-1}) [7].

Hagen et al. [8] demonstrated multi-element tagging (chlorofluoroalkylation) and element ratioing (fluorine-to-carbon and chlorine-to-carbon) for qualitative and quantitative analysis. A reduced pressure helium m.i.p. was used. Haas and Caruso [9] used a moderate power (190 W) helium m.i.p. at atmospheric pressure. The results of element ratioing for chlorine-to-carbon and bromine-to-carbon were improved by an off-line background correction technique. Although these ratios were valid to within 1% absolute, the system suffered from a lack of sensitivity in comparison to the typical low power (atmospheric pressure) helium m.i.p.

While the previous groups reported both feasible and unfavorable element ratio results, only a few investigators have evaluated empirical-formulae determination by reduced pressure [10—14] and atmospheric pressure [15, 16] g.c./m.i.p.; all used multichannel spectrometers with a similar optical alignment and spectral resolution. Gough et al. [10] applied simultaneous carbon, hydrogen, nitrogen and fluorine-specific detection to determine, partially, the formula of a compound resulting from a pyridine catalyzed reaction. Brenner [11] determined favorable empirical formulae for a mixture of

diols (carbon, hydrogen and oxygen) and sulfur compounds (carbon, hydrogen and sulfur) but less accurate values for polychlorinated biphenyl isomers (carbon, hydrogen and chlorine) whose identities were verified by gas chromatography/mass spectrometry (g.c./m.s.). Bonnekessel and Klier [12] evaluated alkanes and compounds containing carbon, hydrogen, chlorine, nitrogen and oxygen. Because oxygen was used as a scavenger gas (0.2% O_2 v/v), it was not included in the element ratios. Most of the element-to-carbon ratios had a 10% error. Yu et al. [13] evaluated empirical formulae for a select number of halogenated (fluorine, chlorine, bromine and iodine) organics. Zeng et al. [14] determined empirical formulae for oxygenated compounds (carbon, hydrogen and oxygen) for which nitrogen was used as a scavenger gas. In both studies, most experimental values, when rounded off to the nearest integer, confirmed the corresponding known empirical formulae.

Slatkavitz et al. [15, 16] have reported the only empirical and molecular formulae determinations using a low power (50—100 W), atmospheric pressure helium m.i.p. An initial study [15] demonstrated the feasibility of the technique for chlorinated and non-chlorinated organic compounds. Calculated empirical and molecular formulae were within 2% relative. Another study [16] determined the stoichiometry of carbon, hydrogen and silicon-containing compounds. Formulae accuracies for hydrocarbons and mixed alkylsilanes were within 1.3% and 2.5%, respectively.

A helium m.i.p., maintained at atmospheric pressure, has certain advantages over its reduced pressure counterpart. These include enhanced sensitivity for most elements and the ability to view the plasma emission axially instead of transversely through the walls of the quartz discharge tube [17]. Therefore, the spectrometer's view of the atomic emission in the plasma will not be subject to interference by the formation of carbonaceous deposits on the walls of the discharge tube.

The determination of empirical formulae has also been investigated with i.c.p. as the plasma excitation source. Windsor and Denton [18] evaluated g.c./i.c.p., relative errors being approximately equivalent to those obtained by reduced- and atmospheric-pressure m.i.p.'s Unfortunately, g.c./i.c.p. affords less favorable detection limits. Morita et al. [19] also used h.p.l.c./i.c.p. to determine the empirical formula of vitamin B12.

This paper reports the evaluation of a low power (50—100 W) atmospheric pressure helium m.i.p. for the empirical-formulae determination of high resolution gas chromatographic eluates. Accuracy and precision (mean relative standard deviation) are established for various types of compounds containing carbon, hydrogen and chlorine. These examples include a series of alkanes (C_{10}—C_{24}), alkanes/alkenes and chlorinated organic compounds (aromatics, chloroalkanes). From the chromatographic retention chracteristics of the various compounds, molecular formulae can be readily deduced from empirical formulae. Factors which affect the determination of empirical formulae are discussed.

Also reported is the determination of empirical and molecular formulae of

unknown components of the chlorination products of fulvic acid. Humic substances, like humic and fulvic acids, are naturally occurring soil-derivatized organic macromolecules which are probably at innocuous concentrations in natural water. Various carcinogenic or other undesirable compounds can be derived from the chlorination of humic materials.

EXPERIMENTAL

Instrumentation

The atmospheric pressure g.c./m.i.p. multichannel spectrometer system was as described previously [15]. The microwave plasma was induced and sustained inside a TM_{010} cylindrical resonance cavity. A Model SL-10N tuner (Microlab/FXR, Livingston, NJ) was used to match the frequency and impedance between the cavity and 2.45 GHz microwave generator (Microtherm Model CMD5, Raytheon Systems Co., Lexington, MA). The photocurrent was monitored by operational amplifiers and displayed on a stripchart recorder (Omniscribe, Houston Instruments, Houston, TX) and/or acquired by a PRO-350 Micro-computer (Digital Equipment Corp., Marlboro, MA) complete with RTI (Real-Time Interface) and 16-bit ADMPC (Analog/Digital converter module). Data acquisition was performed in FORTRAN while all data analysis utilized PRO/BASIC routines.

Materials

Separations were provided by a 25-m (0.31 mm i.d., 0.52-μm film) cross-linked SE-30 (methylsilicone) fused silica capillary column (Hewlett-Packard, Avondale, PA). Regular grade helium (Union Carbide, Linde Division, Somerset, NJ) was purified by passing it through molecular sieve (5 Å)/drierite (Alltech Associates, Deerfield, IL). Quartz tubes (5 cm, 6.2 mm o.d. × 1 mm i.d.) were obtained from Quartz Scientific Co. (Fairport, OH).

Chemicals were obtained as follows. All alkanes and alkenes (Aldrich Chemical Co., Milwaukee, WI); sodium hypochlorites (J. T. Baker Chemical, Phillipsburg, NJ); 1,1,1-trichloroethane, 1,4-dichlorobutane, 1,1,2,2-tetrachloroethane, dichlorobenzene, 1,2,4-trichlorobenzene (Eastman, Rochester, NY); diethyl ether (anhydrous), chloroform, carbon tetrachloride, methylene chloride, 1,1-dichloroethane, trichloroethane, *o*-chlorotoluene, toluene (Fisher Scientific Co., Fairlawn, NJ); sodium thiosulphate (Mallincroat Chemical Works, St. Louis, MO). Diazomethane was generated from *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (Aldrich). The fulvic acid sample used in the study was extracted from local soil (Hop Brook, Amherst, MA [20]).

Analytical procedures

The empirical formulae and, in some cases, molecular formulae, were determined by g.c./m.i.p. for various carbon, hydrogen and chlorine-containing compounds (typically at sample concentrations between 1 and 5 μl ml^{-1}). The responses for simultaneous multi-element detection were displayed on

stripchart recorders and peak heights were measured. Element ratios were obtained as follows. Element-to-carbon ratios were determined from the expression:

$$\frac{E}{C} = \frac{E \text{ response of unknown}}{C \text{ response of unknown}} \times \frac{C \text{ response of reference}}{E \text{ response of reference}} \times \frac{E \text{ atoms in reference}}{C \text{ atoms in reference}}$$

In each chromatogram, one compound was designated as the "reference" and all others were treated as "unknowns". For the chlorinated organic compounds (Figs. 1—3), specific element responses were concurrently taken from 10-mV outputs (attenuated) and transmitted to the chart recorders as well as from 1-V outputs (unattenuated) to a PRO-350. Data acquisition was performed at both 2 and 10 points per s. Peak heights and peak areas, obtained from the stored signals, were used to calculate empirical formulae. These results were compared to those generated by hand. Unless otherwise stated, plasma operating conditions were 58-W microwave input power and 80 ml/min^{-1} helium. Chromatographic conditions depended on the separation scheme. In order to minimize hydrogen background response, all fittings were carefully sealed with graphite ferrules. Air leakage into the g.c./m.i.p. system was checked by

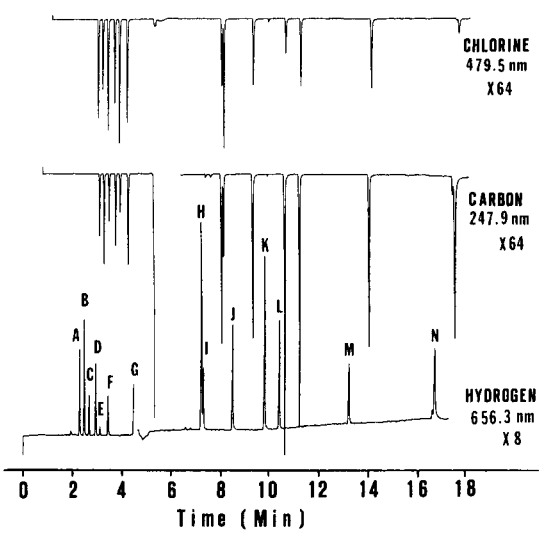
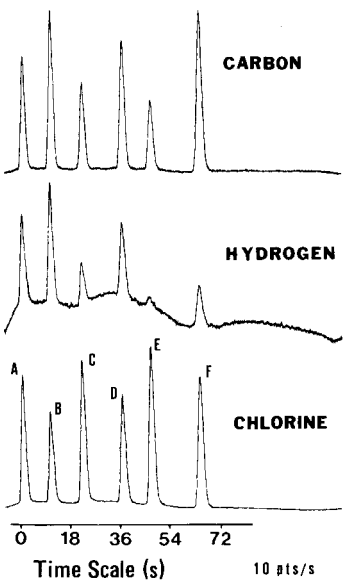


Fig. 1. Chromatograms of simultaneous carbon-, hydrogen- and chlorine-specific detection. Chromatographic conditions as in Fig. 4. Temperatures: injection, 250°C, column, 70°C for 4 min then 10° min^{-1} to 200°C. Plasma conditions as in Fig. 4. Compound concentrations were 1—5 ppt in toluene: (A) methylene chloride; (B) 1,1-dichloroethane; (C) chloroform; (D) 1,1,1-trichloroethane; (E) carbon tetrachloride; (F) trichloroethene; (G) toluene (solvent); (H) 1,4-dichlorobutane; (I) tetrachloroethane; (J) 1,4-dichloro-2-butyne; (K) *o*-chlorotoluene; (L) 1,2-dichlorobenzene; (M) 1,2,4-trichlorobenzene; (N) *o*-chloronaphthalene.



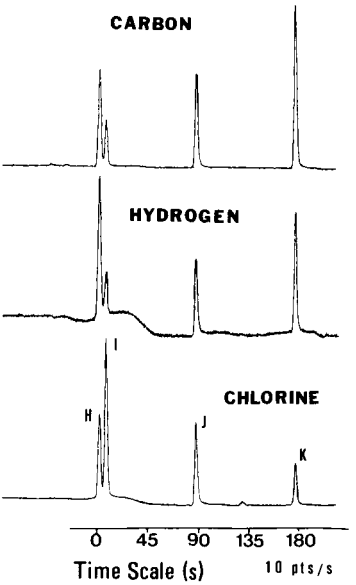


Fig. 2. Computer-generated chromatograms obtained by simultaneous carbon-, hydrogen- and chlorine-specific detection. Data acquired at 10 pts s^{-1} and plotted by a PRO-350 (16-bit A/D). Chromatographic and plasma spectroscopic conditions as in Fig. 1: (A) methylene chloride; (B) 1,1-dichloroethane; (C) chloroform; (D) 1,1,1-trichloroethane; (E) carbon tetrachloride; (F) trichloroethene.

Fig. 3. Computer-generated chromatograms obtained by simultaneous carbon-, hydrogen- and chlorine-specific detection. Chromatographic plasma spectroscopic and data acquisition conditions as in Fig. 1: (H) 1,4-dichlorobutane; (I) tetrachloroethane; (J) 1,4-dichloro-2-butyne; (K) *o*-chlorotoluene.

holding a small piece of solid carbon dioxide at the various connections and monitoring the 247.9 nm carbon emission in the plasma background. Any leaks in the system resulted in a rise in the carbon atomic emission. The chromatographic column was preconditioned at a temperature 20°C higher than the operating temperature.

The chlorinated fulvic acid sample was prepared according to Christman et al. [21], with minor modifications by Young [22]. Fulvic acid was chlorinated in water and extracted into diethyl ether. The sample (1×10^{-3} M) was buffered to pH 6.9 and reacted with aqueous sodium hypochlorite at a 5:1 (w/w) chlorine-to-carbon ratio for 24 h. This reaction was quenched with excess sodium thiosulfate at pH 0.5 and extracted into 100 ml of ether. After concentrating to 0.5 ml in a Kuderna Danish apparatus, 1.5 ml of diazomethane was used as a derivatizing agent. A 0.8-μl injection was made with a 20:1 split. Because the fused silica column was positioned to within 5 mm of the plasma, solvent venting could not be employed. The plasma, after being momentarily extinguished, was therefore reignited immediately following

elution of the solvent. Empirical formulae were obtained for these "unknown" chlorination products.

RESULTS AND DISCUSSION

Certain spectroscopic and chromatographic conditions affect accuracy and precision in determining empirical formulae. Precision is limited by plasma instability, which results from fluctuations in the microwave input power and plasma flow rate. In addition, accuracy is limited by linearity of elemental responses and any effect of chemical structure on particular element response. In determining the empirical formulae of unknown compounds, it must be assumed that the calibration graph for each element passes through the origin, is linear for the concentrations being examined, and is identical for both the unknown and reference compounds. Although elemental responses are assumed to be largely independent of chemical structure, no exhaustive study has been undertaken to verify this.

In any event, the factors which affect precision and accuracy must be considered in evaluation of empirical formulae. The g.c./m.i.p. combination has demonstrated 2—3% short- and long-term precision for operating conditions similar to those used for determining empirical formulae. Therefore, a similar precision would be expected for the results in this study.

Each element has its own set of optimal g.c./m.i.p. operating conditions, which include the helium plasma flow rate and the microwave input power. Also, the optimum atomic emission is viewed at a different position in the plasma for each element. The optimization of this set of conditions is necessary to maximize elemental sensitivity and selectivity, but compromise conditions must be selected for simultaneous multi-element detection. The goal is to retain as much sensitivity and selectivity as possible for each element detected.

In this study, the emission lines used for carbon, hydrogen and chlorine were C(I) 247.9 nm (2nd order), H(I) 656.3 nm, and Cl(II) 479.5 nm. Conditions for these three elements were all optimized at a high microwave input power (60—70 W) and a low helium flow rate (60—80 ml min^{-1}). Little compromise should result, therefore, between operating conditions for simultaneous detection and elemental detection limits and selectivities. The best viewing position for chlorine in the plasma differs slightly. Empirical and molecular formulae were determined for elemental quantities above the detection limits of carbon (2.4 pg s^{-1}), hydrogen (6.0 pg s^{-1}) and chlorine (56 pg s^{-1}).

Hydrocarbons

Simultaneous carbon- and hydrogen-specific element chromatography for the C_{10}—C_{24} alkanes, with the exception of C_{21}, is depicted in Fig. 4. Alkane concentrations were 1—3 μl ml^{-1}. Empirical formulae, in this case obtained simply from the hydrogen-to-carbon ratios, were determined by using hexa-

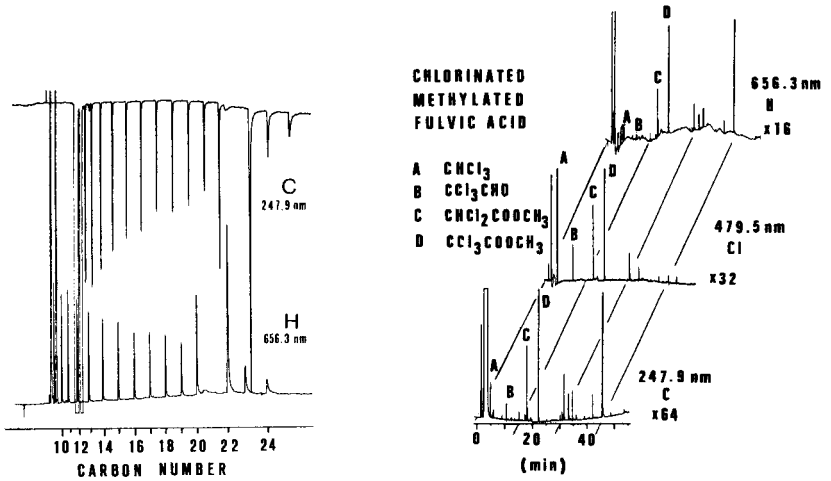


Fig. 4. Chromatograms of simultaneous carbon- and hydrogen-specific detection of C_{10}–C_{24} alkanes. Chromatographic conditions: 25 m crosslinked SE-30 fused silica capillary column, 1.0 ml min^{-1} helium, 1—80 column split, 0.1 μl sample injection. Temperatures: injection, 300°C; column, 170°C for 2 min then 10° min^{-1} to 280°C; interface, 300°C. Plasma conditions: 58 W microwave input power, 80 ml min^{-1} helium. Alkane concentrations were 1—3 ppt in toluene or ethylbenzene.

Fig. 5. Chromatograms representing simultaneous carbon-, hydrogen- and chlorine-specific detection of fulvic acid chlorination and methylation products. Column and flow rates as in Fig. 4, 0.8 μl column injection. Temperatures: injection, 250°C; column, 25°C for 10 min then 4° min^{-1} to 200°C; interface, 200°C. Plasma conditions: 60 W microwave input power and 80 ml min^{-1} helium.

decane, $C_{16}H_{34}$, as the reference and by treating the other alkanes as "unknowns". Hydrogen-to-carbon ratios are shown in Table 1. The corresponding empirical and molecular formulae are listed in Table 2. Molecular formulae were readily deduced from empirical formulae by determination of the chromatographic retention behavior of the various compounds. Each formula, determined experimentally, is the mean value of three results.

The mean relative standard deviation for the determination of 13 formulae was 1.3%. Relative errors between the known and experimental formulae ranged between no detectable difference to 2.3%. The mean relative error was 0.7%. With the exception of that of decane, all formulae were $\leqslant$1% of the correct values. Examination of the specific element chromatograms (Fig. 4) shows that decane eluted only a few s after the ethyl benzene, solvent peak. This, and the lower formula accuracy obtained for decane, suggested that the microwave plasma had not fully regained stability from solvent perturbation when decane entered the discharge region.

The results of three decane trials gave a 1.5% relative standard deviation indicating that the plasma is repeatably less stable for the evaluation of any compound eluting immediately after the solvent. Undecane, which eluted

TABLE 1

Hydrogen-to-carbon ratios for alkanes measured by g.c./m.i.p.

Compound	Element ratios		
	Known	Determined[a]	R.s.d.[b] (%)
$C_{10}H_{22}$	2.20	2.15	1.5
$C_{11}H_{24}$	2.18	2.15	0.4
$C_{12}H_{26}$	2.17	2.16	1.8
$C_{13}H_{28}$	2.15	2.14	2.4
$C_{14}H_{30}$	2.14	2.12	1.1
$C_{15}H_{32}$	2.13	2.13	0.7
$C_{16}H_{34}$	2.13	Reference	
$C_{17}H_{36}$	2.12	2.10	1.1
$C_{18}H_{38}$	2.11	2.10	1.7
$C_{19}H_{40}$	2.11	2.10	1.4
$C_{20}H_{42}$	2.10	2.08	1.4
$C_{22}H_{46}$	2.10	2.09	1.5
$C_{23}H_{48}$	2.09	2.09	0.8
$C_{24}H_{50}$	2.08	2.08	1.2

[a]Average of 3 results. [b]Relative standard deviation of a single result.

TABLE 2

Empirical and molecular-formulae determination of alkanes by g.c./m.i.p.

Compound	Formula[a]	R.s.d.[b] (%)
$C_{10}H_{22}$	$C_{10}H_{21.5}$	1.5
$C_{11}H_{24}$	$C_{11}H_{23.7}$	0.4
$C_{12}H_{26}$	$C_{12}H_{2.9}$	1.8
$C_{13}H_{28}$	$C_{13}H_{27.8}$	2.4
$C_{14}H_{30}$	$C_{14}H_{29.7}$	1.1
$C_{15}H_{32}$	$C_{15}H_{31.9}$	0.7
$C_{16}H_{34}$	Reference	
$C_{17}H_{36}$	$C_{17}H_{35.7}$	1.1
$C_{18}H_{38}$	$C_{18}H_{37.8}$	1.7
$C_{19}H_{40}$	$C_{19}H_{39.9}$	1.4
$C_{20}H_{42}$	$C_{20}H_{41.5}$	1.4
$C_{22}H_{46}$	$C_{22}H_{46.0}$	1.5
$C_{23}H_{48}$	$C_{23}H_{48.0}$	0.8
$C_{24}H_{50}$	$C_{24}H_{49.9}$	1.2

[a]Mean of 3 results. [b]Relative standard deviation of a single result. The mean value for all results is 1.3%.

30 s after decane, had better accuracy, 1.3%, although this value was still above the mean relative error. It appears, in this example, that a 30 s recovery time is adequate for plasma restabilization.

The advantage in solvent venting is that large quantities of analyte are not allowed to saturate the plasma discharge tube. Quantities of analyte in excess of 50 μl will typically extinguish a low power (50—100 W) m.i.p. Because a fused silica capillary column was used in these empirical-formula determinations, the sample injection (0.1 μl) and column split ratio (1:80) were such that only 1 nl of analyte entered the plasma at any given time. This quantity is not enough to extinguish the plasma, but is evidently sufficient to create plasma instability. Solvent venting, however, has its disadvantages. Components which elute immediately after the solvent are also vented. In venting, the end of the chromatographic column must be positioned before the venting T. As a result, chromatographic resolution will be lower than when the column is placed within 5 mm of the TM_{010} cavity.

In order to chromatograph the high boiling, high molecular weight hydrocarbons successfully, high injection port, column and interface temperatures were necessary. The carbon and hydrogen emission background levels were greater at these high operating temperatures. Column bleed and the out-gasing of high molecular weight oils from the stainless steel fittings and injection port contributed to this problem. It was necessary to condition the g.c./m.i.p. system overnight at these high operating temperatures to achieve more stable, lower carbon and hydrogen emission backgrounds.

The accuracy of these empirical and molecular formulae also suggests that, at least over a range of 14 carbon and 28 hydrogen atoms, elemental responses are not significantly affected by chemical structure. If structural dependences were more important, an accuracy much poorer than 0—2% would have been obtained. Dingjan and deJong [6] recommended the use of a post-column/pre-plasma pyrolysis unit to give a more complete fragmentation of high molecular weight compounds. Chiba and Haraguchi [23] designed such a system to operate at 900°C. Such a pyrolysis unit was unnecessary for the series of alkanes studied in this work (Table 2) and it is concluded that provided that there are no cold-spots along the g.c./m.i.p. interface, a pyrolysis unit should not be needed for alkanes of up to at least C_{24}. It remains possible that the complete fragmentation of high molecular weight biomolecules may require the use of an additional heating zone.

Microanalysis for carbon, hydrogen and nitrogen by combustion techniques, usually demands a ±0.3% accuracy in order for a positive formula identification to be made. The accuracies obtained by g.c./m.i.p. were, on average, ±0.6%. However, g.c./m.i.p. has the advantage of a separation technique, and retention characteristics of a compound can provide added information that microanalysis cannot give. The latter is also dependent on the purity of a compound, but in g.c./m.i.p. contaminants may elute at different times from the compound of interest. Also, because element ratios are used to determine formulae in g.c./m.i.p., the exact concentrations of each compound (unknown and reference) need not be known.

In separate studies, for which the chromatographic and spectroscopic conditions were somewhat different [15, 16], formulae were obtained for alkanes and alkenes. Different reference compounds, tridecane and pentadecane, were used in each case. These studies showed that accurate formulae could be obtained for compounds with the same carbon number but different hydrogen-to-carbon ratios.

Chlorinated organic compounds

The empirical formulae of a number of chlorinated organic compounds were determined. Simultaneous carbon-, hydrogen- and chlorine-specific element chromatography is shown in Fig. 1, where species with a range of chemical structures were included in this test solution. Empirical and molecular formulae were obtained with 1,1-dichloroethane as the reference and the other compounds as unknowns. The experimental results are listed in Table 3.

Good accuracies were obtained for all chlorine-to-carbon ratios, but although rounded hydrogen values agreed with expected formulae, the absolute and relative errors were higher than those for the hydrocarbons. This may reflect a minor dependence of specific element response on chemical structure, but empirical formulae determinations are still useful. It is also interesting to note that the mean relative standard deviation for the hydrogen results (1.8%) was twice that for chlorine (0.9%). Both of these values are close to that for the formulae determination of alkanes (1.3%).

TABLE 3

Empirical- and molecular-formulae determination of chlorinated organic compounds by g.c./m.i.p.

Compound	Formula[a]	R.s.d. (%)[b]	
		H	Cl
A CH_2Cl_2 (dichloromethane)	$CH_{2.1}Cl_{2.0}$	0.2	0.7
B $C_2H_4Cl_2$ (1,1-dichloroethane)	Reference	--	—
C $CHCl_3$ (chloroform)	$CH_{1.3}Cl_{2.9}$	2.0	0.6
D $C_2H_3Cl_3$ (trichloroethane)	$C_2H_{3.1}Cl_{3.0}$	2.0	0.7
E CCl_4 (carbon tetrachloride)	$CCl_{4.0}$	—	1.1
F C_2HCl_3 (trichloroethene)	$C_2H_{1.4}Cl_{2.9}$	3.3	1.0
G Solvent (toluene)			
H $C_4H_8Cl_2$ (1,4-dichlorobutane)	$C_4H_{7.6}Cl_{2.1}$	0.6	0.8
I $C_2H_2Cl_4$ (tetrachloroethane)	$C_2H_{2.3}Cl_{4.0}$	1.6	1.3
J $C_4H_4Cl_2$ (1,4-dichloro-2-butyne)	$C_4H_{4.0}Cl_{2.1}$	1.9	0.5
K C_7H_7Cl (*o*-chlorotoluene)	$C_7H_{6.7}Cl_{1.1}$	2.2	0.8
L $C_6H_4Cl_2$ (dichlorobenzene)	$C_6H_{4.0}Cl_{2.1}$	0.9	0.9
M $C_6H_3Cl_3$ (trichlorobenzene)	$C_6H_{3.3}Cl_{3.1}$	2.6	1.2
N $C_{10}H_7Cl$ (*o*-chloronaphthalene)	$C_{10}H_{6.8}Cl_{1.1}$	2.5	1.6

[a]Mean of 3 results. [b]Relative standard deviation of a single result. The mean values for all results are 1.8% (C) and 0.9% (Cl).

The effect of non-ideal chromatographic resolution on the determination of empirical formulae is also demonstrated in Fig. 1. Baseline resolution was obtained for all compounds except 1,4-dichlorobutane and tetrachloroethane. In order to determine empirical formulae, peak heights were measured because, for overlapping peaks, this approach is much easier than the measurement of peak areas. The chlorine-to-carbon ratios showed no detectable absolute error and, in general, the accuracy and precision for the formulae determination of these overlapping peaks did not differ from those obtained for the resolved peaks.

A small response for carbon tetrachloride was observed at the hydrogen 656.3-nm emission line. This type of non-selective response was also reported by Bonnekessel and Klier [12] for alkane responses at the chlorine emission line. The carbon tetrachloride non-selective response at the hydrogen channel results in a $CCl_{4.0}H_{0.3}$ empirical formula. The chromatographic retention of this compound makes a $C_3Cl_{12}H$ formula most unlikely and thus CCl_4 is a suitable empirical-formula choice.

For this mixture of chlorinated organic compounds, specific element responses were simultaneously output to chart recorders as well as to a PRO-350 microcomputer, where data acquisition was performed at both 2 and 10 points per s. Peak heights and peak areas, obtained from the stored responses, were used to calculate empirical formulae. Figure 2 demonstrates the computer generated element specific chromatograms for the lower boiling compounds and Fig. 3 shows the less volatile ones. In both cases, data were acquired at 10 points per s. Much more baseline drift is observed in Figs. 2 and 3 than in Fig. 1, particularly for the hydrogen emission line. This may be due to noise pick-up because of incomplete grounding and shielding.

Tables 4 and 5 show computer generated empirical formulae for comparison with those obtained from the stripchart recorders. Both peak height and peak area measurements are obtained at 2 and 10 points per s. These values were used to calculate the stoichiometry. Results obtained by hand calculation using peak heights compared well with those obtained using computer generated peak heights. The computer generated peak areas were also close to these values but were more dependent on which data points were used to begin and end integration. Although a clear difference in peak shape could be observed for the 2 and 10 points per s plots, reasonable agreement in stoichiometry was found when the peak height and area measurements were used. While hand measurements of peak heights on stripchart recorders provide a viable means for determining stoichiometry, a computer data-acquisition/manipulation system affords the added ability to recall and re-evaluate data, an important feature for analyzing complex chromatograms. The major requirement here is the need to develop a good algorithm for chromatographic analysis.

Fulvic acid chlorination products

Humic substances (humic and fulvic acids) are naturally occurring soil-derived organic macromolecules present in natural water. Chlorination, done

TABLE 4

Comparison of computer-acquired peak height and area measurements for the calculation of empirical- and molecular-formulae

Compound formula	Peak height[a]				Peak area[a]			
	I	II	III	IV	I	II	III	IV
Methylene chloride								
C 1	1	1	1	1	1	1	1	1
H 2	2.2	2.1	2.2	2.1	2.2	2.1	2.2	2.0
Cl 2	1.9	2.0	1.8	2.0	1.6	2.0	2.0	1.9
1,1-Dichloroethane								
C 2								
H 4			Reference					
Cl 2								
Chloroform								
C 1	1	1	1	1	1	1	1	1
H 1	1.3	1.3	1.2	1.3	1.3	1.3	1.2	1.2
Cl 3	2.8	2.9	2.8	2.9	2.7	2.9	2.9	2.9
Trichloroethane								
C 2	2	2	2	2	2	2	2	2
H 3	3.3	3.1	3.1	3.2	3.2	3.2	3.1	3.0
Cl 3	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Carbon tetrachloride								
C 1	1	1	1	1	1	1	1	1
H 0	—	—	0.4	0.3	0.6	0.3	0.4	0.3
Cl 4	4.0	3.9	3.9	4.0	4.1	3.9	4.1	4.0
Trichloroethene								
C 2	2	2	2	2	2	2	2	2
H 1	1.5	1.4	1.3	1.4	1.4	1.4	1.4	1.4
Cl 3	2.9	2.9	2.9	2.9	2.9	2.9	2.8	2.8
1,4-Dichlorobutane								
C 4	4	4			4	4		
H 8	7.3	7.6			7.3	7.7		
Cl 2	2.0	2.0			1.9	2.0		
Tetrachloroethane								
C 2	2	2			2	2		
H 2	2.3	2.4			2.2	2.5		
Cl 4	3.9	4.0			3.8	3.9		
1,4-Dichloro-2-butyne								
C 4	4	4			4	4		
H 4	3.9	4.0			4.0	4.0		
Cl 2	2.0	2.1			2.1	2.1		
o-Chlorotoluene								
C 7	7	7			7	7		
H 7	6.9	6.7			7.5	6.8		
Cl 1	1.1	1.0			1.1	1.0		
Dichlorobenzene								
C 6	6				6			
H 4	4.0				3.9			
Cl 2	2.0				2.0			

[a]Trials I and II, 10 pt s^{-1}; trials III and IV 2 pt s^{-1}.

TABLE 5

Empirical- and molecular-formula determinations of unknown chlorination products of fulvic acid by g.c./m.i.p.

Compound	Formula[a]	R.s.d. (%)[b]	
		H	Cl
$C_3H_3Cl_3O_2$ (trichloroacetate)	Reference		
$C_3H_4Cl_2O_2$ (dichloroacetate)	$C_3H_{3.9}Cl_{2.0}O_x$	1.8	3.6
$C_2H_1Cl_3O_1$ (chloral)	$C_2H_{0.9}Cl_{2.9}O_y$	8.7	1.1
$CHCl_3$ (chloroform)	$C_1H_{1.1}Cl_{2.9}$	11.1	0.4

[a]Mean of 3 results. [b]Relative standard deviation of a single result.

for disinfection of drinking water, can cause these humic materials to give rise to various carcinogenic or otherwise undesirable compounds, such as dichloroacetic acid, trichloroacetic acid, chloroform, trichloroacetaldehyde (chloral) and dichloroacetonitrile as well as mixed halomethanes. The chlorination products of humic and fulvic acids have been examined by chlorine- and carbon-specific detection by g.c./m.i.p. [24—27].

The g.c./m.i.p. method was evaluated to determine the stoichiometry of unknown compounds from the chlorination and methylation of fulvic acid [21, 28, 29]. The simultaneous carbon-, hydrogen- and chlorine-specific chromatograms for this sample are shown in Fig. 5. Methyl trichloroacetate was identified by its retention time, and using it as a reference, empirical formulae of other major components were determined. The results are present in Table 5, methyl dichloroacetate, chloral and chloroform were positively identified from their calculated stoichiometries.

In order to maintain the plasma when the solvent (ca. 0.07 μl of ether) entered the microwave cavity, the input power was raised momentarily to 80—90 W and then lowered to the 60-W operating level. Although the precision of the chlorine-to-carbon ratio for three trials appears to be similar to previous results (see Table 3), the hydrogen-to-carbon precision was initially poor after solvent peak elution. If variation of the microwave input power caused this lack of precision, then the carbon-to-chlorine rate should also have been affected. This complication is more likely to be due to "saturation" of the discharge tube walls by solvent. While the relative standard deviation was greater than 10%, the accuracy for these three trials still permitted a positive identification of chloroform.

Chlorinated organic compounds are often detected by an electron-capture detector (e.c.d.). Although the e.c.d. is more sensitive than carbon- or halogen-specific m.i.p. detection for many species, this sensitivity depends on the nature of the electronegative compound. This significant response dependence on chemical structure necessitates the use of a separate calibration graph for each compound.

On the other hand, g.c./m.i.p. has been established as an element-specific

detector, high sensitivities and selectivities being observed for both metals and non-metals. In theory, if fragmentation and excitation are complete or at least reproducibile, then quantitation could be obtained by use of any one of a number of compounds to produce a representative calibration graph. In practice, however, depending on the required accuracy, it may or may not be satisfactory to use a single calibration graph for the quantitation of several structurally different compounds.

The g.c./m.i.p. method can be valuable for the determination of the formula of an unknown compound; however, the less that is known about the analyte, the greater the number of element-specific wavelengths that must be monitored. In this study, carbon, hydrogen and chlorine compounds were evaluated. Several other elements might readily be added to this list for routine microanalysis. Oxygen and nitrogen would be the two elements to have the greatest impact when extending the practical use of g.c./m.i.p. for empirical- and molecular-formulae determination.

Oxygen-specific detection at atmospheric pressure has been evaluated recently [30]. A hydrogen-rich helium plasma was used to transform any oxygen background emission to OH molecular emission. A detection limit of 212 pg s^{-1} was reported. The feasibility of nitrogen-specific detection has not been addressed by atmospheric pressure g.c./m.i.p. The results presented thus far, however, indicate that the advantages far outweigh any disadvantages of g.c./m.i.p. as an analytical technique. High resolution g.c. with a simultaneous, multielement, specific detector may become one of the most valuable analytical methods for the identification of unknown compounds.

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THE SIMULTANEOUS DETERMINATION OF COPPER AND NICKEL BY LIQUID-LIQUID EXTRACTION AND GAS-LIQUID CHROMATOGRAPHY WITH (ACETYLPIVALYLMETHANE)-(TRIFLUOROACETYLACETONE)ETHYLENEDIIMINE AS REAGENT

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SUMMARY

The unsymmetrical tetradentate ligand, 1,1,1-trifluoro-1',1',1'-trimethyl-4,4'-(ethane-1,2-diyldiimino)bis(pent-3-en-2-one), derived from the condensation of trifluoroacetylacetone and acetylpivalylmethane and trivially named (acetylpivalylmethane)(trifluoroacetylacetone)ethylenediimine has been isolated and its copper(II) and nickel chelates have been prepared. These chelates are readily separated by gas chromatography and a method is proposed for the simultaneous determination of copper and nickel by liquid-liquid extraction of their chelates with the new reagent followed by gas chromatography on a column of OV-1 supported on Gas Chromb Q at 230°C. The method is applied to the determination of the composition of cupro-nickel coinage metal.

The early development of the gas chromatography of metal chelates centred on the fact that most of the metal chelates so utilised were derived from β-diketones [1]. The need for alternative ligands to β-diketones for the gas chromatography of metal chelates became evident when, in attempts to improve such factors as thermal stability, volatility and resolution of a mixture of metal chelates of a given ligand showed deficiencies in chelate characteristics. One of the most interesting related ligand systems involves the tetradentate-β-ketoamines, and it is now well established that, in terms of thermal stability and gas chromatographic applicability, chelates of these β-ketoamines have many superior features for the analysis of divalent transition metals such as copper, nickel and palladium [2—7] and vanadium [8], over the simpler β-diketonates.

A recent development has involved the mixed fluorinated/non-fluorinated tetradentate-β-ketoamines as alternative ligands [2, 9], e.g., (acetylacetone)-(trifluoroacetylacetone)ethylenediimine, H_2[en(AA)(TFA)], 1,1,1-trifluoro-4,4'-(ethane-1,2-diyldiimino)bis(pent-3-en-2-one). A number of advantages

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are envisaged [6]. The compromise in structure, thermal stability, reaction rates and chromatographic behaviour can work to analytical advantage, and the molar flame-ionisation response is higher than that of the fluorinated chelates and is almost as high as that for the wholly non-fluorinated species. The present paper describes the preparation of the mixed fluorinated/non-fluorinated tetradentate-β-ketoamine, (acetylpivalylmethane)(trifluoroacetylacetone)ethylenediimine H_2[en(APM)(TFA)], 1,1,1-trifluoro-1′,1′,1′-trimethyl-4,4′-(ethane-1,2-diyldiimino)bis(pent-3-en-2-one), and its copper and nickel chelates to assess the usefulness of this ligand as an analytical reagent. The nonsystematic nomenclature of the ketoamine is used to indicate structure, mode of preparation and relationship with the parent diketones, and is used subsequently in this paper.

In the development of H_2[en(APM)(TFA)] as an analytical reagent, the thermogravimetric, spectrophotometric and gas chromatographic characteristics of its copper(II) and nickel(II) chelates were studied together with the solvent extraction of these chelates from aqueous solution. The synthesis of the reagent is complicated by the fact that, unlike the preparation of H_2[en(AA)(TFA)], H_2[en(APM)(TFA)] is not produced as a major product in the condensation of ethylenediamine with the two β-diketones. The reagent was isolated by converting the crude reaction product into the mixed copper chelates, which were then separated by column chromatography. The desired reagent was then regenerated from its copper chelate by precipitation with hydrogen sulphide.

EXPERIMENTAL

Preparation of (acetylpivalylmethane)(trifluoroacetylacetone)-ethylenediimine

About 1 g of the purified copper chelate of this compound (for preparation see below) was dissolved in benzene (25 cm^3) and hydrogen sulphide from a lecture bottle (Matheson) was bubbled through it for 30 min. The black CuS precipitated was filtered with a No. 3 sintered glass crucible. The solvent was driven away at about 60°C under reduced pressure, leaving behind a yellowish compound (crude yield, 0.74 g). The crude product was recrystallized twice from ethanol; its melting point was 112—113°C. Elemental analysis gave 56.5% C, 7.5% H, 8.8% N and 18.0% F; $C_{15}H_{23}F_3N_2O_2$ requires 56.2% C, 7.24% H, 8.75% N and 17.8% F.

Preparation of (acetylpivalylmethane)(trifluoroacetylacetone)-ethylenediimine-copper(II)

Acetylpivalylmethane, H(APM), (2.8 g; 0.02 mole) prepared by the condensation of pinacolone with ethylacetate in the presence of sodium hydride was dissolved in ethanol (45 cm^3) contained in a 100-cm^3 beaker. Pure reagent-grade ethylenediamine (1.2 g; 0.02 mole) was dissolved in 15 cm^3 of ethanol and trifluoroacetylacetone, H(TFA), (3.1 g, 0.02 mole) was dissolved

in 45 cm^3 of ether, and these solutions were added dropwise from two small separating funnels to the mechanically stirred solution of H(APM) and the stirring was continued for 30 min. The solvents were evaporated off under reduced pressure leaving behind an orange viscous liquid. The crude product was dissolved in ethanol (20 cm^3) contained in a 100-cm^3 beaker. Copper(II) perchlorate (3 g) dissolved in ethanol (40 cm^3) was slowly added with stirring to the solution of the crude product. Water (30 cm^3) was then added with stirring to precipitate the violet-coloured copper complex, which was filtered off and dissolved in 20 cm^3 of ether. Silica gel 60 (7 g; 70-230 mesh ASTM) was added and the ether was carefully evaporated on a warm water bath in a fume-cupboard. The complex adsorbed on the silica gel was added to a chromatographic column containing about 70 g of the same silica gel, and the chelate was eluted with ether. During development of the column, three different bands were observed, a slow-moving one above two fast-moving bands. Fortunately, the latter two can be separated. The second violet band eluted from the column was collected and a violet material was obtained from the evaporated solution (crude yield, 0.55 g). The crude product was recrystallised twice from acetone where violet crystals of the copper(II) complex were obtained (m.p. 184—185°C). Elemental analysis gave 46.6% C; 5.5% H; 7.3% N; 15.0% F; $C_{15}H_{21}F_3N_2O_2$ Cu requires 47.2% C, 5.54% H, 7.34% N and 14.9% F. The complex gave a single peak when examined by gas chromatography.

Preparation of (acetylpivalylmethane)(trifluoroacetylacetone)-ethylenediimine-nickel(II)

The corresponding nickel complex was prepared by adding nickel(II) chloride hexahydrate (0.24 g; 1 mmol) dissolved in ammonia (1 M; 5 cm^3) to an acetone solution of the ligand (0.32 g, 1 mmol), and the reaction mixture was heated on a steam bath for 15 min. The brown chelate precipitated was extracted with ether. The solvent was evaporated under reduced pressure leaving behind a fluffy brown compound (crude yield, 0.15 g). The nickel chelate was recrystallised twice from acetone. The nickel chelate melted at 208—209°C. Elemental analysis gave 48.0% C, 5.7% H, 7.5% N, 14.7% F; $C_{15}H_{21}F_3N_2O_2$ Ni requires 47.8% C, 5.61% H, 7.43% N and 15.1% F. The complex gave a single peak when examined by gas chromatography.

Solutions

Solutions of the ligand H_2[en(APM)(TFA)] (0.02 M) were prepared in ethylacetate and in ethanol. Solutions of the metal chelates (about 0.002 M) were prepared in ethylacetate. Metal salts were of analytical reagent grade. Organic solvents were redistilled from laboratory reagent-grade products.

Instrumentation

A Unicam SP 800 recording spectrophotometer with 10-mm silica cells was used to record the spectra of the chelates. A Perkin-Elmer Model TGS-1

thermobalance was used to trace the thermogravimetric curves of the two chelates, a nitrogen (oxygen-free) flow rate of 50 cm^3 min^{-1} was used, and the temperature scan rate was 20°C min^{-1}.

A Varian series 3700 gas chromatograph equipped with flame ionization detection was used with a Houston Super Scribe chart recorder. Hydrogen and air were used for the detection system. The flow rate of the nitrogen (oxygen free) carrier gas was controlled by an automatic flow controller. The glass column (6 ft. × 2 mm i.d.) used was packed with 3% OV-1 (or QF-1) on Gas Chromb Q (100—120 mesh) (Applied Science Division, Milton Roy Co. Laboratory Group). The column was preconditioned for 16 h at the maximum operating temperature. Samples were injected into the column from a 5-μl syringe with a removable needle (Scientific Glass Engineering Pty. Ltd., Australia). For the chromatography of the individual metal complexes, a column temperature of 230°C was generally used with injection port and detector oven temperatures at 240 and 250°C, respectively. Carrier gas flow was 10 cm^3 min^{-1}. A gas chromatogram of H_2[en(APM)(TFA)] is shown in Fig. 1.

Simultaneous determination of copper and nickel

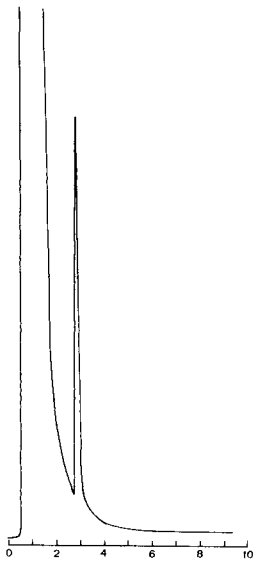
Solutions (1 cm^3) containing copper and nickel (0.02—0.12 mg cm^{-3}) were taken in 5-cm^3 volumetric flasks, and 1 cm^3 of 0.1 M potassium acetate buffer solution was added to give pH 7.0. A solution of H_2[en(APM)(TFA)] in ethanol (1 cm^3) was added and the mixture was heated on a boiling water bath for 15 min. Ethyl acetate (2 cm^3) was added to the cooled mixture and the flasks were tightly closed with glass stoppers. The copper and nickel chelates were extracted by shaking the flasks for 2 min on an ultrasonic cleaner. The mixtures were allowed to stand for about 30 min so that the aqueous and organic phases separated. Portions (1—5 μl) of the organic phase were subjected to gas chromatography with the standard column, maintained at 230°C and a carrier gas flow rate of 10 cm^3 min^{-1}.

For the analysis of the coins, the sample (ca. 5.7 g) was dissolved in aqua regia with warming to expel free chlorine and nitrous oxide fumes. The solution was diluted to 500 cm^3, and 5 cm^3 of this solution were diluted to 250 cm^3. Finally, 10 cm^3 of the diluted solution were further diluted to 250 cm^3, after adjusting the pH to 6.0—7.0 with 0.1 M sodium hydroxide. An aliquot (1 cm^3) of this solution was taken for analysis as above.

RESULTS AND DISCUSSION

Spectrophotometry of the metal chelates

Freshly prepared solutions of the copper and nickel chelates of H_2[en(APM)(TFA)] in chloroform showed, for the copper complex, a simple broad band with a maximum at 550 nm and for the nickel complex, a narrower band with peaks at 356 and 371 nm. The molar absorptivities were calculated to be 250.3 l mol^{-1} cm^{-1} for the copper complex at 550 nm, and 4009 and 3838 l mol^{-1} cm^{-1} for the nickel complex at 356 and 371 nm, respectively.



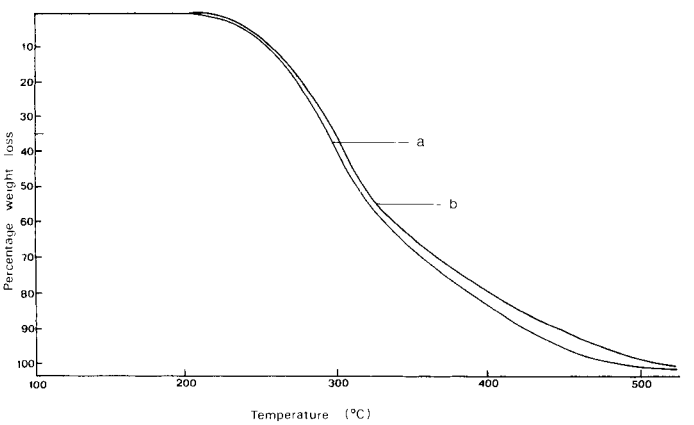


Fig. 1. Gas chromatogram of H_2[en(APM)(TFA)]. Glass column (6 ft. × 2 mm i.d.) packed with 3% OV-1 on Gas Chromb Q (100—120 mesh). Column at 230°C; injection port 240°C; detector 250°C; nitrogen flow, 10 cm^3 min^{-1}.

Fig. 2. Thermogravimetric curves for: (a) copper(II); (b) nickel(II) chelates of H_2[en-(APM)(TFA)].

Since the molar absorptivities of these two complexes are comparatively small, they were not considered to provide a basis for spectrophotometric determinations of copper or nickel.

Thermogravimetric analysis

The thermogravimetric curves for the copper and nickel chelates of H_2[en-(APM)(TFA)] (Fig. 2) showed complete volatilization of these chelates. Both chelates started to sublime around 210°C. The weight loss for both chelates at 330°C was found, by separate experiments, by stopping heating at 330° and reweighing the aluminium pan after cooling it to room temperature; the weight loss at 330°C was 100% for both chelates.

A closer look at the thermobalance during the heating cycle revealed that in both cases some crystals were seen to start to accumulate around 250°C on the metal baffle plate as well as on the suspension wire, but they later disappeared completely around 500°C. This is perhaps why the thermogravimetric curves for the copper and nickel chelates dropped only gradually after volatilization began and why these curves dropped eventually to the baseline.

Liquid-liquid extraction of Cu[en(APM)(TFA)] and Ni[en(APM)(TFA)]

A study of the effectiveness of the extraction of the copper and nickel chelates from the aqueous phase to the organic phase showed that ethyl

acetate was the best organic solvent. Since both chelates were insufficiently soluble in n-hexane, this solvent was not good for the extraction. The chelates were quite soluble in both chloroform and ether, but ether was too volatile and the separation of aqueous and organic phases was poor when chloroform was used. Neither of these organic solvents was, therefore, suitable for the extraction of the copper and nickel chelates.

Belcher et al. [7] found that nickel was not extracted at pH <8.0, nor was there any evidence for the extraction of such metal ions as chromium(III), manganese(II), iron(II), iron(III), cobalt(II) and zinc(II), when the ions were subjected to the general treatment in amounts 10-fold in excess of that of copper using H_2[en(APM)$_2$] as the ligand. This was also observed with H_2-[en(APM)(TFA)] as the ligand using borate buffers. However, when the pH of the aqueous phase was adjusted to pH 10—11, using 1 M ammonia, nickel could be extracted without difficulty. The discrepancy can be explained by the fact that nickel was precipitated at pH >8 and chelate formation was thus prevented. However, ammonia can keep nickel in solution by complex formation and Ni[en(APM)(TFA)] can then be formed when the ammonia ligands are displaced. It was observed that the amount of metal chelates that could be extracted was substantially decreased in the presence of such ions as chromium(III) and manganese(II), which were precipitated as "hydroxides" at pH 10. Thus, although ammonia is good as a buffer for extraction of binary mixtures of copper and nickel, it is not suitable when other metal ions forming insoluble "hydroxides" are present. For this reason, pH 7 was taken as the recommended pH, since the interferences caused by such ions as chromium(III), manganese(II), iron(II), cobalt(II) and zinc(II) were negligible when these ions were present in amounts two-fold in excess of those of copper or nickel. Iron(III) interfered seriously with the determination because neither copper nor nickel chelate was extracted in the presence of iron(III). The interference could be minimized by reducing iron(III) with hypophosphite; when 70—80 mg of sodium hypophosphite was added to 25 μg of copper or nickel in 1 ml of sample solution, 50 μg of iron(III) could be tolerated.

The extent of extraction was studied by comparing the area of the gas chromatographic peak obtained by injecting a suitable aliquot of the organic phase, after extraction, into the chromatograph with those expected from a solution of the metal chelate dissolved directly in the same solvent. The extraction of both copper and nickel chelates by ethyl acetate following the procedure described above was found to be quantitative when the pH of the aqueous phase was 7.0. To avoid any error caused by less than quantitative extraction, calibration graphs for the determination of copper and nickel in real samples should be prepared by extracting the copper or nickel chelate from aqueous solutions containing known amounts of the respective metal.

Gas chromatography of Cu[en(APM)(TFA)] and Ni[en(APM)(TFA)]

The instrument used for these studies was a standard commercial equipment employing an unmodified flame ionization detector. In view of the

relatively high temperatures needed to volatilize the two metal complexes, suitable liquid phases with high upper temperature limits were selected. In qualitative experiments with Cu[en(APM)(TFA)], successful chromatography was obtained on a glass column (6 ft. × 2 mm i.d.) containing Gas Chromb Q (100—120 mesh) coated with OV-1, at a 3% loading. The carrier gas flow of nitrogen was optimized at 10 cm^3 min^{-1} and the injection port and detector oven temperatures were maintained at 10° and 20°C, respectively, above the temperature of the column.

Unlike the case for Cu[en$(APM)_2$] [7], the shapes of the eluted peaks were not temperature dependent in the range 220—260°C; sharp, symmetrical peaks were obtained, and the resolution of complex and solvent peaks was good. A typical gas chromatogram of the copper chelate is shown in Fig. 3a; Ni[en(APM)(TFA)] was also satisfactorily eluted under these conditions (Fig. 3b). Good Gaussian shaped elution peaks were obtained for both copper and nickel chelates, with no indication of decomposition or sample loss on the column, and with good base-line return. At 230°C, the retention times for the two chelates were 417 s (copper) and 456 s (nickel).

Figure 4 depicts a chromatogram of Cu[en$(TFA)_2$], Cu[en(APM)(TFA)] and Cu[en$(APM)_2$] under the conditions mentioned above. It can be seen

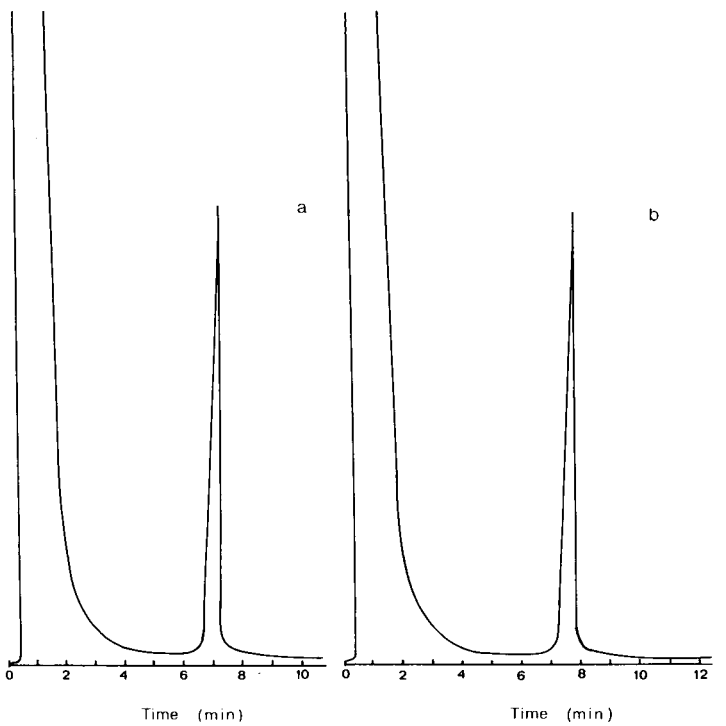


Fig. 3. Gas chromatograms of: (a) copper(II); (b) nickel(II) chelates of H_2[en(APM)-(TFA)]. Chromatographic conditions as in Fig. 1.

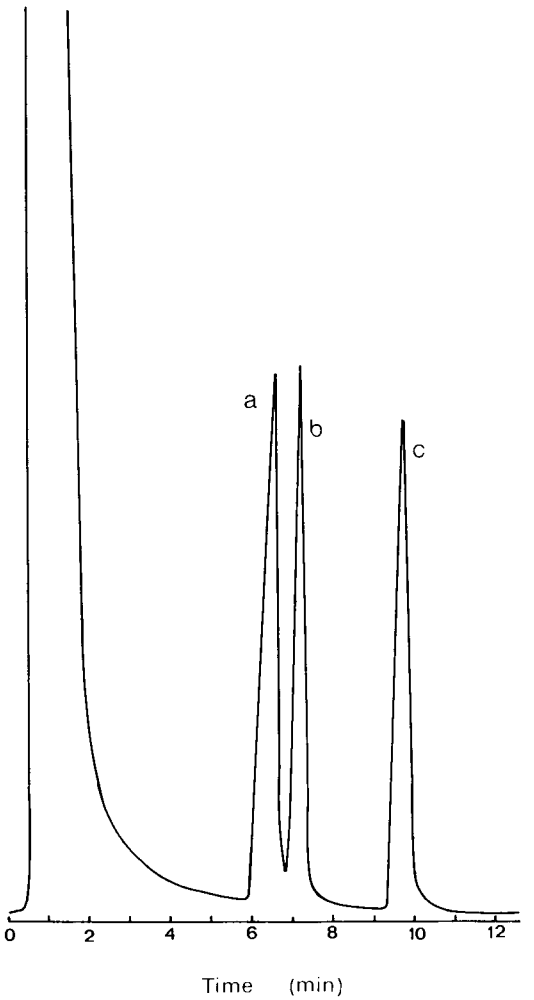


Fig. 4. Gas chromatographic separation of copper(II) chelates of: (a) $H_2[en(TFA)_2]$; (b) $H_2[en(APM)(TFA)]$; (c) $H_2[en(APM)_2]$. Chromatographic conditions as in Fig. 1.

that the volatility of Cu[en(APM)(TFA)] lies between those of Cu[en-$(TFA)_2$] and Cu[en$(APM)_2$], but is closer to that of Cu[en$(TFA)_2$]. The same observation holds for the analogous nickel chelates (see Fig. 5) and, for these chelates, the similarity in volatility of Ni[en$(TFA)_2$] and Ni-[en(APM)(TFA)] is even greater. Note that the column temperature used here was 260°C in order to obtain a sharp and symmetrical peak for the chelate of $H_2[en(APM)_2]$. The behaviour of the copper and nickel chelates of H_2[en(APM)(TFA)] is exactly analogous to that of Ni(enAA.TFA) [2]. Thus, chelates of the tetradentate β-ketoamine ligand obtained by the condensation of a diamine with trifluoroacetylacetone and other non-fluorinated diketone is expected to behave in a similar manner.

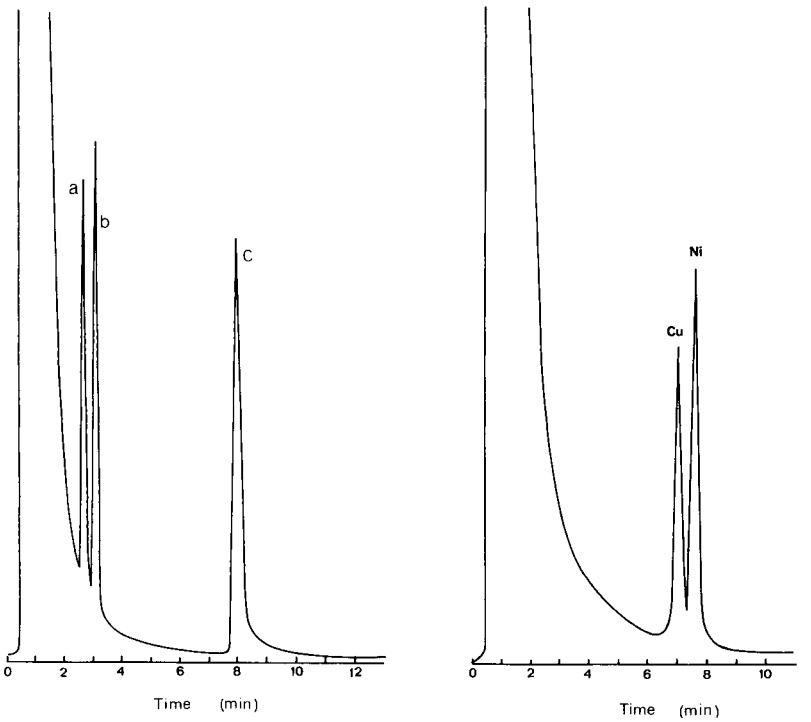


Fig. 5. Gas chromatographic separation of nickel(II) chelates of: (a) $H_2[en(TFA)_2]$, (b) $H_2[en(APM)(TFA)]$ and (c) $H_2[en(APM)_2]$. Chromatographic conditions as in Fig. 1, except for column at 260°C, injection port at 270°C and detector at 280°C.

Fig. 6. Gas chromatographic separation of Cu[en(APM)(TFA)] and Ni[en(APM)(TFA)] (60 ng Cu; 48 ng Ni). Chromatographic conditions as in Fig. 1.

Gas chromatographic separation of Cu[en(APM)(TFA)] and Ni[en(APM)(TFA)]

The chromatographic behaviour of binary mixtures of the two metal chelates under conditions similar to those optimized for the individual chelates, shows that the best resolution (resolution factor 1.21) of these chelates can be achieved at a column temperature of 230°C (Fig. 6). With these conditions, it was possible to achieve separations of both chelates, when either was present in up to a 10-fold excess. Furthermore, it was observed that adjustments of such parameters as temperature and gas flow rate could not further improve the resolution factor. Nevertheless, the resolution of these chelates is sufficient for the simultaneous determination of copper and nickel by gas chromatography of the chelates formed with $H_2[en(APM)(TFA)]$. It may be noted that the copper and nickel chelates of $H_2[en(TFA)_2]$ could not be completely resolved when OV-1 was used as the stationary phase [2].

It is of interest to note that in changing the stationary phase from OV-1 (a methylsilicone oil) to the more polar fluoropropyl silicone oil QF-1, a pro-

found effect on the relative retention time for Ni[en(TFA)$_2$] and Cu[en(TFA)$_2$] [2] was observed, and these chelates can be resolved completely on a QF-1 column. However, the corresponding change for the chelates of H_2[en(APM)(TFA)] is not great, as the retention data in Table 1 show, and the resolution factor was increased by 10% only on changing the stationary phase from OV-1 to QF-1. Furthermore, while Cu[en(TFA)$_2$] elutes before Ni[en(TFA)$_2$] on the OV-1 column and a reversal occurs on the QF-1 column, Cu[en(APM)(TFA)] elutes before Ni[en(APM)(TFA)] on both columns.

Quantitative gas chromatography of Cu[en(APM)(TFA)] and Ni[en(APM)(TFA)]

Quantitative studies of the two chelates were made with the standard column at 230°C and a carrier gas flow rate of 10 cm^3 min^{-1}. Peak areas were measured by triangulation; the mean of three determinations was calculated for each quantity of chelate injected into the chromatograph, and suitable linear calibrations were obtained over the range 10^{-7}—10^{-8} g of each metal (Fig. 7), linearity being achieved to a lower limit of ca. 5 ng of metal in both cases.

Application to metal analysis

The liquid-liquid extraction/gas chromatographic determination of the copper and nickel chelates of H_2[en(APM)(TFA)] was used for the simultaneous determination of copper and nickel in two samples of copper/nickel alloy (U.K. coinage metal and H.K. coinage metal) (Fig. 8). Calibration graphs were prepared by extracting the copper (or nickel) complex from 1 cm^3 of aqueous solution containing known amounts of copper (or nickel) in the range 10—100 μg. The linear graphs obtained were used to determine the amount of copper or nickel in the test samples. The results were checked by atomic absorption spectrometry; the agreement was good (Table 2).

TABLE 1

Retention data for metal chelates of H_2[en(APM)(TFA)] in ethyl acetate on OV-1 and QF-1[a]

Stationary phase	Chelate	t_R (s)[b]	Resolution factor
OV-1	Cu[en(APM)(TFA)]	417	1.21
	Ni[en(APM)(TFA)]	456	
QF-1	Cu[en(APM)(TFA)]	378	1.33
	Ni[en(APM)(TFA)]	420	

[a]Chromatograms obtained on a 6 ft. × 2 mm i.d. glass column packed with either 3% w/w OV-1 or QF-1 on Gas Chromb Q (100—120 mesh); column temperature 230°C; injection port and detector at 250°C; nitrogen flow rate 10 ml min^{-1}. [b]Retention time.

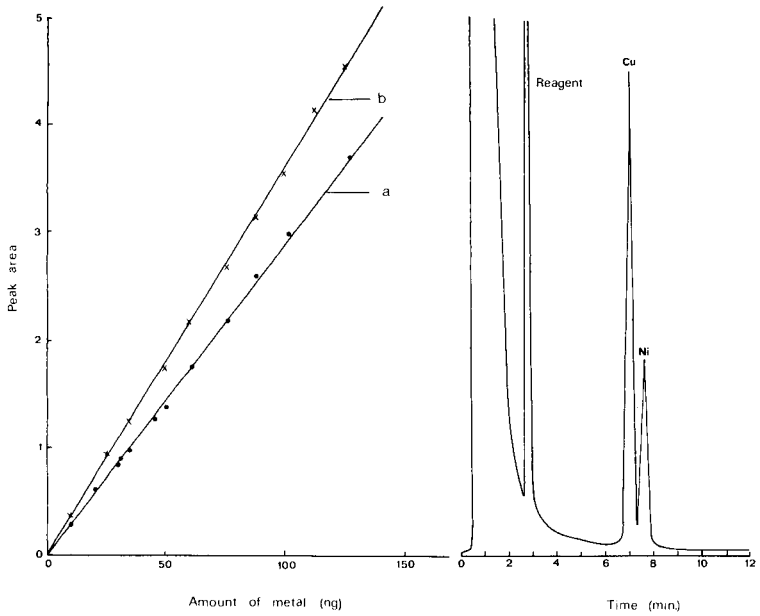


Fig. 7. Calibration graphs for: (a) Cu[en(APM)(TFA)]; (b) Ni[en(APM)(TFA)].

Fig. 8. Gas chromatogram of copper and nickel extracted from a solution of a coin with H_2[en(APM)(TFA)].

TABLE 2

Analysis of metal alloys

Material	Copper		Nickel	
	A.a.s. (%)[a]	Proposed method (%)[a]	A.a.s. (%)[a]	Proposed method (%)[a]
Coin (U.K.)	74.8	75.3	24.4	24.8
Coin (H.K.)	74.8	75.1	24.9	25.1

[a]Mean of two determinations.

CONCLUSIONS

The present work indicates the analytical potential of the tetradentate β-ketoamine, H_2[en(APM)(TFA)]. The volatilities of the copper and nickel chelates of this ligand are close to and slightly lower than those of the corresponding chelates formed with H_2[en$(TFA)_2$] and higher than those of the corresponding chelates formed with H_2[en$(APM)_2$]. The advantages of using this reagent include: the better resolution of the gas chromatographic peaks of the copper and nickel chelates when compared with the reagent H_2-

[en(TFA)$_2$]; the sharp and symmetrical peaks of the copper and nickel chelates; and the better resolution obtainable at lower column temperatures than those for the H$_2$[en(APM)$_2$] ligand. The proposed method is best suited to the simultaneous determination of copper and nickel in copper-nickel alloys where these metals exist as the major constituents, or in samples such as waters where the amounts of foreign ions present are comparatively low.

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DEVELOPMENT AND EVALUATION OF CONDITIONS FOR THE KINETIC QUANTITATION OF ACETOACETATE IN BODY FLUIDS

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SUMMARY

This paper describes a detailed study of the kinetic behavior of reactions used in the quantitation of acetoacetate in body fluids. Reactions studied are those between acetoacetate and glycine to form a transiently stable enamine intermediate, reaction of the enamine intermediate with nitroprusside, and simultaneous reaction of acetoacetate, glycine, and nitroprusside (nitrosopentacyanoferrate(III)). Variables studied include pH, temperature and concentrations of all reactants. The primary reaction between acetoacetate and glycine follows pseudo-first-order kinetics at high pH (> 8.6) at which the enamine is transiently stable; the other reactions deviate from first-order behavior at longer times because the reaction product is unstable. Several options involving nonlinear curve-fitting methods applied to all reaction sequences above and an initial-rate method applied to the combined reactions are evaluated. Also, the two-step and combined-reaction sequences are evaluated for quantitation of acetoacetate in urine and serum samples. For acetoacetate in the concentration range 0.5—4.25 mmol l^{-1}, the methods yield relative standard deviations in the range 1.0—1.8% with absolute standard deviations of 3.5—35 μmol l^{-1}.

Acetoacetate is frequently determined in body fluids for the detection and management of ketosis. Abnormally high concentrations of acetoacetate in sera and urines can result from diabetes mellitus, diets low in carbohydrates, and starvation [1]. Procedures for the determination of acetoacetate are generally less reliable than similar procedures for many other species, primarily because of special features of reactions used in the determination.

The most common procedures used for the determination of acetoacetate (AA) involve its reaction with glycine (G) and nitroprusside (NP) to produce a colored product with an absorption maximum near 550 nm [1—4]. This reaction is used in commercially available dipstick- and tablet-type determinations, as well as homogeneous solution reactions. Problems with the reaction system result from the insufficient stability of the reaction product which means that an equilibrium measurement cannot be made, and from the kinetics of the reaction system which are very complex. Despite these facts, no detailed kinetic study of this reaction system has been reported. The primary goals of this investigation were to study the kinetic behavior of the reaction system in some depth and to use the kinetic information to

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develop improved methods for the determination of acetoacetate. A brief review of the presumed mechanisms for reactions between ketoacids and amines will aid a description of the rationale for the study.

Amines can act as catalysts in the decarboxylation of ketoacids [5]. This is a multistep process involving the formation first of a ketimine which can decay to form carbon dioxide and regenerate the amine or react further to form an enamine. It is believed that the colored product in the acetoacetate-glycine-nitroprusside reaction results from the reaction of nitroprusside with the enamine intermediate; however, the reaction product has not been identified. In the remainder of this paper, the reaction of acetoacetate and glycine to produce the enamine is identified as the primary reaction and the reaction of nitroprusside with the enamine is identified as the indicator reaction.

The rate at which the ketimine reacts to produce carbon dioxide is highly dependent on pH, being relatively fast at pH <7 and relatively slow at pH >8. Also, the enamine absorbs radiant energy in the near u.v. region with an absorption maximum near 285 nm.

The above information led the authors to consider three options to quantify acetoacetate with this reaction sequence. The options are: (a) to use the absorbance of the enamine at 285 nm, (b) to permit the primary reaction to proceed to completion and then add nitroprusside and monitor the absorbance at 540 nm, and (c) to mix all three reactants simultaneously and monitor the absorbance at 540 nm. Each option is expected to offer different advantages and limitations.

EXPERIMENTAL

Instrumentation

A variety of photometers, including a diode-array-based spectrophotometer (Hewlett Packard 8450A), a highly stabilized filter photometer [6], and a multichannel centrifugal mixing/measurement system (Aminco Rotochem IIa), was used in different phases of this study. These instruments were interfaced to a variety of computers including a Digital Equipment Corporation PDP-12 minicomputer, a Hewlett Packard 2100A minicomputer, and, most recently, a Masscomp MC-500 supermicro computer. Kinetic data (absorbance vs. time) were collected on-line by the computers. With the Rotochem IIa instrument, solutions were mixed centrifugally in temperature-controlled cells; with the other photometers, solutions were mixed manually in 1-cm, water-jacketed, quartz cells. Linear and nonlinear least-squares methods [7, 8] were used to process the absorbance vs. time data.

Reagents

All solutions were prepared in distilled deionized water. Phosphate buffer solution, 0.17 mol l^{-1} with ionic strength of 0.50, was prepared by dissolving 44.7 g of $Na_2HPO_4 \cdot 7H_2O$ in water and diluting to 1.0 l. The resulting solution was filtered through a 5.0-μm SM type Millipore filter and the pH was

adjusted to 8.60 by addition of 1 mol l^{-1} hydrochloric acid. Unless stated otherwise, results were obtained with phosphate buffer.

Aqueous acetoacetate standards were prepared by distilling ethylacetoacetate (Matheson, Coleman and Bell, Cincinnati, OH 45212), taking 3.0 ml of the distillate boiling between 176—177°C, and diluting to 100.0 ml with 0.2 mol l^{-1} sodium hydroxide. The ethylacetoacetate was allowed to hydrolyze in a refrigerator at 4°C for 48 h to form a solution nominally 0.236 mol l^{-1} in acetoacetate. Spectrophotometric studies of the hydrolysis product indicated the presence of residual ethylacetoacetate in the hydrolysate at the 3—12 μmol l^{-1} level. Aqueous standards were prepared by diluting the 0.236 mol l^{-1} stock solution to a 9.44 mmol l^{-1} solution with phosphate buffer and by further diluting this latter solution with phosphate buffer to appropriate concentrations. Stock standards were refrigerated at 4°C and renewed weekly.

A 0.8 mol l^{-1} solution of glycine in a 0.1 mol l^{-1} phosphate buffer was prepared by dissolving 60.0 g of glycine and 26.8 g of $Na_2HPO_4 \cdot 7H_2O$ in water and adjusting the pH to 8.60 by addition of 3 mol l^{-1} sodium hydroxide whilst diluting to exactly 1 l. A 0.8 mol l^{-1} solution of glycine in a 0.1 mol l^{-1} carbonate buffer was prepared by dissolving 60.0 g of glycine and 10.6 g l^{-1} of Na_2CO_3 in water and adjusting to pH 10.0 whilst diluting to exactly 1 l. A 1.6 mol l^{-1} solution of glycine with ionic strength 0.50 in 0.14 mol l^{-1} phosphate buffer was prepared by dissolving 120.1 g of glycine and 37.5 g of $Na_2HPO_4 \cdot 7H_2O$ in water and adjusting to pH 8.60 with 3 mol l^{-1} sodium hydroxide whilst diluting to exactly 1 l. All solutions were stored at 4°C.

Sodium nitroprusside (sodium nitrosopentacyanoferrate(III)) solutions of 0.1 and 0.05 mol l^{-1} (29.8 and 14.9 g l^{-1} $Na_2[Fe(NO)(CN)_5] \cdot 2H_2O$, Sigma Chemical Co.) were prepared fresh daily in phosphate buffer and stored at room temperature in amber bottles to decrease photochemical reaction.

Serum and urine specimens. Urine samples obtained from healthy adult males were pooled, adjusted to pH 8.60 with 3 mol l^{-1} sodium hydroxide, and filtered through a 5.0-μm SM type Millipore filter. Standard addition specimens were prepared by adding desired amounts of 9.44 mmol l^{-1} acetoacetate standard solution to 5.0-ml aliquots of the filtered urine sample and diluting to 10.0 ml with phosphate buffer. An acetoacetate concentration range from 0.0 to 4.25 mmol l^{-1} was used. Sera were obtained from local hospitals and pooled, and standard addition specimens were prepared by adding desired amounts of acetoacetate solution to measured volumes of serum.

Procedure

Primary reaction. The primary reaction was initiated by mixing 0.50 ml of acetoacetate solution with 0.50 ml of glycine solution in the thermostated reaction cuvet. Absorbance values at 285 nm were recorded at appropriate time intervals from 10 s after the reaction was initiated until the reaction

had reached equilibrium. The 10-s initial delay allowed time for mixing perturbations to subside. In the studies of the effect of variables, a fitting range from 10 s to time of equilibrium was used. In the quantitation studies, a fitting range from 10 s to the time corresponding to one half-life was used. Because the primary reaction obeyed pseudo-first-order kinetics, the choice of the fitting range did not have any significant effect on the results, as long as it was not shorter than one half-life.

Indicator reaction. Aliquots (0.50 ml each) of acetoacetate sample and glycine solution were mixed in the cuvet and incubated to equilibrium (8—9 half-lives). The indicator reaction was then initiated by adding 0.2 ml of sodium nitroprusside solution to the cuvet. The indicator reaction exhibited pseudo-first-order behavior only during the first two half-lives. However, data from t = 10 s to equilibrium were used to compute apparent rate constants. Computed absorbance changes, $\Delta\hat{A}_\infty$, were obtained by fitting data between 10 s and 300 s to a first-order model.

Combined reactions. In the studies with the stabilized photometer, the reaction was initiated in the cuvet by the addition of 0.25 ml of sodium nitroprusside solution to a stirred solution containing 2.5 ml of glycine in phosphate buffer and 0.25 ml of standard acetoacetate solution or specimen. Data acquisition was initiated immediately after addition of the nitroprusside solution, and absorbance at 540 nm was measured at 1.20-s intervals for 300 s. Data recorded during the first 12 s were discarded to avoid complications resulting from mixing processes. Because the reaction followed pseudo-zero-order kinetics between 12 and 60 s after mixing, data during that period were used in a linear least-squares computation of the slope of the absorbance vs. time response curve during that period. Data obtained between 12 and 300 s after mixing, during which time the reaction approached pseudo-first-order kinetics, were used to generate computed absorbance changes as before. Because a short fitting range of 10 to 300 s was used, rate constants are not judged to be reliable and are not reported for the simultaneous reactions.

In the studies with the Rotochem IIa system, 0.30 ml of glycine solution was mixed with 0.30 ml of acetoacetate and 0.12 ml of nitroprusside solutions. Absorbance (measured with a filter centered at 550 nm) was monitored over a range 10 to 301 s after the reaction was initiated, which was also used as a fitting range for the calculation of absorbance changes, $\Delta\hat{A}_\infty$.

RESULTS AND DISCUSSION

Results of preliminary studies were used to establish conditions for pseudo-first-order behavior whenever possible. To provide as much consistency as possible among results, the effects of variables were studied with uniform values of the variables not being studied. Unless stated otherwise, conditions used for the various studies were pH 8.60, 25° C and 8.3 mmol l^{-1} nitroprusside. In the studies of the primary and indicator reactions, unless otherwise noted, glycine and acetoacetate concentrations were 0.8 mol l^{-1}

and 0.472 mmol l^{-1}, respectively. In the combined-reaction studies, glycine and acetoacetate concentrations were 0.67 mol l^{-1} and 0.295 mmol l^{-1}, respectively, unless noted otherwise. All concentrations are reported as those in the reaction cuvet except for acetoacetate in urine and serum samples and situations in which acetoacetate concentration is varied, where the concentrations reported are those in the original samples (before mixing).

Because the primary aim of this phase of the study was to establish and evaluate conditions for the quantitation of acetoacetate, kinetic results are presented with minimal mechanistic interpretation. Studies designed to better understand the kinetic behavior are continuing and will be reported later. Rate constants reported for the primary reaction are judged to be reliable because the reaction closely follows pseudo-first-order kinetics; rate constants reported for the indicator reaction are less reliable because the decay of the reaction product causes the reaction to deviate from first-order behavior. Rate constants are not reported for the combined reactions because they deviate badly from first-order behavior.

Although no attempt is made at this stage to develop rate equations to describe the observed kinetic behavior, it is worthwhile to provide empirical mathematical descriptions of some nonlinear responses. To do this, quadratic equations of the form $y = b_0 + b_1x + b_2x^2$ are used, with numerical values for the coefficients b_0, b_1, and b_2 that give the best (least-squares) fit to the data being reported for kinetic responses (y) and parameters such as concentration, temperature, pH, etc. (x).

Response curves

Figure 1 presents both experimental and fitted (first-order) response curves for the three reaction options. The response for the primary reaction is fitted very well by a first-order model while both the indicator and combined reactions deviate from first order behavior at longer times where product instability becomes significant. This is typical behavior for all acetoacetate concentrations examined with these conditions. In Figs. 1B and C, the calculated data at longer times represent the absorbances expected if the reaction product were stable. In each case, it is the computed absorbance change, $\Delta\hat{A}_\infty = \hat{A}_\infty - \hat{A}_0$, that is related to acetoacetate concentration, where $\hat{A}_0$ and $\hat{A}_\infty$ are the computed values of absorbance at $t = 0$ and ∞, assuming the products are stable (projected values in Figs. 1B and C).

Primary reaction

pH dependence. The effects of pH on the apparent first-order rate constant and computed absorbance change for the formation of the enamine in the primary reaction are illustrated in Fig. 2. It is apparent from the figure that it will be necessary to select a pH that will represent a compromise between maximum sensitivity and minimum reaction time. The choice of pH is also influenced by its effect on the stability of the enamine. Data similar to those in Fig. 1A, but at pH 8.0 and 7.5, exhibit similar behavior at short times but show a decrease in absorbance at longer times. These results suggest that the

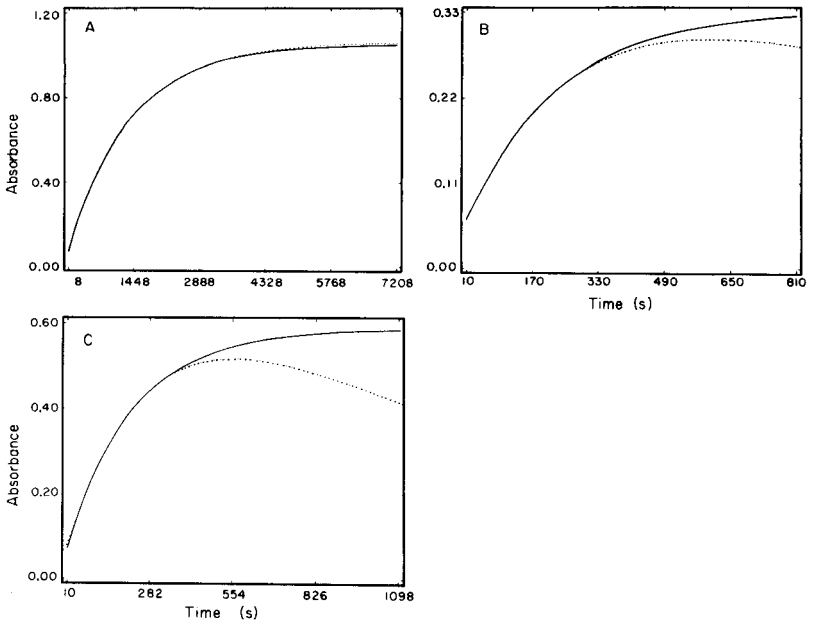


Fig. 1. Response curves for primary, indicator and combined reactions. (• • •) Experimental data; (—) calculated data. (A) Primary reaction: fitting range 8—1898 s, pH = 8.60, 25° C, 0.4 mol l^{-1} glycine, 0.45 mmol l^{-1} acetoacetate. (B) Indicator reaction: fitting range 10—270 s, pH = 8.60, 30° C, 0.8 mol l^{-1} glycine, 0.47 mmol l^{-1} acetoacetate, 8.3 mmol l^{-1} nitroprusside. (C) Combined reaction: fitting range 10—262 s, pH = 8.60, 30° C, 0.67 mol l^{-1} glycine, 0.39 mmol l^{-1} acetoacetate, 8.3 mmol l^{-1} nitroprusside.

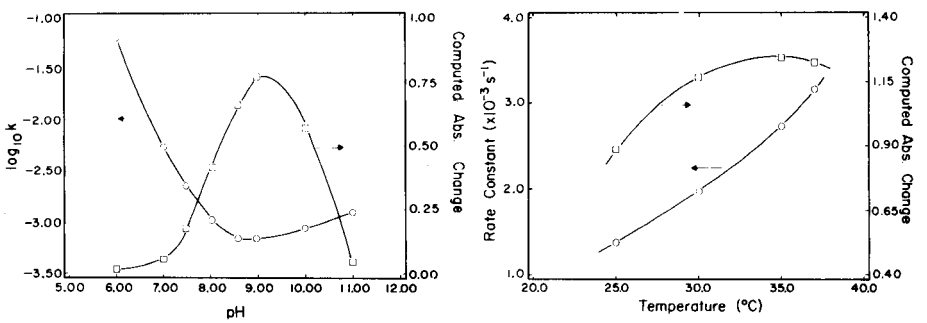


Fig. 2. Effects of pH on the apparent first-order rate constant (k) and computed absorbance change for the primary reaction (25° C, 0.4 mol l^{-1} glycine, 0.346 mmol l^{-1} acetoacetate.)

Fig. 3. Effects of temperature on the apparent first-order rate constant and computed absorbance change for the primary reaction. (pH = 8.60, 0.8 mol l^{-1} glycine, 0.472 mmol l^{-1} acetoacetate.)

enamine is unstable for pH values lower than about 8.6, probably because of the acid-catalyzed decarboxylation of the ketimine [5]. To ensure formation and retention of maximal amounts of the reactive intermediate, the remainder of this study was done at pH 8.6 unless stated otherwise. The rate constant is near a minimum value at this pH and, accordingly, reaction velocity (and time) is sacrificed in favor of sensitivity.

Temperature. Effects of temperature on the rate constant and absorbance change are illustrated in Fig. 3. The temperature dependence of the rate constant follows the expected exponential behavior. For the limited temperature range examined, both dependencies can be represented quite well by quadratic equations. For the specific conditions of this study, the quadratic coefficients b_0, b_1 and b_2 (see above) for the rate constant are 1.9, -0.14, and 4.6×10^{-3}, respectively and the coefficients for absorbance change are -3.5, 2.8, and -4.0×10^{-3}, respectively. The temperature recommended for quantitation of acetoacetate will be discussed after data for the other reactions are presented.

Glycine concentration. Figure 4 shows that the pseudo-first-order rate constant varies linearly with glycine concentration in the range studied while the maximum absorbance change becomes independent of glycine concentrations above about 0.8 mol l^{-1}. Linear least-squares coefficients (b_0 and b_1) for the rate constants are 4.4×10^{-4} s^{-1} and 1.3×10^{-3} l mol^{-1} s^{-1} respectively, and the quadratic coefficients for absorbance change are 0.115, 1.27 and -0.54. The data for absorbance suggest a reversible reaction being forced to equilibrium with increasing glycine concentration. The glycine concentration of 0.8 mol l^{-1} chosen for the quantitation of acetoacetate is near the minimum value required to yield near complete conversion of acetoacetate to the reactive intermediate; it does not represent the maximum achievable rate.

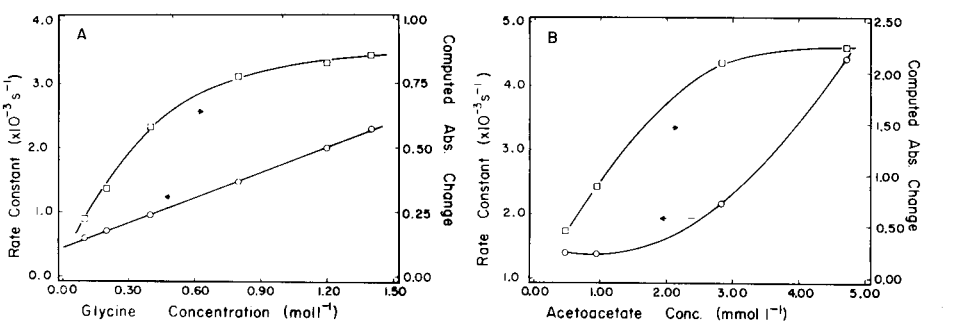


Fig. 4. Effects of glycine concentration on the apparent first-order rate constant and computed absorbance change for the primary reaction. (pH = 8.60, 25° C, 0.346 mmol l^{-1} acetoacetate.)

Fig. 5. Effects of acetoacetate concentration on the apparent first-order rate constant and computed absorbance change for the primary reaction. (pH = 8.60, 25° C, 0.8 mol l^{-1} glycine.)

Acetoacetate concentration. For acetoacetate concentrations from 0.1 to 1.5 mmol l^{-1} at 30° C, the rate constant was virtually independent of acetoacetate concentration (k_a (s^{-1}) = -3.4×10^{-5} C^0_{AA} (mmol l^{-1}) + 3.2×10^{-3} s^{-1}), where C^0_{AA} is the initial AA concentration. The computed absorbance change varied linearly with acetoacetate concentration up to 1.5 mmol l^{-1} ($\Delta\hat{A}_\infty$ = 0.93 C^0_{AA} (mmol l^{-1}) + 0.016) with a standard error of the estimate (S_{yx}) of 0.009 and r = 0.999.

At higher acetoacetate concentrations (0.47—4.7 mmol l^{-1}) the rate constant became dependent on acetoacetate concentration and the absorbance change varied nonlinearly with concentration (see Fig. 5). Quadratic coefficients (b_0, $-b_2$) for the rate constant dependency are 1.5, —0.35 and 0.20 and those for absorbance change are —0.078, 1.17 and —0.14. These dependencies probably reflect the fact that the glycine concentration (0.8 mol l^{-1} used in these studies was inadequate to force the primary reaction to completion with the higher acetoacetate concentrations.

Indicator reaction

In these studies, the primary reaction was permitted to proceed to completion before the solution was mixed with nitroprusside to evaluate effects of variables on the indicator reaction.

pH and temperature. The effects of pH on the apparent first-order rate constant and computed absorbance change (fitting range of $2t_{1/2}$) were evaluated at several temperatures from 25 to 35° C and several concentrations of acetoacetate from 0.47 to 4.7 mmol l^{-1}. Results are summarized in Figs. 6A and 6B. The rate constant at each temperature is relatively constant between pH 7.5 and 8.0, but increases significantly above pH 8.0. At each pH, the rate constant varies exponentially with temperature, as expected (see Fig. 3). In all cases, the computed absorbance change varied linearly with acetoacetate concentration (0.47—4.7 mmol l^{-1}). Figure 6B shows the effects of pH and temperature on the least-squares slopes of computed

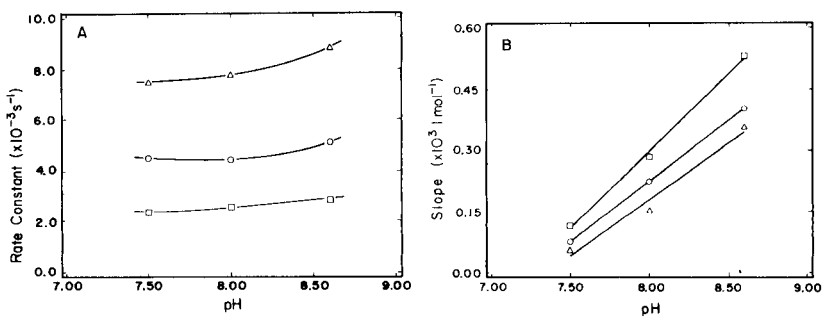


Fig. 6. Effects of pH and temperature on: (A) the apparent first-order rate constant for the indicator reaction. (B) Least-squares slopes of computed absorbance change vs. acetoacetate concentration. (△) 35°C; (○) 30°C; (□) 25°C. (0.8 mol l^{-1} glycine, 0.47—4.7 mmol l^{-1} acetoacetate, 8.3 mmol l^{-1} nitroprusside.)

absorbance change ($\Delta\hat{A}_\infty$) vs. acetoacetate concentration ($\Delta\hat{A}_\infty = b_0 + b_1 C^0_{AA}$ (mmol l^{-1})). The calibration slopes increase linearly with increasing pH in most cases and decrease with increasing temperature. Higher sensitivity is achieved with higher pH and lower temperature; recommended choices are discussed below.

Glycine. For glycine concentrations between 0.1 and 1.4 mol l^{-1}, both the apparent first-order rate constant and computed absorbance change vary linearly with glycine concentration. For the rate constant, the slope and intercept (k_a vs. C^0_G) are 0.69×10^{-3} l mol^{-1} s^{-1} and 2.4×10^{-3} s^{-1}, respectively, with $S_{yx} = 3.2 \times 10^{-5}$ s^{-1} and $r = 0.997$. The intercept is given only to permit interested readers to locate the data on the ordinate; it has no physical significance because there is no reaction in the absence of glycine. For the absorbance change, the slope and intercept ($\Delta\hat{A}_\infty$ vs. C^0_G) are 0.47 l mol^{-1} and 0.035 respectively, with $S_{yx} = 0.02$ and $r = 0.997$. Again, the intercept has no physical significance because there is no absorbance change without glycine present.

Nitroprusside. Effects of nitroprusside concentration on the pseudo-first-order rate constant and computed absorbance change for the indicator reaction are illustrated in Fig. 7. After an initial sharp rise, the rate constant varies only gradually with nitroprusside concentration. The variation in computed absorbance change is more gradual with nitroprusside and at higher concentrations the sensitivity is relatively independent of concentration. Quadratic coefficients for the data are 0.19, 0.01 and -2.7×10^{-4} for the rate constant and 0.036, 0.032 and −0.99 for the absorbance change, with nitroprusside concentration expressed as mmol l^{-1}.

Acetoacetate concentration. The rate constant for the indicator reaction is virtually independent of acetoacetate concentration. Linear least-squares slope and intercept for a fit of k_a vs. C^0_{AA} are 4×10^{-5} l $mmol^{-1}$ s^{-1} and $3.9 \times$

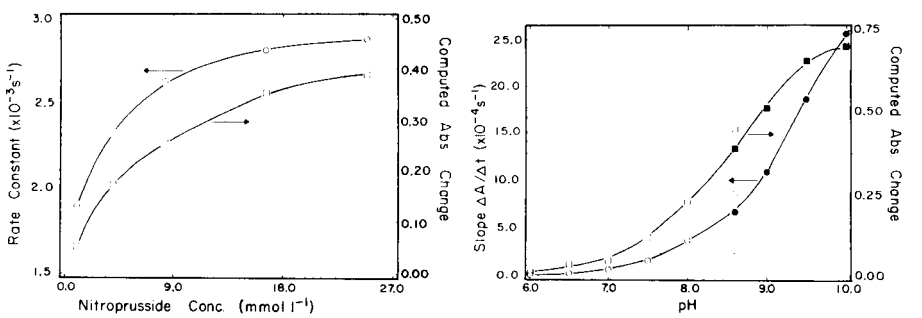


Fig. 7. Effects of nitroprusside concentration on the apparent first-order rate constant and computed absorbance change for the indicator reaction. (pH = 8.60, 25° C, 0.8 mol l^{-1} glycine, 0.3 mmol l^{-1} acetoacetate.)

Fig. 8. Effects of pH on initial rate and computed absorbance change for the combined reaction: (○, □) phosphate buffer; (●, ■) carbonate buffer. (25° C, 0.67 mol l^{-1} glycine, 0.295 mmol l^{-1} acetoacetate, 8.3 mmol l^{-1} nitroprusside.)

10^{-3} s^{-1}, respectively, with the small value of the slope reflecting the small dependence on concentration. As was noted above, the computed absorbance change varies linearly with acetoacetate concentration up to 4.7 mmol l^{-1}. At 30°C and other conditions identified earlier, the least-squares equation for absorbance change vs. concentration is:

$$\Delta\hat{A}_{\infty} = (0.447 \pm 0.005)\, C^{0}_{AA}\ (\text{mmol l}^{-1}) + 0.008 \pm 0.015$$

with $S_{yx} = 0.018$ and $r = 0.999$. Given the fact that the absorbance change for the primary reaction tends to vary nonlinearly with higher acetoacetate concentrations, the linear relationship observed here for the indicator reaction is somewhat surprising. One possible rationalization is that consumption of the enamine by the indicator reaction disrupts an equilibrium condition and forces the primary reaction to completion in the time frame in which the indicator reaction is monitored. Whatever the reason, use of the indicator reaction yields wider linear dynamic range than use of the primary reaction alone.

Combined reactions

Because the simultaneous reactions of acetoacetate, glycine and nitroprusside deviate from first order behavior, it is judged to be misleading to report apparent rate constants. Accordingly, the effects of variables on initial rates ($\Delta A/\Delta t$) are reported instead.

pH. Figure 8 shows the effects of pH on the initial rate and computed absorbance change for the combined reactions. The sensitivities for both initial-rate and computed absorbance change increase with pH throughout the range examined. These data suggest that both sensitivity and dependence on pH changes can be optimized at relatively high pH (>10).

Temperature. Figure 9 shows the effects of temperature on the initial rate and computed absorbance change for all reactions proceeding simultaneously. The temperature dependence of the initial rate does not follow the exponential behavior that might be expected. The apparent insensitivity to temperature above 30°C may be a result of compensating factors resulting from near equivalent increases in rates of formation and decay of the colored product being monitored. This increased rate of decay of the colored product at higher temperatures probably accounts for the fact that the computed absorbance change decreases with increasing temperature.

Glycine concentration. Both the initial rate and computed absorbance change vary linearly with glycine concentration between 0.1 and 1.4 mol l^{-1}. For the rate data the slope and intercept ($\Delta A/\Delta t$ vs. C^{0}_{G}) were 1.5×10^{-3} l mol^{-1} s and -2.2×10^{-4} s^{-1}, respectively, with $r = 0.994$. For computed absorbance change, the slope and intercept were 0.56 l mol^{-1} and 0.002, respectively, with $r = 0.995$.

Nitroprusside concentration. Effects of nitroprusside on the initial rate and computed absorbance change for the combined reactions are illustrated in Fig. 10. After an initial sharp rise, both plots become relatively independent of nitroprusside concentration above ca. 18 mmol l^{-1}.

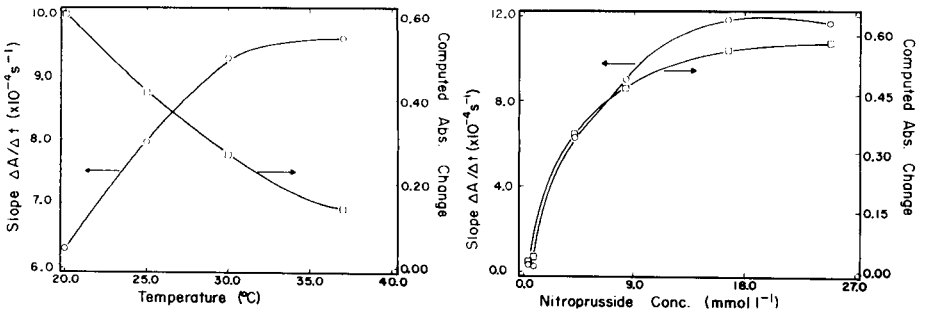


Fig. 9. Effects of temperature on the initial rate and computed absorbance change for the combined reaction. (0.67 mol l^{-1} glycine, 0.295 mmol l^{-1} acetoacetate, 8.3 mmol l^{-1} nitroprusside, pH = 8.60.)

Fig. 10. Effects of nitroprusside concentration on the initial rate and computed absorbance change for the combined reaction. (25°C, pH = 8.60, 0.67 mol l^{-1} glycine, 0.295 mmol l^{-1} acetoacetate.)

Acetoacetate concentration. For the conditions described above, both the initial rate and computed absorbance change varied linearly with acetoacetate concentration between 0.02 and 4.7 mmol l^{-1}. Sensitivities are ca. 2.5×10^{-3} l mmol^{-1} s^{-1} for rate data and 0.6 l mmol^{-1} for absorbance change. More complete results for acetoacetate concentration are presented below.

Selection of quantitative conditions

It is not possible to identify a single set of conditions that is optimum for the quantitation of acetoacetate. The choice of pH is perhaps the most clear-cut. A pH of 8.6 was chosen as a compromise between lower rate constants at higher pH and instability of the enamine intermediate at low pH. Although rate constants for the primary and indicator reactions increased with temperature, results became less precise above 30°C, so this temperature was chosen as a compromise between increased rate constant and decreased precision. Glycine concentrations above 0.4 mol l^{-1} yield near-maximum sensitivity, and values of 0.4 and 0.8 mol l^{-1} were chosen for different parts of this study.

Conditions used to obtain quantitative data are summarized in Table 1. It is re-emphasized that many other combinations of conditions will yield similar performance, but it is unlikely that other conditions will yield substantial improvements relative to results reported below.

Quantitation of acetoacetate

The predictive kinetic [7, 8] and initial-rate methods were evaluated for the quantitation of acetoacetate in aqueous standards and urine samples to which known amounts of acetoacetate were added.

For the predictive kinetic method, all three reaction modes were evaluated for acetoacetate concentrations up to 4 mmol l^{-1}. The indicator and combined

TABLE 1

Conditions selected for the quantitation of acetoacetate

Variable	Predictive-kinetic method			Initial-rate method	
	Primary	Indicator	Combined	Low[a]	High[a]
pH	8.6	8.6	8.6	8.6	10
Temp. (°C)	30	30	30	25	30
Glycine (mol l^{-1})	0.8	0.8	0.67	0.67	0.83
NP (mmol l^{-1})	—	8.3	8.3	8.3	8.3
Buffer	PO_4^{3-}	PO_4^{3-}	PO_4^{3-}	PO_4^{3-}	CO_3^{2-}

[a]See text for explanation.

reactions exhibited linear behavior between computed absorbance change ($\Delta\hat{A}_\infty$) and acetoacetate concentration up to 4 mmol l^{-1}; the primary reaction exhibited linear behavior only up to about 1.5 mmol l^{-1}, with the plot curving toward the concentration axis at higher concentrations. Results obtained by the three reaction modes are summarized in Table 2 as least-squares statistics of $\Delta\hat{A}_\infty$ vs. concentration of acetoacetate.

Comparing the slopes, it is noted that the primary reaction is the most sensitive and the indicator reaction is the least sensitive. Unfortunately, attempts to utilize the primary reaction to quantify acetoacetate in urine were unsuccessful because of the very high background absorbance at 285 nm. Also, for reasons not yet understood, there is a gradual (ca. 1% per day) decrease in the slopes of calibration plots with time for each new set of standards. This change is not observed with the other options. Both the indicator and combined reactions yielded similar performance for aqueous and urine samples. In each case, the slopes are similar for the two sample types; for the indicator reaction, the larger intercept for the urine sample probably reflects acetoacetate in the unspiked sample. Comparing the standard errors of estimate, it is noted that the scatter about the linear least-squares line is substantially worse for the combined reactions than for either of the other modes.

To evaluate the imprecision of the indicator and combined reactions, multiple runs were made on aqueous and urine samples containing four different acetoacetate concentrations. Nine runs were made on each sample with the indicator reaction and fifteen runs were made on each sample with the combined reactions. In every case, the standard deviations increased as the computed absorbance change increased and in fact, for each concentration the standard deviations were very similar for each of the four data sets (aqueous and urine samples run with indicator and combined reactions). For concentrations of 0.47, 1.89, 3.78 and 4.25 mmol l^{-1}, the average values of the four standard deviations of absorbance at each concentration were 0.0035, 0.012, 0.027 and 0.035 mmol l^{-1}. These absolute deviations correspond to relative standard deviations between 1.0 and 1.8% with relative imprecision

TABLE 2

Least-squares statistics for fits of computed absorbance change vs. acetoacetate concentration (3 runs at each of 5 concentrations)

Sample type	Slope ± s.d. (1 mmol^{-1})	Intercept ± s.d. (absorbance)	Std. error (absorbance)	Correlation coefficient
Primary reaction[a]				
Aqueous	0.935 ± 0.005	0.016 ± 0.004	0.009	0.999
Indicator reaction[b]				
Aqueous	0.447 ± 0.005	0.008 ± 0.014	0.018	0.999
Urine	0.454 ± 0.001	0.037 ± 0.003	0.005	0.9999
Combined reactions[b]				
Aqueous	0.69 ± 0.026	−0.067 ± 0.068	0.08	0.997
Urine	0.69 ± 0.023	−0.013 ± 0.053	0.08	0.997

[a]0.1—1.5 mmol l^{-1}. [b]0.5—4.25 mmol l^{-1}.

being somewhat smaller for the combined reactions because of the slightly larger absorbance change (see Table 2). Least-squares statistics for these data were similar to those in Table 2, with the indicator reaction exhibiting lower uncertainties.

Although linear least-squares statistics are presented above for results ($\Delta\hat{A}_\infty$ vs. C_A^0) obtained with the combined reactions, there is good reason to question whether this is the best model. In every set of experiments, least squares statistics for results with the combined reactions were degraded relative to results with the indicator reaction, and plots of $\Delta\hat{A}_\infty$ vs. C_A^0 appeared to curve away from the concentration axis. Each of the data sets for the combined reactions did give excellent fits with a quadratic model. The least-squares statistics for the urine samples represented in Table 2 are:

$$\Delta\hat{A}_\infty = 0.0525 + 0.50\,C_A^0 + 0.036\,(C_A^0)^2$$

with S_{yx} = 0.036 mmol l^{-1} and r = 0.9995 (15 points). The plots (not shown) all exhibit excellent fits to the experimental data by the quadratic model. Although analytical scientists tend to be conditioned to prefer linear calibration results, the use of computers makes it just as easy and reliable to use a quadratic model as a linear model.

Some useful generalizations can be made from these results. Although the primary reaction yields the highest sensitivity, and would likely be most attractive for situations in which samples contain little or no background absorbance in the near u.v., it is not useful for urine and serum samples unless a separation step is included to remove acetoacetate from other strongly absorbing components. For direct quantitation without a separation step, either a procedure involving a preliminary incubation step (primary reaction) followed by direct monitoring of the indicator reaction or a procedure

involving the combined primary and indicator reactions is necessary. The former two-step procedure yields substantially better linearity for the range examined; however, the sensitivity and detection limits are slightly degraded ($<\times 2$) relative to the results with the combined reaction and the incubation period is time-consuming and troublesome. Given the facts that the $\Delta\hat{A}_\infty$ vs. C data for the combined reaction are easily fitted to a quadratic model, that it offers the best sensitivity and detection limits, and is faster and more straight-forward, it is likely to be the method of choice.

The combined reaction sequence was also evaluated by an initial-rate method in which slopes of A vs. t data during the early part of the reaction were computed with a linear least-squares model. Two sets of conditions identified as "low" and "high" conditions (see Table 1) were evaluated. For the "low" conditions, slopes were computed from data obtained between 12 and 60 s; for the "high" conditions, slopes were computed from data obtained between 12 and 24 s. Plots of initial rate vs. acetoacetate concentration in aqueous samples were linear with slopes of 0.243×10^{-3} and 1.18×10^{-3} s l mmol^{-1} for the "low" and "high" conditions respectively and with intercepts of -0.005×10^{-3} and -0.04×10^{-3} s^{-1}, respectively.

Known amounts of acetoacetate were added to 21 serum and urine samples, and concentrations were determined by the initial-rate method and a published [4] method. The results are summarized in Table 3 and show reasonable agreement.

It is concluded that this reaction sequence offers several viable options for quantifying acetoacetate in biological samples. An advantage of the predictive kinetic method when applied to reactions that follow well-defined kinetic behavior (e.g., first-order or Michaelis Menten) is that it compensates for variations in experimental variables (e.g., pH and temperature) that influence rate constants but not the kinetic order. That advantage is unlikely to apply with the indicator and combined reactions because they deviate from first-order behavior. Therefore, the choice of measurement/data-processing approach will be influenced primarily by subjective issues. All other factors

TABLE 3

Least-squares statistics for fits of results obtained by initial rate vs. published method [4]

Conditions[a] (Table 1)	Slope ± s.d.	Intercept ± s.d. (mmol l^{-1})	Std. error (mmol l^{-1})	Correlation coefficient
Serum				
Low	1.08 ± 0.01	0.09 ± 0.01	0.04	0.9997
High	1.05 ± 0.01	0.17 ± 0.04	0.11	0.998
Urine				
Low	1.04 ± 0.01	0.08 ± 0.01	0.04	0.999
High	1.03 ± 0.01	0.01 ± 0.02	0.06	0.999

[a]21 Samples, 0—5 mmol l^{-1} acetoacetate added.

being equal, it is probable that an initial-rate method applied to the combined reactions will likely be the method of choice because it is the fastest of the several options.

As was noted earlier, studies designed to give a better understanding of the chemical implications of the kinetic behavior reported above are continuing and will be reported later.

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SELECTIVITY IN MULTICOMPONENT ANALYSIS

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SUMMARY

Figures of merit for judging the selectivity of procedures for multicomponent analysis are compared with respect to their performance under different experimental conditions. The condition number of the calibration matrix is evaluated as being the most general selectivity measure. Practical selectivities based on the condition number are given for atomic and molecular spectroscopy and for electroanalytical methods. The most promising data reduction schemes, such as factor analysis or partial least squares in latent variables are discussed for systems with unselective sensors.

Judging the performance of analytical procedures applicable to multicomponent analysis is almost tantamount to asking for high selectivity of the analytical system. As a measure of the selectivity of multicomponent procedures numerous selectivity criteria have been proposed ranging from simple quantification of interferent effects on the analyte signal [1] to quality measures of linear algebra such as the condition number [2, 3].

With respect to the semantic definition of selectivity a major breakthrough was made by Kaiser in 1972 [4]. Based on his suggestion the term selectivity of an analytical procedure is used here in the sense that it is a figure of merit for multicomponent analysis; i.e., the procedure should at least enable two components to be determined simultaneously by using two or more responses. In addition, since multicomponent analysis, according to the linear calibration model in Eqn. 1, is based on predicting the sought concentrations, **c**, by regressing the calibration (response) matrix on the signal vector, **r** (Eqn. 2 or 3), the measure of selectivity should be related by fundamental linear algebra.

$$\mathbf{CK} = \mathbf{R} \tag{1}$$

$$\mathbf{K}^t\mathbf{c} = \mathbf{r} \tag{2}$$

$$\mathbf{c} = (\mathbf{K}\mathbf{K}^t)^{-1}\mathbf{K}\mathbf{r} \tag{3}$$

The concentration matrix **C** consists of n columns (mixtures) and m rows

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(components), the response matrix **K** having m rows and s columns (sensors), the signal matrix **R** is a $n \times s$ matrix and the t stands for the transpose of the matrix. It must be emphasized that the given selectivity is valid for a definite analytical procedure only and should be reported together with the conditions applied for that procedure.

At a glance not all procedures for multicomponent analysis are carried out by solving Eqns. 1—3: with highly selective multicomponent methods such as atomic emission spectroscopy the relationship between signal and concentration can frequently be estimated for the analytes independently of each other. Very often, also, only particular pairs or groups of components are to be considered with respect to mutual interferences.

These simplifications, of course, do not affect the validity of Eqn. 1 describing multicomponent analysis as a problem of multivariate calibration. Highly selective procedures enable simplifications to be made in such a manner that, ideally, only the diagonal elements of the response matrix, **K**, would be different from zero and no corrections for the effect of matrix elements are necessary.

In order to judge the degree of selectivity of multicomponent procedures and to link the selectivity of a system with precision and accuracy, the present work is aimed at: comparing the selectivity definitions with respect to their general validity; computing typical selectivities in atomic and molecular spectroscopy, in electroanalysis and in chromatography with unresolved peaks by using the most general criterion, the condition number of a matrix; and deducing the most promising data reduction scheme from selectivity considerations.

EXPERIMENTAL

Ultraviolet spectra were recorded on a model Specord M 40 two-channel spectrophotometer (VEB Carl Zeiss Jena, G.D.R.). Energy-dispersive x-ray fluorescence was measured on a Philips/Edax EXAM VI with zirconium as the secondary fluorescer in the energy range 4.02—10.74 keV. Theoretical intensities were calculated using the NRLXRF software [5]. This program does not permit L_γ lines to be studied; measured relative emission intensity values were inserted instead. Computations were run on a UNIVAC 1100 or SM4/20 computer.

For calculation of the condition number the norms of the **K** matrix and of its inverse were used: $\mathrm{Cond}(\mathbf{K}) = \|\mathbf{K}\| \cdot \|\mathbf{K}^{-1}\|$. These norms were computed from the highest and the reciprocal lowest singular values of the **K** matrix. In order to report the smallest Cond(**K**) values attainable the original data were scaled sensorwise to unit variance. The singular values were computed as described previously [6]. Other definitions for calculation of matrix norms have also been applied (cf., [3]). Multiple regression analysis for multivariate calibration and prediction of concentrations was based on the partial least squares (PLS) algorithm [7] as described previously [8].

COMPARISON OF SELECTIVITY DEFINITIONS

A collection of formulae for characterization of selectivity is summarized in Table 1. Kaiser's selectivity criterion (no. 1) is deduced from the idea that fully selective analysis is feasible if all but the diagonal elements of the transposed response coefficient matrix $\mathbf{K}^t$ are zero. For example, at different wavelengths only the analyte to be determined gives a response to the system:

$$\mathbf{K}^t = \begin{matrix} k_{11} & 0 & \cdots & 0 \\ 0 & k_{22} & \cdots & 0 \\ \vdots & & & \\ 0 & 0 & & k_{pm} \end{matrix}$$

where the number of sensors is assumed to be equal to the number of components, i.e., $p = m$.

From this understanding of selectivity, Kaiser defines the ratio of the sensitivity of the analyte (k_{ii}) to the sum of the sensitivities of all other com-

TABLE 1

Definitions or criteria of selectivity in multicomponent analysis

No.	Figure of merit	Mathematical expression	Ref.
1	Selectivity	$\Xi = \underset{i=1}{\overset{n}{\text{Min}}} \dfrac{\lvert k_{ii}\rvert}{\sum_{j=1}^{n} \lvert k_{ij}\rvert - \lvert k_{ii}\rvert} - 1$	4
2	Total selectivity	$\Delta_c = \underset{i=1}{\overset{n}{\text{Min}}} \dfrac{\lvert k_{ii}\rvert c_i}{\sum_{j=1}^{n} \lvert k_{ij} + \mu_{ij}\rvert c_j - \lvert k_{ii}\rvert c_i} - 1$	10
3	Non-specificity coefficient	$S_L = \underset{j=1}{\overset{m}{\text{Min}}} \dfrac{1}{\sum_{i=1}^{n} \mathbf{K}_{p_i}} - 1$ where $\mathbf{K}_{p_i} = k_{ij}/k_{ii}$	11
4	Degree of non-selectivity	$\Delta_i = 100 \dfrac{\Sigma k_j c_j}{k_i c_i}$	12
5	Selectivity product	$S_p = k_{1,A} k_{2,B} / k_{1,B} k_{2,A}$	13
6	Distension	$\Theta = \dfrac{1}{n(n-1)} \sum_{i=1}^{n} \sum_{j=1}^{n} \beta_{ik}$	14
7	Determinant	$\text{Det}(\mathbf{K})$ or $\text{Det}(\mathbf{K}\mathbf{K}^t)$	3, 15, 16
8	Condition number	$\text{Cond}(\mathbf{K}) = \lVert\mathbf{K}\rVert \cdot \lVert\mathbf{K}^{-1}\rVert$	2, 3, 8, 15 16, 17

ponents in the calibration matrix as being the partial "specificity" for the actual component. Evaluation of the minimum partial "specificity" of all rows of the calibration matrix reveals the selectivity of the system (cf., Table 1).

Although the Kaiser criterion was at the time of its submission the most general, it has some drawbacks from today's perspective. It is not clear how to handle overdetermined systems that are commonly encountered, e.g., in molecular spectroscopy [3, 7, 8]. Furthermore, as the performance of the system is judged by its weakest link, some of the properties that a criterion should have [9] are lacking from Kaiser's definition. To mention only the most important ones:

(1) injectivity, i.e., any change of a single element in the calibration matrix should result in changes of the criterion itself;

(2) continuity, i.e., minor or major changes in the partial sensitivity of components should induce minor or major changes in the selectivity criterion, respectively;

(3) monotony, i.e., if a single element in the matrix rises the criterion should rise too; and

(4) a distinct value for full selectivity; with Kaiser's criterion a fully selective system has the distinct value of "infinity", a somewhat fuzzy result.

In the light of the properties required, definitions 2—5 in Table 1 do little to overcome the limitations of Kaiser's approach. In definition 2 Kaiser's criterion was modified in order to take interelement (chemical) effects in atomic spectroscopy into consideration and to obtain a measure for characterizing the analytical procedure and the sample quality. (In the Eqn., c_i refers to the analyte concentration, c_j is the concentration of interferent species j, and μ_{ij} is the slope of the calibration curve for the interferent at analyte concentration c_i). With this criterion, the authors obtain for atomic absorption spectrometric determinations of K, Na, Mg, Ca, Sr, Cu, Zn and Fe total selectivity values of 2.94 in sea water, 3.88 in orange juice and 3.47 in blood serum. From the almost equal selectivities one would expect that the performance of the analytical procedure is independent of the sample quality. This, however, is not true because there are mutual interferences between alkaline and alkaline earth elements at sea water concentration levels [10]. This interference can easily be predicted with the use of definition no. 1 (Table 1) giving values for sea water, orange juice and blood serum of 67.8, 9.76×10^5 and 9.37×10^4, respectively.

In addition, it should be mentioned that intercomponent effects could be modelled by concentration terms of higher or mixed order giving a calibration matrix that is still linear in the parameters (responses or sensitivities) and can be characterized, e.g. by criteria 7 and 8. More general definitions have been proposed. Criterion no. 6, the distension, is based on calculating the angle β_{ik} between a given row vector (one sensor) and all other row vectors of the calibration matrix. This criterion has been used to decide upon the choice of optimal measuring points in multicomponent analysis.

According to the authors' suggestion the criterion is applicable to describe the quantitative relation of the selectivity of any single point of a spectrum with respect to the others [14]. A broader application of criterion 6 for characterizing the selectivity of a whole system is not known to the present authors.

As demonstrated earlier [2, 3, 15, 16], the most general criteria related to the selectivity of an analytical procedure are the determinant (no. 7) and the condition number (no. 8) of the calibration matrix, because they apply to exactly determined and overdetermined systems, and properties such as injectivity, continuity and monotony are also obeyed by both criteria.

The performance of criteria 7 and 8 under various experimental conditions is shown below by means of computer simulations along with their relationship to realistic error bounds in multicomponent analysis.

SELECTIVITY AND ITS RELATIONSHIP TO ACCURACY AND PRECISION

Simulations of multicomponent systems of different complexities were based on Gaussian shaped profiles (bands or peaks). The calibration matrix **K** obtained (cf. Eqn. 1) was used to compute the determinant and the condition number as selectivity criteria. In order to relate the various values of the criteria to the precision and accuracy of multicomponent analysis, an entire analytical procedure consisting of calibration and determination of unknowns (prediction) was simulated and the data were analysed by multiple regression analysis based on the PLS algorithm (see below).

In a typical experiment, calibration of 2 components was carried out by means of 10 mixtures, each containing both elements at levels ranging from 0.1 to 1.0 concentration units in increments of 0.1. The precision and accuracy of the procedure is estimated by prediction of 10 mixtures of similar composition. Averaged over 5 complete calibration/prediction runs, a mean relative prediction error (r.p.e.) is calculated as r.p.e. = $100[\Sigma_i\Sigma_j(c_{ij} - \hat{c}_{ij})^2/\Sigma_i\Sigma_j c_{ij}^2]^{1/2}$, where the $\hat{c}_{ij}$ are predicted concentrations from the ith run and the jth component and the c_{ij} are the actual concentrations in the mixtures. The r.p.e. is a measure of accuracy and precision since the differences between predicted and actual concentrations characterize the accuracy of the method and the 5 repetitions of the full calibration/prediction cycle give an estimate of precision. Details of the simulated experiments together with the results are summarized in Table 2.

The resolution of the Gaussian signals was investigated for two- and five-component systems of constant and non-constant resolution. Both the determinant and the condition number reflect correctly the decrease of resolution and thus of selectivity as the criteria monotonously increase or decrease, respectively.

The r.p.e. increases continuously with decreasing selectivity of the system and, as has already been shown [17], there is a linear relationship between the r.p.e. and the condition number normalized to the square root of the

TABLE 2

Dependences of precision and accuracy on determinant and condition number of the calibration matrix[a]

Variable	Cond.(K)	Det.(KK^t)	R.p.e. (%)
Resolution ($\Delta\sigma$)			
2 components			
1.0	2.84	19.66	0.56
0.2	14.3	1.40	2.08
0.1	28.7	0.364	4.15
0.05	59.6	0.093	8.39
0.04	87.8	0.0597	10.48
0.03	146.2	0.00337	13.95
5 components			
2.0	2.08	138.7	1.24
1.0	14.94	5.80	4.75
0.8	34.98	0.494	9.19
0.7	60.90	0.0821	14.2
0.6	108.9	0.0091	20.6
5 components, non-constant resolution			
2, 0.7, 0.7, 0.7	32.79	0.773	6.76
2, 2, 2, 0.7	4.91	50.67	2.54
Number of sensors ($\Delta\sigma$ = 0.05)			
5	59.0	0.0038	19.42
10	58.5	0.0235	10.31
20	59.6	0.0929	8.39
40	59.2	3.69	4.99
Intensity ratio			
1	2.84	1.97×10^1	0.465
10	16.1	1.97×10^3	0.858
100	160.1	1.97×10^5	5.70
1000	1570	1.97×10^7	59.9

[a]2 Components, 1σ band separation, 10 measuring points (sensors), band intensities 1 to 1, 10 calibration mixtures, 1% relative signal noise, unless otherwise specified.

component number m ($\mathrm{Cond}(\mathbf{K})/m^{1/2}$). Such a relationship is to be expected from the error propagation scheme of linear algebra according to which the upper bound of the relative error for prediction of concentrations ($\|\delta\mathbf{c}_o\|/\|\mathbf{c}_o\|$) results from the sum of relative errors due to signal ($\|\delta\mathbf{r}_o\|/\|\mathbf{r}_o\|$) and calibration measurements ($\|\delta\mathbf{K}\|/\|\mathbf{K}\|$) multiplied by the condition number as follows [2, 18]:

$$\frac{\|\delta\mathbf{c}_o\|}{\|\mathbf{c}_o\|} = \mathrm{Cond}(\mathbf{K}) \left(\frac{\|\delta\mathbf{r}_o\|}{\|\mathbf{r}_o\|} + \frac{\|\delta\mathbf{K}\|}{\|\mathbf{K}\|} \right) \tag{4}$$

where $\|\cdot\|$ stands for the norm of a vector or that of a matrix and o characterizes the prediction phase.

From the selectivity values of systems with varying resolution it can be concluded that the criteria give a more sensible picture of the system's selectivity than Kaiser's measure would do as the resolution of all component pairs is of importance and not only the worst resolved pairs.

The influence of the number of sensors (measuring points) on selectivity and r.p.e. was investigated next (Table 2). Varying the number of sensors between 5 and 40 does not change the condition number apart from numerical effects. This seems to be plausible since the selectivity of a system that consists of symmetric Gaussian bands should not depend on the number of measuring points. The decrease in r.p.e. with increasing sensor number is attributed to the averaging effect over the signal intensities revealing a lower r.p.e. according to the error propagation scheme in Eqn. 4.

Another result is found for the determinant. The determinant decreases with increasing sensor number and one deduces from this dependence that the selectivity of a multicomponent procedure can be changed by varying the number of sensors. This conclusion, although correct in principle, is unjustified if systems with symmetric components profiles are used. The number of sensors affects the selectivity of a system only if asymmetrically-shaped bands or peaks exist. Then the condition number varies with a varying number of sensors, too (cf. [8, 15, 16]).

As a third variable, the intensity ratio of the component bands was studied. The r.p.e. increases with increasing intensity of the second band because of decreasing selectivity of the system. The decreased selectivity is reflected by increased condition numbers (Table 2). The determinant also changes in dependence on the increase in intensity ratio, but in an unexpected way. Normally, the determinant becomes smaller if the selectivity of the system decreases (cf. Table 2, resolution study). Here, the determinant is enhanced with decreasing system selectivity, i.e., optimizing the selectivity by maximizing the determinant of the system would result in a completely unselective system with highly different intensity ratios.

Summarizing, this comparison of different criteria reveals the condition number as being the most appropriate selectivity measure. The condition number relates the variables resolution, sensor number and intensity ratio correctly to analytical selectivity and there is a theoretically based relationship to the accuracy and precision of the method according to Eqn. 4. Criteria 1 to 6 are not general enough and the determinant (no. 7, Table 1) gives a wrong picture of selectivity if the intensity ratio or the number of sensors with symmetric bands varies.

PRACTICAL SELECTIVITIES

In the following section, the condition number was used to characterize the selectivity of practically used analytical procedures. In some cases the condition number was already published. Otherwise the number was computed from the response data given or the responses were measured as part

of this investigation. Table 3 contains the results. The data given are by no means general nor can they be regarded as being typical for the analytical principle outlined. They just represent worked examples refering to definite analytical procedures. The selectivity values are expected to vary between instruments, laboratories and experimenters and therefore they will have different values if the experimental conditions, e.g., the resolution of a spectrometer, change.

In atomic spectrometry, the very selective atomic emission spectrometry

TABLE 3

Practical selectivities characterized by the condition number

Analytes	Number of sensors	Cond.(K)	Ref.
Atomic spectroscopy			
I.c.p./a.e.s.			
Cu, Ni, Zn	3	3.20	18
	3	1.08	19
Al, As, Cd	3	8.04	18
Lanthanoids (14 elements)	14	1.29	20
X.r.f.			
Lanthanoids (14 elements)	65 (w.d.x.r.f.)	14.8	21
	84 (e.d.x.r.f.)	1960	—[a]
Molecular spectroscopy			
I.r.			
o-, *m*-, *p*-Xylene	3	1.23	—[a]
U.v.			
Caffeine, propyphenazone, phenacetine	13	112	—[a]
Adenylic, cytidylic, guanylic, uridylic acid	36	12.7	15
Visible			
Dithiocarbamates of Co, Ni, Cu, Fe, Pd	14	50.6	8
4-(2-Pyridylazo)resorcinol chelates of 8 metal ions	14	853	8
Electroanalytical methods			
Anodic stripping voltammetry			
Zn, Cd	2	2.68	22
Potentiometric stripping			
Pb, Tl, In, Cd, Zn, Cu	6	1.74	23
Potentiometry			
K, Na, Ca, Mg	4	2.10	24
Amperometry			
Hypoxanthine, inosine, inosine-5-phosphate, adenosine-5-phosphate	4	19.5	25

[a]This work.

(a.e.s.) is to be expected to reveal condition numbers of 1, i.e. full selectivity. Mutual interferences (spectral or intercomponent effects) of some element combinations cannot be ruled out, however, as seen for measurements of Cu, Ni, Zn or Al, As, Cd or the lanthanoids in the inductively coupled plasma (i.c.p.). The condition number remains lower than 10, and those interferences could be easily overcome by means of multivariate calibration combined with multiple regression analysis (see below).

Lower selectivity is a feature of x-ray fluorescence analysis (x.r.f.), especially when used in the energy-dispersive mode. For example, the condition number for the 14 lanthanoids amounts to 14.8 in wavelength-dispersive (w.d.) x.r.f. compared to 1.29 for i.c.p./a.e.s. (Table 3). Determination of overlapping coefficients becomes a prerequisite for analysis.

In general, x.r.f. is theoretically comparable with other spectroscopic methods only if the area weight of the sample can be considered to be small. In cases of infinite sample thickness the picture is more complicated since additional matrix effects, due to absorption and secondary fluorescence, affect the component spectra. The selectivity of such systems has to be calculated for every sample composition separately. Table 3 contains as an example the condition number for determination of the lanthanoids by e.d.x.r.f. analysis in an infinitely thick sample consisting of calcium fluoride as the matrix. The system was based on a sample that contains 0.1% by weight of each of the lanthanoids. The obtained condition number of 1960 (Table 3) refers to a resolution of 180 eV, but the value could be decreased if the detector resolution is improved.

Data for molecular spectroscopy are given for the i.r. and u.v./visible spectral ranges (Table 3). The especially low selectivity in the u.v./visible range is depicted in Fig. 1 for one of the examples. This is the reason why u.v./visible spectrophotometry is only recently becoming a practical tool for multicomponent analysis after microprocessor-controlled instrumentation linked to powerful laboratory computers has been introduced. The number of components that can be determined, however, is limited.

A look at electroanalytical methods shows some interesting features. Voltammetric and potentiometric stripping methods have so far been used as multielement techniques almost only when full selectivity was achieved, but recently systems of lower selectivity have also been considered, the peak (signal) overlap being corrected by multivariate calibration (Table 3). Likewise multiple channel sensors of limited selectivity are emerging in potentiometry for the determination of the alkaline or alkaline earth elements [24] and have been proposed for enzyme electrodes based on amperometric monitoring of oxygen [25].

In chromatography the condition number has been applied for characterizing chromatographic selectivity in cases of overlapping peaks [17]. From the condition numbers obtained the upper error bounds for curve resolution based on multivariate calibration could be evaluated.

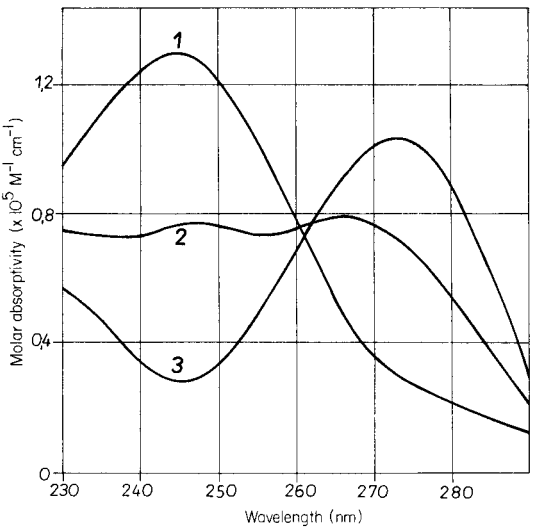


Fig. 1. Ultraviolet spectra of: (1) phenacetine; (2) propyphenazone; (3) caffeine.

DATA REDUCTION SCHEMES FOR MULTIVARIATE CALIBRATION

Multivariate calibration that is based on regressing calibration mixtures on the signal matrix can be done with use of different mathematical algorithms. Highly selective and accurately measurable systems can be handled by any algorithm; in the simplest case Eqn. 1 is handled by means of matrix inversion for both calibration ($\mathbf{K} = \mathbf{R}\mathbf{C}^t(\mathbf{C}\mathbf{C}^t)^{-1}$) and prediction of the concentrations sought, according to Eqn. 3. It is to be noticed from this last Eqn. that in the calibration step the condition number of the concentration matrix is of prime importance [2]. For less ideal systems, several data-reduction schemes have been proposed in order to tackle problems, such as multicolinearity, drift, noise, unknown compounds or unknown background. Of these, mathematical analysis is sometimes carried out by regressing the signal matrix **R** directly on the concentration matrix **C** [27], i.e. $\mathbf{RP} = \mathbf{C}$. To estimate the unknown concentrations of a sample its signal vector is then multiplied by **P** and no further regression analysis as needed for prediction with Eqn. 3 is necessary.

In practice, however, it has not yet been shown that regression of **R** on **C** would be better than regressing **C** on **R**. Brown [26] concluded from theoretical considerations and practical studies that there is little to choose between the two methods. The conditioning in the **P**-matrix approach ($\mathbf{RP} = \mathbf{C}$) is obviously not improved because the precision of the method now depends on the condition number of the signal matrix **R** which is worse conditioned than the concentration matrix.

Real improvements are achieved by a group of related techniques called singular value decomposition, principal components analysis, rank annihila-

tion, eigenvector projection or factor analysis. With these techniques the original signal matrix, **R**, is represented by a product of two smaller matrices $\mathbf{R} = \mathbf{F_R L_R}$, where $\mathbf{F_R}$ is the factor and $\mathbf{L_R}$ the loading matrix, and the regression on the concentration matrix is done as follows: $\mathbf{F_R T} = \mathbf{C}$, with **T** now being the response coefficient matrix. With the PLS approach [7, 8], the **C** matrix is also decomposed into smaller matrices taking the correlation of concentration and signal matrix additionally into consideration: $\mathbf{C} = \mathbf{F}_c \mathbf{L}_c$, and the regression is based on relating the projected signal matrix $\mathbf{F_R}$ to that of the concentration projection $\mathbf{F}_c = \mathbf{F_R V}$, where **V** is a diagonal matrix of d elements, the number of abstract components.

There may be an enhancement in selectivity with the latter methods if the conditioning of the system is improved after linear projection of the signal or concentration matrix or both. As an example the condition numbers of the original and projected signals and concentration matrices are given in Table 4 for the two component Gaussian-shaped systems as influenced by the number of components. As seen from Table 4, both the conditioning of the signal matrix and of the concentration matrix are improved, giving a basis for more accurate data analysis.

CONCLUSIONS

The increasing need for simultaneous multicomponent analysis has led to the use of fully selective analytical procedures and of systems of limited selectivity as well. For judging the performance of non-selective systems, the condition number is recommended as the most general selectivity measure and it is hoped that this criterion will be applied by the analytical chemist independently of the analytical principle employed. Continuous improvements in data reduction schemes raise hopes that in future analytical procedures based on unselective sensors will enable multicomponent analysis to be carried out with similar accuracy and precision as it is now feasible with fully selective procedures.

TABLE 4

Dependence of conditioning of signal and concentration matrices on component number after rotating into latent variables[a]

Component number	Cond.(**R**)	Cond.($\mathbf{F_R}$)	Cond.(**C**)	Cond.($\mathbf{F}_c$)
2	101.5	1.15	2.27	1.0
3	107.6	3.32	6.32	2.15
4	101.6	10.81	12.84	4.86
5	118.2	13.09	16.31	4.35

[a]Conditions: Gaussian-shaped components, 1σ band separation, band intensities 1 to 1, 20 sensors, 10 calibration mixtures.

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NON-LINEAR MULTIPARAMETER ESTIMATION IN SPECTROPHOTOMETRY

Part I. The Selection of Close-to-Linear Model Functions with Easy Convergence using Least Squares Algorithms

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SUMMARY

Least squares optimization offers an objective function which, compared to other computational strategies, is favourable for non-linear models. Generally, iterative procedures using approximations of the objective function are used to find the global minimum and its parameter set. Convergence is a crucial aspect in these calculations. In this respect it is not always realized that for a specific model many model functions can usually be raised, each exhibiting a different degree of non-linearity and, on substitution in the objective function, an associated ease of convergence. For good convergence, a close-to-linear model function with a low intrinsic non-linearity should be selected. The remaining parameter non-linearity can be decreased by change of parameters. The measures of non-linearity are elucidated geometrically in the sample space in relation to possible non-Gaussian behaviour and bias of the estimated parameters.

An unequivocal explanation of the shapes of titration curves of high accuracy complexiметric titrations of metals at low concentration (10^{-5} M) with photometric end-point detection [1—3] requires that detailed information be obtained about the individual spectrophotometric absorption curves of the expected metal/indicator species MI, MI_2, M_2I and M_2I_2 and about their formation constants. The possibilities for investigating these metal/indicator systems are restricted, firstly to low concentrations ($<10^{-4}$ M) because of the high molar absorptivities and the limited solubility of the free indicator and the metal/indicator species, and secondly to distinct pH regions because of the hydrolysis, especially of metal ions in high oxidation states [4, 5]. These restrictions are such that only u.v.-visible spectrophotometry is able to provide sufficient information.

Job curves and mole ratio experiments have frequently been advocated for studying the composition of coloured compounds, but in principle they are suitable only when one strongly dominating species is present in solution. A multicomponent system is too complicated to be handled [6] in this way. The basic idea was to extend the Job-curve measurements to more wavelengths. Experimentally this multicomponent system in which the components

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are in equilibrium with each other can be featured as follows. Suppose the absorbances of 80 of the metal/indicator mixtures are each measured at 25 wavelengths thus giving 2000 absorbance readings. Including the free indicator, five absorbing species (I, MI, MI_2, M_2I and M_2I_2) are present in each solution. The contribution of each component to the absorbance at any wavelength is determined by its concentration, which depends upon the analytical concentration of metal and indicator and the formation constant of the component, and by its molar absorptivity at that wavelength. The number of parameters involved in the system is counted as follows. At each wavelength every component has its own molar absorptivity, so there are 5×25 absorbance parameters. The initial (analytical) concentrations are known; the real concentrations are determined by the 4 conditional equilibrium (formation) constants. Adding these gives a total of 129 parameters to be estimated. Because of the four equilibrium equations underlying the model function, the whole model is essentially non-linear.

In this paper, the authors discuss the lines followed in order to avoid the pitfalls that can occur in non-linear multiparametric estimation and how the many parameters were successfully estimated from the absorbance data by a suitable choice of model function. In a later paper the results obtained with thorium and xylenol orange and semixylenol orange will be presented.

MODEL, MODEL FUNCTION AND PARAMETER SET

An immense literature on methods for finding least-square estimators or minimum variance estimators is available [7, 8], and with the proliferation of computer facilities in laboratories, the number of users of non-linear regression models is rapidly growing. Nevertheless, non-linear regression modelling is still in its infancy. Only in the last decade have serious attempts been made to study systematically the properties of the estimators found [9—11], and the number of resulting papers has been remarkably small.

In general, modellers wish to fit a model to a set of data either to predict response values Y for a given fixed value of the regressor variable X, or for making inferences with the estimated parameters. Their interest fades either because convergence is not attained or, if it is attained, because they have reached their goal (minimum variance, least squares sum, maximum likelihood or some other criterion) not aware of the fact that there is an interrelation between the ease of reaching the criterion and the statistical properties of the parameters found, such as bias and skewness of distribution.

In fitting the model to the data it is necessary to distinguish between the objective function, the model, the model function associated with the model and the set of parameters related to the particular model function. Different model functions can be associated with a given model [9]. To illustrate this consider parameter estimations in spectrophotometry. The sum of squares is adopted as the objective function and the least squares sum is adopted as the criterion for optimization. The sum of squares of the differences between

measured and calculated absorptions (and transmissions), indexed m and c, can be minimized by $S_{tion} = \Sigma_i(I_i^m - I_0 10^{-A_i^c})^2$ hence using $I_0 10^{-A^c}$ as the model function in which A^c is further a function containing the parameter set. A second option is to minimize the differences between measured and calculated absorbances, $S_{ance} = \Sigma_i(A_i^m - A_i^c)^2$, in which case A^c itself is the model function (the objective function S is the same!).

Within each model function, the parameter set can still be selected. Consider A^c to be linearly dependent on the molar absorptivities, ϵ_{pq}, where p and q are the numbers of metal and indicator species in the metal/indicator complex. By intuition it is expected that this linearity will promote convergence, but how should the formation constants be introduced? They could be introduced as K_{pq}, but after change of parameters $\log K_{pq}$ may lead to better convergence. Both K_{pq} and $\log K_{pq}$ have a fundamental chemical significance and are acceptable. They do not give an extra barrier to understanding, provided that the relationship between both parameter types satisfies a few simple criteria such as monotonicity and single-valuedness, which are satisfied in this problem. Within a given model function the parameter set can be transformed into a different set without changing the model function. The foregoing suggests that there is much freedom in selecting the model function and in choosing the parameter set. This freedom can be used to improve the optimization.

Non-linear models differ from linear regression models in their estimation properties in that, given the usual assumption of independent and identically normally-distributed experimental data, linear models give rise to unbiased, normally-distributed, minimum-variance estimators for both the parameter set and the predicted response values [7, 8, 12, 13], whereas non-linear models tend generally to do so only under certain conditions and when the sample size is not too small.

Bates and Watts [9] have shown that non-linearity can be quantized with two measures: intrinsic and parameter non-linearity. The intrinsic non-linearity of a model/data set combination depends upon the model function and determines the bias of the predicted response values. The parameter non-linearity is related to the bias of the parameters. This measure does not depend upon the model function selected and can be improved by change of parameters.

The importance of this discussion is that it is desirable to seek a model function with low intrinsic non-linearity and, by suitable change of parameters, with low parameter non-linearity. If such a (reparameterized) model function has been found, the model will behave like a linear model in that the estimators are unbiased and normally distributed with minimum variance. Most important, however, is that when dealing with such a "close-to-linear" model, least squares algorithms such as the Gauss-Newton algorithm converge rapidly, yielding the parameters belonging to the global minimum. Even with badly chosen, distant starting points the algorithms find the estimates in a few iterations.

These algorithms for non-linear parameter estimation will be considered, and later reconsidered more mathematically in relation to the convergence problem.

COMPUTATIONAL METHODS FOR NON-LINEAR REGRESSION

The parameter set can be represented by a vector $\theta = (\theta_1, \theta_2, \theta_3, \ldots \theta_p)$. The parameter vector θ is related to a model function $\mathbf{y}^c = \mathbf{f}(\theta, \mathbf{x})$ in which $\mathbf{x} = (x_1, x_2, x_3, \ldots x_n)$ is the n-vector regression variable and $\mathbf{y}^c$ the n-dimensional response variable. In the present case, x is the vector representation of the different metal/indicator ratios and $\mathbf{y}^c$ is the vector of the corresponding calculated absorbances or absorptions (depending on the model function chosen). In general, for a given set of $\mathbf{x}$, $\mathbf{y}$ is found experimentally and the best fit of $\mathbf{y}^c$ to $\mathbf{y}$ is sought by adapting θ.

In cases where many experimental data are dealt with, least-square (LSQR) algorithms have some advantages [7, 8] for non-linear regression. Therefore, the LSQR scalar objective function is considered, $S(\theta) = \Sigma_i^n [y_i - f_i(\theta, \mathbf{x})]^2$, which, in vector notation, is $S(\theta) = [\mathbf{y} - \mathbf{f}(\theta, \mathbf{x})]^T [\mathbf{y} - \mathbf{f}(\theta, \mathbf{x})]$ (T indicates transposed (= row) vector). The basic idea is that the best fit of $\mathbf{y}^c = \mathbf{f}(\theta, \mathbf{x})$ to $\mathbf{y}$ is attained when the minimum of $S(\theta)$ is reached. The best computational scheme for finding this minimum $S(\hat{\theta})$ is to make an initial guess, θ_1, and to calculate $S(\theta_1)$. A step $\Delta\theta_j$ is made in the parameter space to a new point $\theta_{j+1} = \theta_j + \Delta\theta_j$ for which $S(\theta_{j+1}) < S(\theta_j)$ holds. This process is iterated until the global minimum $S(\hat{\theta})$ is approached sufficiently. It means that convergence should occur.

Convergence has three strategic aspects. First, from the starting point the computation should provide a pathway which should roughly proceed in the direction of the global minimum. Second, the minimum should be reached within a reasonably small number of iterations and, third, it has to be proved that the correct minimum has been reached. Consider the last point first. In practice certain assumptions are made for termination of the iteration process: $S(\theta_{j+1}) - S(\theta_j)$ is significantly small compared to the usual statistical uncertainty in $S(\theta_j)$ (F-test), or $(\hat{\theta}_{j+1} - \hat{\theta}_j)$ is small enough for the purpose required, or both.

Generally it is difficult to verify, by using only computational criteria, that the true (global) minimum has been approached sufficiently and that the system has not located a (nearby) local minimum. In practice the results are cross-linked with the original field from which the data originated (spectrophotometry in this case). It is easier to compare the residual sum of squares with the experimental error, to plot $[y_i - f_i(\mathbf{x}, \hat{\theta})]$ vs. x_i and thereby to check whether the obtained results are consistent.

With respect to the other convergence aspects it is usually a more serious problem to get convergence within a reasonable number of iterations. The probability that convergence does not occur increases exponentially with increasing number of parameters in the set. Several computational strategies have been developed to find the best step $\Delta\theta_j$ in each iteration step [7].

Steepest descent with a step length related to the steepness is very inefficient. It requires a large number of steps and approaches the global minimum asymptotically. The method is not recommended for practical applications when it is used alone. It is sometimes used in combination with other methods to escape from non-convergence. The most active work has been based on the use of approximating forms of the objective function $S(\theta)$ and by far the majority uses a quadratic function for approximation.

The iteration process through the quadratic approximation T

The procedure starts together with the initial guess θ_1. If T is the quadratic approximation, $T(\theta_1) = S(\theta_1)$ in the starting point. The shape of T can be deduced either from the objective function S, from the model function $\mathbf{f}(\theta, \mathbf{x})$ or from the model in some other way. Leaving for a while which method is to be followed, the minimum of the approximation T suggests a better parameter set θ_2 and an estimate, $S(\theta_2)$, of the minimum for the actual objective function S. Generally the estimate $S(\theta_2)$ is not adequately close and a new approximation is started with θ_2 as a "better" initial guess and with a new quadratic approximation, T, for which $T(\theta_2) = S(\theta_2)$. The shape of T is again deduced as previously and the minimum of T is again determined giving θ_3 as a better guess, etc. The procedure is iterated until the global minimum has been reached, which in practice means until the criterion for termination is fulfilled.

Approximating the real objective function, S, by a quadratic function, T, has several advantages for optimization. First, the LSQR calculations from T are relatively simple. Standard routines in computer libraries can be used and large numbers of experimental data and parameters can be handled. Second, compared to the steepest descent procedure the method exhibits accelerated convergence near the global minimum sought, and high numerical accuracy is possible. Third, it provides asymptotic estimates of variance. As a whole these advantages give the LSQR algorithm preference over other algorithms such as maximum likelihood and minimum variance.

But what about convergence from bad initial guesses? Two aspects play an important role in achieving convergence. The first is the deviation from linearity and the second is the deduction of the quadratic approximation, T, either from S (Newton-Raphson method) or from the model function (Gauss-Newton method).

When a model is linear, starting with independent, identically normally-distributed experimental data, the different parameter estimation methods fall together [12, 13]. Maximum likelihood estimators, minimum variance estimators and least squares estimators become identical and calculation procedures also become identical. In that case the model function is linear with respect to θ. So the objective function, S, is quadratic and T becomes identical to S. The consequence is that the LSQR computational method will find the minimum in one step and that the result becomes independent on the starting point (see below for the proof).

Changing from linear to non-linear models is not a discontinuity as might be thought. The closer a model function is to linearity, the less disadvantageous are bad starting points, and a larger number of parameters is admitted before non-convergence arises. Thus a model function as close to linearity as possible should be selected. Therefore absorbance is far to be preferred to absorption data in this case. The second aspect is the quadratic approximation, T, of the objective function, S.

The Newton-Raphson procedure

In this procedure, T is deduced directly from S. If θ_j is the starting point in a specific iteration, $T(\theta)$ can be written as:

$$T(\theta) = T(\theta_j) + (\theta - \theta_j)^T P(\theta_j) + 1/2[(\theta - \theta_j)^T Q(\theta_j)(\theta - \theta_j)]$$

Considering the $(p + 1)$ dimensional parameter space with an extra dimension for the sum of squares S and its approximation T, S will be a hyperplane with, in general, a global minimum, side minima, saddle points and other deviations from the simple hyperparaboloid which occurs for a linear model function. The initial guess of the j^{th} iteration step θ_j resembles a point in the "parameter + S" space where S and T coincide. In the neighbourhood of θ_j, S can be developed in a Taylor series:

$$S(\theta) = S(\theta_1) + (\theta - \theta_1)G(\theta_1) + 1/2[(\theta - \theta_1)^T H(\theta_1)(\theta - \theta_1) + \ldots]$$

Neglecting terms higher than quadratic leads to the quadratic approximation, T:

$$T(\theta) = S(\theta_1) + (\theta - \theta_1)G(\theta_1) + 1/2[(\theta - \theta_1)^T H(\theta_j)(\theta - \theta_1)]$$

It is obvious that $\mathbf{P}$ equals the gradient vector $G(\theta_1)$ built up from the first derivatives in θ_1 and that matrix $\mathbf{Q}$ is equal to the so-called Hessian matrix, $\mathbf{H}$, consisting of the second derivatives $(\partial^2 S/\partial\theta_i \partial\theta_j)_{\theta_1}$ ($\mathbf{Q}$ and $\mathbf{H}$ are symmetric matrices). The step towards the minimum of T is found in length and direction by differentiation of T and putting the derivative equal to zero; the step vector is $\Delta\theta_j = (\theta_{j+1} - \theta_j) = -\mathbf{Q}^{-1}\mathbf{P}$, and contains the inverse of matrix $\mathbf{Q}$. The Newton-Raphson procedure has one main drawback, i.e., local irregularities play a dominant role in $\mathbf{Q}$ and so in $\mathbf{Q}^{-1}$ and $\Delta\theta_j$ which is comparable to the differentiation of noisy signals. While integration improves the signal-to-noise ratio, differentiation degrades it. Second derivatives are sharp measures for local irregularities in the hyperplane S. To demonstrate the disproportionate influence of the local irregularities on the estimation of the step vector, a one-dimensional example was synthesized (Fig. 1) which consists roughly of two parabolic segments connected by a circular segment. θ_1 on the circular segment gives $(\partial^2 S/\partial\theta^2)_{\theta_1} < 0$. The consequence is that the step is made in the wrong direction, to the maximum in T.

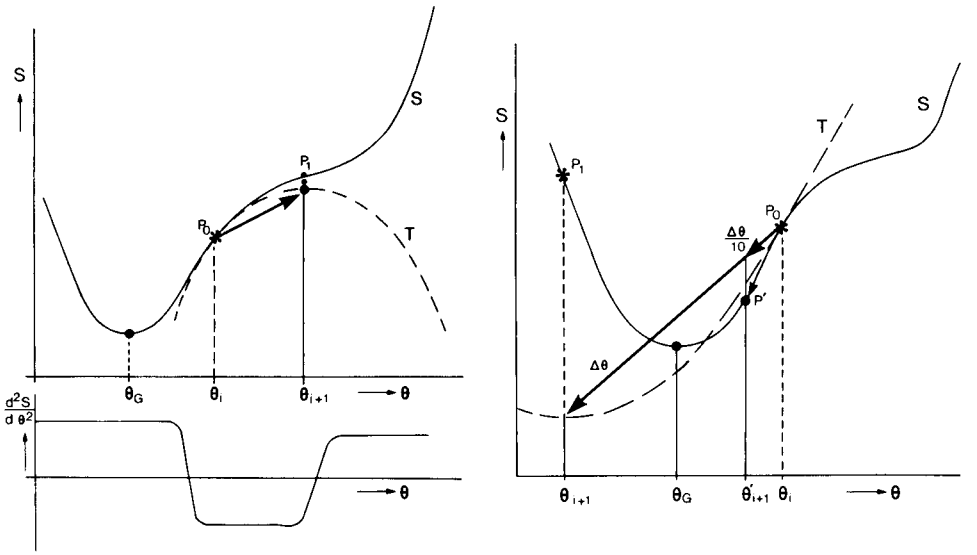


Fig. 1. A two-dimensional Newton-Raphson approach showing the influence of local irregularities. Starting with θ_i and P_0 in a range where the second derivative of S is negative, the next starting point θ_{i+1} (P_1) lies opposite the global minimum θ_G.

Fig. 2. A two-dimensional Gauss-Newton approach showing a worse-defined approximate surface T and a correct step vector direction. As the next starting point P_1 is higher than P_0 the step vector is decimated until P' lies lower. The risk of non-convergence is lower as in Fig. 1.

In general the Hessian matrix should be definitely positive to prevent a step in the wrong direction, but the real plane, S, usually has many "irregularities" which are inherent in the combination data set/model function/objective function. If only a few parameters are to be estimated, the risk of being sent in a wrong direction will be small and it if occurs it is not disastrous. After some (wrong) steps, there will arise a situation where $H(\theta)$ becomes definitely positive, and it may be expected that with only a few parameters the track ends at the global minimum θ_G. With an increasing number of parameters, however, the irregularity density in the S plane increases. The risk of going astray increases sharply and generally convergence is not attained within a reasonable time-span.

Several modifications have been proposed for the Newton-Raphson method in order to prevent negative diagonal elements in the Hessian matrix. The modification of Marquardt [7, 8, 14] has been reasonably successful. The principle is to add a factor λ, a definite positive diagonal matrix, to the Hessian matrix with λ chosen large enough to make all diagonal elements positive. For this diagonal matrix the gradient vector is usually used. By this method the step vector is a combination of the (steep descent-)gradient

vector and the calculated Newton-Raphson vector. It increases the chances of escaping from non-convergence. In the authors' experience, however, the Marquardt modification is a nice refinement near the "parameter limit" of non-convergence or, in accidental cases, to escape from it, but it does not lead to much progress in the number of parameters that can be estimated.

Other modifications such as constraints to θ to areas of physical meaning and reduction of the step size necessary to prevent overshooting (as near inflection points $\Delta\theta$ tends to infinity) are discussed below, where a much more promising optimization procedure is elucidated.

The Gauss-Newton method

In this method the model function $\mathbf{f}(\mathbf{x}, \theta)$ is used for deducing the coefficients of T. The model function is expanded in a Taylor series around θ_i, the starting point of the i^{th} iteration. Only the first two terms are retained:

$$\mathbf{f}(\theta) = \mathbf{f}(\theta_i) + J(\theta_i)(\theta - \theta_i) \tag{1}$$

note that, $\mathbf{f}(\mathbf{x}, \theta)$ is written as $\mathbf{f}(\theta)$ since the $(n \times p)$ matrix $\mathbf{X}$ is constant.

The $n \times p$ matrix $J(\theta_i)$ is the so-called Jacobian matrix defined by $J_{kj}(\theta_i^*) = (\partial f_k/\partial\theta_j)_{\theta_i^*}$ where $k = 1, 2 \ldots n$ and $j = 1, 2 \ldots p$. Substitution in $S = (\mathbf{y} - \mathbf{f}(\theta))^T(\mathbf{y} - \mathbf{f}(\theta))$ yields the hyperparaboloid, T, as:

$$T = [\mathbf{y} - \mathbf{f}(\theta_i)]^T[\mathbf{y} - \mathbf{f}(\theta_i)] - 2[\mathbf{y} - \mathbf{f}(\theta_i)]^T J(\theta_i)(\theta - \theta_i) + (\theta - \theta_i)^T J^T(\theta_i) J(\theta_i)(\theta - \theta_i)$$

The step vector is found, as before, by putting the derivative of T to θ zero:

$$\Delta\theta_i = \theta_{i+1} - \theta_i = (J^TJ)^{-1}J^T[\mathbf{y} - \mathbf{f}(\theta_i)] \tag{2}$$

The Hessian matrix in the Newton-Raphson method is now replaced by the $p \times p$ square matrix J^TJ which has on the diagonal the terms $\Sigma_k^n(\partial f_k/\partial\theta_j)^2 > 0$. Hence J^TJ is definitely positive for all starting points. It implies that steps in the opposite direction no longer take place. This advantage is paid for by a worse defined length of the step vector, which can be understood as follows. If in the Taylor series development of $\mathbf{f}(\theta)$ the next highest term (second derivative) is taken into account, there arises an extra contribution to the quadratic term of $T(\theta)$. This extra term represents the difference between the Hessian matrix in the Newton-Raphson method and the matrix J^TJ in the Gauss-Newton method. The neglect of the extra term with second derivatives makes J^TJ less sensitive to local irregularities in S and $\mathbf{f}(\theta)$ but the values of the matrix terms are farther from the correct value leading to the global minimum.

As a whole the quadratic approximation T provides better values for the direction of $\Delta\theta_i$, but worse values for its length. If the vector is too small the new starting point is always suitable for a new iteration. If the vector is too large, $S(\theta)_{i+1} > S(\theta_i)$ and the new starting point (P_2 in Fig. 2) cannot be accepted. The best way to cope with this problem is to constrain the starting

points θ_i to their region of physical meaning, a multidimensional cube in parameter space, and to decimate the vector length, repeatedly if necessary, until $S(\theta_{i+1}) < S(\theta_i)$.

In a next paper the results of a case study will be shown. The results have been obtained with the Gauss-Newton method; 99 parameters could be estimated. The computer program has been developed such that it was optional to use the Newton-Raphson or the Gauss-Newton method. In both modes the parameter track was constrained to the multidimensional cube and vector decimating was used. Marquardt's method was available for emergency cases to escape from non-convergence or too much vector decimating. It turned out that the Newton-Raphson-Marquardt method never did function from distant starting points, while the Gauss-Newton method always led to the global minimum without using the Marquardt modification. The Gauss-Newton method is strongly to be preferred, but certain conditions, which will be discussed after the following section, have to be fulfilled.

The variance in θ and the residual sum of squares

At convergence the estimate of the asymptotic variance-covariance matrix is found as follows. Suppose θ_t is the true (unknown) vector of the parameter set and $\hat{\theta}$ is its estimator. So $\mathbf{y} - \mathbf{f}(\theta_t) = \epsilon$ is the deviation from the true value. Assume that ϵ is independent, and identically-normally distributed ($\epsilon = N(0, \sigma^2\mathbf{I})$), so with n measurements the expectation of the sum of squares is $E(S(\theta_t)) = E(\epsilon^T\epsilon) = n\sigma^2$ (note that $E(\epsilon\epsilon^T) = \sigma^2\mathbf{I}$ is a matrix). At the global minimum:

$$\hat{\theta} - \theta_t = (J^TJ)^{-1}J^T[\mathbf{y} - \mathbf{f}(\theta_t)] = (J^TJ)^{-1}J^T\epsilon \tag{3}$$

This leads to the estimate of the asymptotic variance-covariance matrix:

$$E(\hat{\theta} - \theta_t)(\hat{\theta} - \theta_t)^T = E[(J^TJ)^{-1}J^T\epsilon]\,[(J^TJ)^{-1}J^T\epsilon]^T = (J^TJ)^{-1}J^TE(\epsilon\epsilon^T)J(J^TJ)^{-1}$$
$$= \sigma^2(J^TJ)^{-1}$$

For an estimation of σ^2 from the data, $S(\theta_t)$ cannot be used as θ_t is unknown. It can be approximated from $S(\hat{\theta})$. The expectation of $S(\hat{\theta})$ is found from Eqns. 1 and 3:

$$E(S(\hat{\theta})) = E(\mathbf{y} - \mathbf{f}(\hat{\theta}))^T(\mathbf{y} - \mathbf{f}(\hat{\theta}))$$
$$= E(\mathbf{y} - \mathbf{f}(\theta_t) - J[(J^TJ)^{-1}J^T\epsilon])(\mathbf{y} - \mathbf{f}(\theta_t) - J[(J^TJ)^{-1}J^T\epsilon])$$
$$= E(\epsilon - J(J^TJ)^{-1}J^T\epsilon)^T(\epsilon - J(J^TJ)^{-1}J^T\epsilon) = E(\epsilon^T\epsilon) - E(\epsilon J(J^TJ^{-1})J^T\epsilon$$

With $J(J^TJ)^{-1}J^T = B$ it is easy to prove that $B^TB = B$ and $(I - B)^T(I - B) = (I - B)$. Considering further, $E(\epsilon^TB\epsilon) = E\Sigma_i\Sigma_j\epsilon_ib_{ij}\epsilon_j = \Sigma_i\Sigma_jb_{ij}E(\epsilon_i\epsilon_j) = \sigma^2\Sigma_ib_i^2 = \sigma^2Tr(B)$. The use of $Tr(AB) = Tr(BA)$ gives $Tr(J(J^TJ)^{-1}J^T) = Tr(I_p) = p$, because the dimensions of J^TJ are $p \times p$. So the final conclusion is that $E\{S(\hat{\theta})\} = \sigma^2(n - p)$.

The best estimate of σ^2 is $s^2 = S(\hat{\theta}_t)/(n - p)$.

$S(\hat{\theta})/\sigma^2$ follows approximately a χ^2-distribution with $(n - p)$ degrees of freedom, $S(\theta_t)/\sigma^2$ follows a χ^2-distribution with n degrees of freedom and consequently $\{S(\theta_t) - S(\hat{\theta}_t)\}/\sigma^2$ follows a χ^2-distribution with p degrees of freedom. As $\{S(\theta_t) - S(\hat{\theta})\}/\sigma^2$ and $S(\hat{\theta})/\sigma^2$ are independent of each other, their ratio follows an F-distribution. Hence [8]:

$$S(\theta_t) = S(\hat{\theta})\{1 + (p/n - p)F\} = S(\hat{\theta}) + \sigma^2 p F_{(p,n-p)}$$

The importance of this relation is that a 95% confidence interval can be defined for $S(\theta)$ wherein $S(\theta)$ cannot be distinguished from $S(\hat{\theta})$. It sets a limit to S and so to the change in θ which cannot be distinguished from $\hat{\theta}$. The extent of the confidence interval is measured in units $\sigma^2 p$, which has a special geometrical meaning, as will be shown later.

The foregoing considerations have been set up as if the system is linear. Strictly speaking the relations do not hold, but when a large number of data are dealt with, ϵ will be normally distributed and when the model function can be chosen as close-to-linear the derived relations can be adopted as good approximations. This will be considered in the next section.

Close-to-linear model function

Consider for a moment that the model function is linear, thus $f(\theta) = \mathbf{X}\theta$, then the Jacobian matrix is equal to the matrix $\mathbf{X}$, i.e., $\mathbf{J} = \mathbf{X}$, and the corresponding equations for the linear model are easily obtained by replacing $\mathbf{J}$ by $\mathbf{X}$. Starting at θ_1, the initial guess, gives:

$$\theta_2 = \theta_1 + (\mathbf{X}^T\mathbf{X})^{-1}\mathbf{X}^T(\mathbf{y} - \mathbf{X}\theta_1) = \theta_1 + (\mathbf{X}^T\mathbf{X})^{-1}\mathbf{X}^T\mathbf{y} - \theta_1 = (\mathbf{X}^T\mathbf{X})^{-1}\mathbf{X}^T\mathbf{y}$$

This result is independent of θ_1. The importance of this result is that from any starting parameter set, θ_1, in one single "iteration" the right LS estimator $\hat{\theta} = (\mathbf{X}^T\mathbf{X})^{-1}\mathbf{X}^T\mathbf{y}$ is attained.

It should be noted that this is not a merit of only the Gauss-Newton method. As all second derivatives of the model function are zero the hyperplane, S, is a pure paraboloid and the Newton-Raphson method will also achieve $\hat{\theta}$ in one iteration. A second conclusion is that if $\mathbf{y}$ is normally distributed, $\hat{\theta}$ will be an unbiased normally-distributed estimator because $\hat{\theta}$ and $\mathbf{y}$ are linearly related. In the $(p + 1)$-dimensional parameter sum-of-squares space the 95% confidence limits correspond to a contour which is found as the intersection of the p-dimensional hyperparaboloid with a p-dimensional flat hyperplane positioned $\sigma^2 pF(p, n - p, d)$ above the paraboloid minimum $S(\hat{\theta})$. The intersection is a symmetrically shaped $(p - 1)$-dimensional ellipsoid.

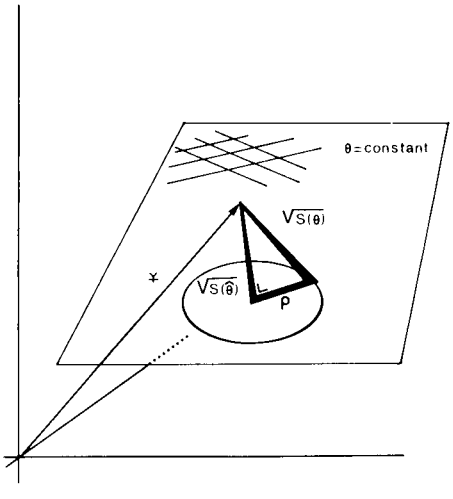
When the model is non-linear a number of results true for the linear case no longer apply. When ϵ is assumed to be normally distributed, in general $\hat{\theta}$ is biased and no longer normally distributed and $s^2 = S(\hat{\theta})/n - p$ is no longer an unbiased estimate of σ^2. Contours of confidence regions can still be defined but will not be ellipsoidic; they become "banana-shaped" or worse. A lot of useful features are lost in principle.

In close-to-linear cases, however, with a suitable choice of model function and a suitable change of parameters, the lost features return. Non-linearity should be quantified for that purpose. Bates and Watts [9] have indicated how to measure the amount of non-linearity.

Geometrical interpretation in the sample space

The n experimental (absorbance) data can be represented by a vector $\mathbf{y}$ in the n-dimensional sample space. On variation of θ model function $f(\theta)$ will develop as a p-dimensional hyperplane, the solution locus, in that sample space. Generally $\mathbf{y}$ does not end up in the solution locus and what is done in the LSQR method is to select $\hat{\theta}$ so that $S(\hat{\theta}) = (\mathbf{y} - \mathbf{f})^T(\mathbf{y} - \mathbf{f})$ is minimal, which can be geometrically interpreted as seeking for a perpendicular from the tip of the arrow head $\mathbf{y}$ to a tangent plane of the solution locus with the perpendicular distance at minimum length.

In this geometrical representation the shape of the solution locus is determined by the model function. If the model function is linear the hyperplane is flat. Considering the contour $S(\theta)$ = constant for the linear model function (Fig. 3) the 95% confidence contour corresponds to a "circle" on the surface with a radius $\rho^2 = S(\theta) - S(\hat{\theta}) = \sigma^2 p F_{(n, n-p, g^s)}$. It marks the region in which θ changes are not significantly different. In the plane $\mathbf{f} = \mathbf{X}\theta$, lines of constant $\theta_1, \theta_2, \ldots \theta_p$ can be drawn. The grid originating on this plane consists of



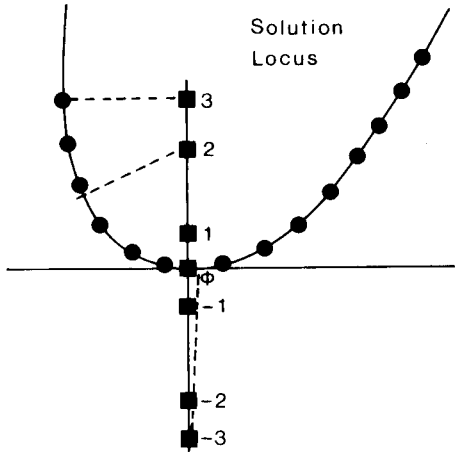


Fig. 3. The sample vector $\mathbf{y}$, its projection on the flat solution locus of the linear model and the circular confidence contour with radius ρ are shown together with the grid θ = constant indicating the confidence limits of $\theta_j, \theta_k \ldots$.

Fig. 4. The influence of strong intrinsic non-linearity on the bias of $\hat{\theta}$. The projections of the upper locations (1, 2 and 3) of the sample vector on the solution locus deviate to the left, while the projections of the locations −1, −2 and −3 remain near the correct value ϕ. The mean of the projections is biased even although the parameter non-linearity is small as follows from the equidistant points θ = constant on the solution locus.

straight lines, parallel and equispaced. If the error in y is normally distributed and the model function is correct, the arrow tip will have a normal distribution spatially arranged on both sides of the solution locus and with its expectation value (true mean) in this surface.

The projection of the arrow tip on the flat solution locus marks the value of $\hat{\theta}$ on that plane. This projection is normally distributed. As the grid of constant θ_j values is parallel, linear and equispaced, $\hat{\theta}$ will be an unbiased estimator normally distributed around its true value. The radius ρ marks also the confidence region of $\hat{\theta}$.

In the following section it will be explained that $\hat{\theta}$ may be regarded as unbiased in non-linear systems if the solution locus is sufficiently flat in the region $\sigma\sqrt{p}$ around the projection and if the grid "θ constant" is sufficiently parallel, linear and equidistant in that region.

Intrinsic non-linearity

When a model function is non-linear, it manifests itself in a curvature of the hyperplane. The influence of this curvature on the distribution of $\hat{\theta}$ follows from Fig. 4. Changing the arrow top position from -3 to $+3$ shifts $\hat{\theta}$ to the left. If the model is correct and y has its arrow tip normally distributed around the point ϕ, $\hat{\theta}$ will be skewly distributed and hence biased, even when the θ-grid is equispaced as in Fig. 4. The bias depends on the extent $\sigma\sqrt{pF}$ of the egg-shaped confidence region of y relative to the curvature of the solution locus, which can be expressed in the radius of curvature R_N perpendicular to the solution locus. Bates and Watts [9] introduced $\gamma_{IN} = \sigma\sqrt{p}/R_N$ as a relative measure for intrinsic non-linearity. Obviously, if $\gamma_{IN} \ll 1$ the solution locus may be regarded as flat in the region of interest. In that case the bias of $\hat{\theta}$ tends asymptotically to zero and the model function behaves as close-to-linear.

Parameter non-linearity

If the model function is linear $\mathbf{f}(\theta) = \mathbf{X}\theta$ the solution locus is flat, the intrinsic non-linearity is zero and the confidence contour in the plane is a circle. Consequently $\hat{\theta}$ will be an unbiased estimator (Fig. 5a). Change of

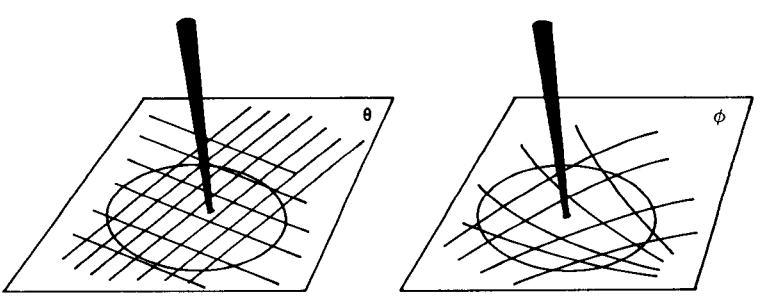


Fig. 5. Even with a flat solution locus (intrinsic non-linearity of zero) considerable parameter non-linearity may occur leading to bias of the parameters.

parameters does not change the shape of the solution locus either in linear or non-linear cases, but it changes the grid in the hyperplane. If $f(\theta) = X\theta$ has its parameters changed, for instance, into $f(\phi) = X\mathrm{e}^{-\phi}$, the grid will no longer be equispaced, parallel and linear (Fig. 5b). The consequence is that, transposed to the parameter space, the distribution of ϕ is skewed and $\hat{\phi}$ will be biased. In spite of the zero intrinsic non-linearity there is a substantial parameter non-linearity. Changes of parameters, conversely, can be used to rescale the grid producing new parameter lines that are more linear, parallel and equispaced, and consequently resulting in a negligibly biased parameter set.

Bates and Watts [9] have indicated in which way non-linearity can be quantified. In linear models the parameter space and the sample space are linearly coupled. A change of θ with a vector $b\mathbf{h}$ (b is a scalar), i.e., $\theta = \theta_1 + b\mathbf{h}$, leads to a translation in the solution locus:

$$f(\theta) - f(\theta_1) = X(\theta_1 + b\mathbf{h}) - X\theta_1 = bX\mathbf{h}$$

A linear translation through the parameter space is coupled with a linear movement along the line $X\mathbf{h}$ lying in the solution locus. With non-linear model functions a constant velocity in the parameter space leads to accelerations, while the point is correspondingly moving through the solution locus in the sample space along the line $f(\theta(b)) = f(b)$. Considering the solution locus is curved itself with the line $f(b)$ in it, there will be an acceleration normal to the plane necessary to keep the "point" in the hyperplane. It is comparable to the centripetal acceleration in classical mechanics $a_N = v^2/R_N$ in which R_N is the radius of the curve followed at that point. Analogously Bates and Watts introduced $R_N = (\mathrm{d}f/\mathrm{d}b)^2/(\mathrm{d}^2f/\mathrm{d}b^2)$ as a measure of the curvature. This relation also holds for the curvature of the θ-lines parallel to the solution locus and even formally for the linear acceleration.

In general the sample space is n-dimensional and the solution locus a p-dimensional surface within it. Hence $(n-p)$ normals to this surface are dealt with here, and $(n-p)$ relative intrinsic non-linearity measures $\gamma_{\mathrm{IN}} = \sigma\sqrt{p}/R_{\mathrm{N}}$ can be defined. In this equation σ^2 can be estimated from $(S(\hat{\theta})/(n-p)$ and R_N from the previous equation whereby $\mathbf{h}$ is selected along the $(n-p)$ orthogonal axes perpendicular to the solution locus. Analogously the relative parameter effect non-linearity measure $\gamma_{\mathrm{PE}} = \sigma\sqrt{p}/R_p$ can be introduced for all accelerations in the hyperplane along the remaining p orthogonal axes. So $(n-p)$ values of γ_{IN} and p values of γ_{PE} are dealt with. When all relative measures are $\ll 1$ (which in practice means <0.10) the model function can be considered as close-to-linear. Strictly, this only holds for the global minimum situation. It has been our experience, however, that even with 100 parameters to be estimated, the Gauss-Newton procedure locates the global minimum in a small number of iterations. It suggests that making non-linearity small will promote convergence. There are no rules as to how to reduce the non-linearity measures, γ. It depends on the model under consideration and the lowest experimental errors achievable in practice.

It is sensible, therefore, to linearize model functions as much as possible,

as it is thought to promote convergence. In spectrophotometry absorbance is usually selected for the model function, so that intrinsic non-linearities are usually small. The parameter non-linearities can be adapted by changing the remaining non-linear parameters in the model. For investigation of the changing of parameters it is often sufficient to guess σ from the experimental errors to be expected and to calculate R from the model function with estimated θ-values. With $\phi = g(\theta)$, $\gamma_{PE}(\phi)$ can be re-estimated from $\gamma_{PE}(\theta)$ after changing parameters, and so some idea can be obtained as to whether such changes will improve convergence. Nevertheless this part of convergence improvement is still partly trial and error.

APPLICATION TO MULTIWAVELENGTH MEASURED MODELS

It is believed by many investigators that spectroscopic data are less precise than the data from other techniques such as potentiometry. If this ever has been true it is certainly no longer true, as excellent apparatus is available with which multiwavelength measurements can rapidly be made. Data acquisition systems allow more data to be handled, which contain considerably more information about the underlying models than any other technique. Most commonly absorption signals are measured, but without a substantial loss in accuracy, these data can easily be transformed into absorbance data with a standard deviation below 0.002 absorbance. The absorbance data are linearly related to concentrations and molar absorptivities. This transformation makes the model function linear for the majority of the parameters (in θ) to be estimated. It lowers the intrinsic non-linearity and so improves convergency. If an equilibrium system containing five absorbing components in solution and determined by four equilibrium constants is dealt with, the parameter set has the form:

$$\theta = \{\epsilon_1, \epsilon_2, \epsilon_3, \ldots . \epsilon_{p-4}, K_{p-3}, K_{p-2}, K_{p-1}, K_p\}$$

in which all ϵ terms appear linearly in the model function $f(\theta) = \mathbf{C}(\mathbf{K})\epsilon$. Considering the model function as being linear in all parameters near the initial guess θ_1 (the Gauss-Newton approach), for neighbouring values for the first rows of the Jacobian matrix, Eqn. 1 gives:

$$\begin{matrix} C_{11} & C_{12} & C_{13} & C_{14} & C_{15} \\ C_{21} & C_{22} & C_{23} & C_{24} & C_{25} \end{matrix} \quad \begin{matrix} 0\ 0\ 0\ 0\ 0\ 0\ 0 \ldots . 0\ 0\ 0\ 0 \\ \text{(zero)} \end{matrix} \quad \frac{d(\epsilon \mathbf{C})}{dK_{p-3}}, \ldots . .$$

The zero elements arise as, at the specific wavelength, only five relevant ϵ values (out of $p - 4$) may contribute in the product $\mathbf{C}\epsilon$, which can only be achieved by making non-relevant contributions zero in J (and in the $n \times (p - 4)$ matrix $\mathbf{C}$). The Jacobian matrix has a block structure with non-zero elements at the shaded areas (Fig. 6). Consequently the $(p \times p)$ matrix J^TJ has a symmetric block structure with many zero elements too (Fig. 7).

In the iterative procedure the inverse of (J^TJ) is needed for the calculation

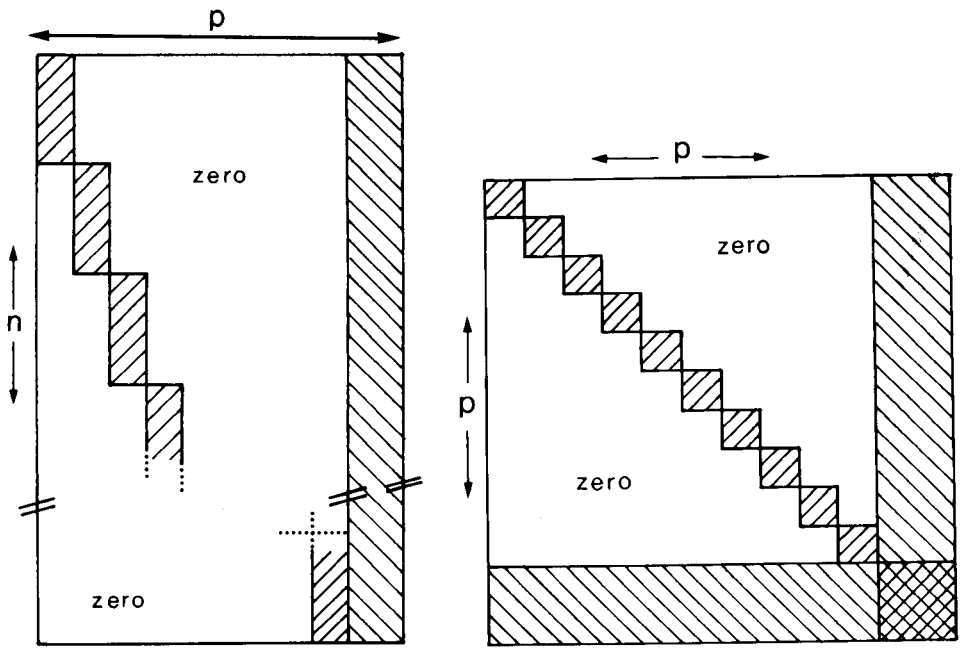


Fig. 6. The Jacobian ($n \times p$) matrix with its block structure.

Fig. 7. The block structure of the positive definite J^TJ matrix allowing a quick inversion procedure based on Cholevsky decomposition.

of θ_{j+1} (Eqn. 2). As (J^TJ) is positive, the most efficient procedure is the Cholesky decomposition. For a fully-filled (125 × 125) matrix the Cholesky inversion method requires about 300 000 operations (ca. $n^3/6$). A special subroutine adapted to the block structure has been developed which performs the inversion in less than 5000 operations. The advantages are a 50-fold increase in speed, less memory required, less rounding-off errors and better numerical safety.

The elements of matrix J can be found in two ways, numerically and analytically. The numerical way has some disadvantages. An important drawback is that there is no straightforward method of determining the optimal step size. A value of $\Delta\theta_j$ that is too large may cause unacceptable contributions of higher derivatives to $(\Delta f_i/\Delta\theta_j)$, a value too small degrades numerical safety. In general there is a substantial risk that in multiparametric systems at least some numerical derivatives will deviate far from reality. It disturbs convergence, as has been observed in the development phase, because the corresponding parameters are constrained by the walls of the multidimensional cube. Moreover the analytical calculation of $df/d\theta$ directly from θ and X is generally faster especially if θ itself is found iteratively as in the present model. It can be concluded that in multiparametric systems it is essential

that the derivatives of the model function, together composing J, are determined analytically. The programming efforts are repaid in accuracy, faster convergence, less computer time needed and in an increase in the number of parameters with which convergence can be achieved.

In the present model the model function is non-linear with respect to only four parameters, the equilibrium constants K. Conversion of K into log K hardly influenced the number of iterations necessary to reach the global minimum. In the test phase the bias of these parameters was checked with synthetic absorbance data to which Gaussian noise was added. Neither K nor log K were unbiased, but in both cases the bias was small compared to their standard deviations. In all cases the relative intrinsic and parameter non-linearities were below 0.1.

The program "Kleikwa" written in Fortran for the mainframe computer of the University of Amsterdam was originally set up from a Letagrop type as originally developed by Sillén and co-workers. Weighing factors were introduced, derivatives numerically derived and the Newton-Raphson model followed. It has been considerably adapted and the current version is not an efficient use of memory. It will be rewritten in the near future and adapted for use on microcomputers, which is possible because of the special block structure of the matrix J^TJ, which allows splitting into small sub-matrices and efficient inversion.

In the next paper applications will be discussed to the thorium/xylenol orange and thorium/semixylenol orange systems.

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TITRATION OF SULPHIDES AND THIOLS IN NATURAL WATERS

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SUMMARY

The concentrations of the species present during the procedure of Boulègue for determination of sulphide, thiols, thiosulphate and sulphite in mineral waters by titration with mercury(II) chloride have been calculated and functions suitable for the evaluation of the equivalence points have been derived. It is shown that the halide ions in seawater interfere only in the titration of sulphite.

Natural waters contain sulphate, and the titration procedure of sulphide with lead perchlorate recommended by Orion Research has the drawback of forming two solid phases, PbS and $PbSO_4$. Boulègue [1] showed that it was possible to titrate sulphide, polysulphides, thiosulphate, sulphite and thiols with mercury(II) chloride in mineral waters. Dyrssen et al. [2] showed that it was possible to titrate sulphide in the 30 to 300 μmol l^{-1} range in Black Sea water by the same procedure. Below 30 μmol l^{-1} Cline's [3] methylene blue method was more accurate. At pH 13, the thiols are titrated when the reaction with sulphide is complete. The purpose of this communication is to calculate the stoichiometry of the titrations by use of selected values of the relevant stability constants, and to derive functions for the evaluation of the equivalence points.

STABILITY CONSTANTS

The solubilities of silver sulphide and black mercury sulphide have been measured by Schwarzenbach and Widmer [4, 5]. The dissociation of hydrogen sulphide in sea water was determined by Almgren et al. [6]. Critical stability constants have been selected by Martell and Smith [7, 8]. For the calculation of the course of the titrations with mercury(II) chloride, the following constants (log units) were used:

$[Hg^{2+}][H_2S][H^+]^{-2}$	−29.93 (solid HgS)
$[H_2S][H^+]^{-1}[HS^-]^{-1}$	6.74
$[H_2S][H^+]^{-2}[S^{2-}]^{-1}$	20.74
$[RSH][H^+]^{-1}[RS^-]^{-1}$	9.34

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$[Hg(OH)_2][H^+]^2[Hg^{2+}]^{-1}$	−6.0
$[Hg(SH)_2][H^+]^2[Hg^{2+}]^{-1}[H_2S]^{-2}$	23.96
$[HgS_2H^-][H^+]^3[Hg^{2+}]^{-1}[H_2S]^{-2}$	17.77
$[HgS_2^{2-}][H^+]^4[Hg^{2+}]^{-1}[H_2S]^{-2}$	9.47
$[Hg(RS)_2][Hg^{2+}]^{-1}[RS^-]^{-2}$	41.6
$[HgOHRS][Hg^{2+}]^{-1}[OH^-]^{-1}[RS^-]^{-1}$	31.6
$[HgSRS^-][Hg^{2+}]^{-1}[S^{2-}]^{-1}[RS^-]^{-1}$	46.6
$[HS_2O_3^-][H^+]^{-1}[S_2O_3^{2-}]^{-1}$	1.6
$[Hg(S_2O_3)_2^{2-}][Hg^{2+}]^{-1}[S_2O_3^{2-}]^{-2}$	29.23
$[Hg(S_2O_3)_3^{4-}][Hg^{2+}]^{-1}[S_2O_3^{2-}]^{-3}$	30.6
$[HSO_3^-][H^+]^{-1}[SO_3^{2-}]^{-1}$	7.18
$[Hg(SO_3)_2^{2-}][Hg^{2+}]^{-1}[SO_3^{2-}]^{-2}$	24.07
$[Hg(SO_3)_3^{4-}][Hg^{2+}]^{-1}[SO_3^{2-}]^{-3}$	25.96

The stability constants for the mixed complexes HgOHRS and $HgSRS^-$ were calculated from the statistical relationship $\beta_{11} = 2\sqrt{{}^L\beta_2 {}^A\beta_2}$ where ${}^L\beta_2$ is the stability constant for $Hg(OH)_2$ or HgS_2^{2-} and ${}^A\beta_2$ is the stability constant for $Hg(RS)_2$ (see [9]). The value of log ${}^A\beta_2 = 41.6$ was selected to fit the titration curve potentials [1, 2]. The value is reasonable if one considers that the log β_2 value for $Hg(SH)_2$ is 37.72 and that H_2S is a somewhat stronger acid than RSH. The constants for the formation of $HgCl_n$ complexes are too small (log β_2 for $HgCl_2$ is 13.22) for these complexes to compete with the formation of $Hg(OH)_2$ and disulphide complexes at pH 13. The titration courses were calculated with our latest version of the HALTAFALL program according to Ingri et al. [10].

TITRATION OF BLACK SEA WATER

A 20-ml sample from a depth of 1800 m obtained on November 20, 1984 at station 239 (43°31′W, 36°30′E) was titrated with 1.011 mM mercury(II) chloride at pH 13. Figure 1 shows how the concentrations of various species change during the addition of mercury(II) chloride. At the first equivalence volume, corresponding to $20 \times 0.245/1.011 = 4.85$ ml, there is a drop in the concentrations of HS^-, HgS_2^{2-} and S^{2-} due to the following three main reactions:

$$HgCl_2 + HS^- \rightarrow HgS(s) + H^+ + 2Cl^-$$

$$HgCl_2 + HgS_2^{2-} \rightarrow 2HgS(s) + 2Cl^-$$

$$HgCl_2 + S^{2-} \rightarrow HgS(s) + 2Cl^-$$

If the equilibrium constants involved are used, the total concentration of sulphide (0.245 mmol l^{-1}) can be expressed by: $[HS^-] + [HgS_2^{2-}] + [S^{2-}] =$

$[S^{2-}](K_1[H^+] + K_{s2} + 1)$. The constants K_1 and K_{s2} denote the equilibria $H^+ + S^{2-} \rightleftharpoons HS^-$ and $HgS(s) + S^{2-} \rightleftharpoons HgS_2^{2-}$, respectively. Thus $(v_1 - v)t = (20 + v)[S^{2-}](K_1[H^+] + K_{s2} + 1)$. Since the potential of the sulphide electrode follows $E = E_0 - 29.5 \log [S^{2-}]$, the straight line $(20 + v)10 \exp (E_0 - E)/29.5$ will give the equivalence point. In Fig. 2 calculated values of $(20 + v)[S^{2-}]$ are plotted against v. The straight line intercepts the horizontal axis at $v = v_1 = 4.85$ ml.

Figure 1 also shows that after the reactions with the sulphide ions are complete, there is an increase in the concentration of $Hg(RS)_2$ and a decrease in the concentration of RS^-. A minor reaction involves $HgSRS^-$. Thus the main reaction that occurs between the two equivalence points is:

$$HgCl_2 + 2RS^- \rightarrow Hg(RS)_2 + 2Cl^-$$

The formation of $Hg(RS)_2$ corresponds to $(20 + v)[Hg(RS)_2] = (v - v_1)t$.

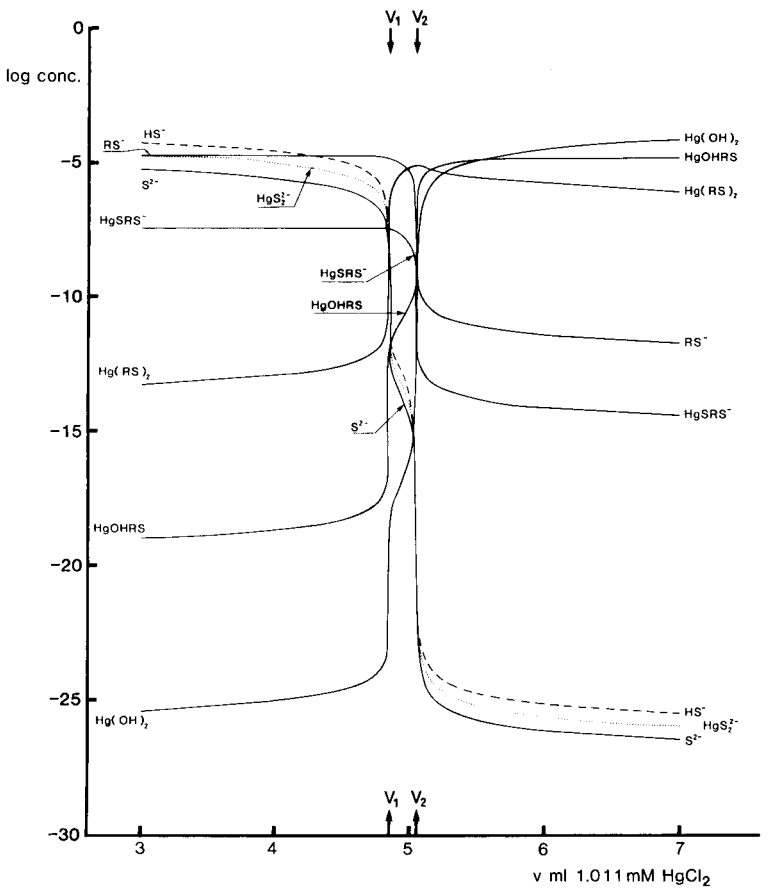


Fig. 1. Titration of 20 ml of Black Sea water from a depth of 1800 m with 1.001 mM $HgCl_2$. The sample contained 0.245 mM sulphide and 0.0212 mM thiols (RSH). The equivalence points for sulphide and thiol are denoted by v_1 and v_2, respectively.

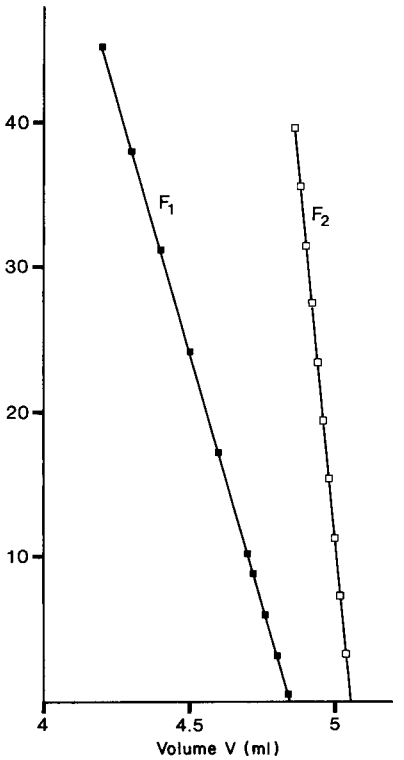


Fig. 2. Linear functions F_1 and F_2 suggested for the evaluation of the equivalence point ($v = v_1 = 4.85$ ml) for sulphide and thiol ($v_2 - v_1 = 0.21$ ml). The vertical axes are $F_1 = 10^6(20 + v)[S^{2-}]$ and $F_2 = (20 + v)[RS^-]$ or, since sulphide is measured, $F_2 = 10^6\sqrt{(20 + v)(v - v_1)[S^{2-}]}$. In practice $[S^{2-}]$ is replaced by $10 \exp (E_0 - E)/29.5$ which is calculated from the potentiometric data.

The total concentration of thiols is $2[Hg(RS)_2] + [RS^-] = 2(v_2 - v_1)t/(20 + v)$. Thus $(20 + v)[RS^-] = 2(v_2 - v)t$. Using the stability constant for $Hg(RS)_2$:

$$\beta_2 = [Hg(RS)_2]\,[Hg^{2+}]^{-1}[RS^-]^{-2}$$

and the solubility product of HgS, $K_{s0} = [Hg^{2+}]\,[S^{2-}]$, the eqns. above give:

$$(20 + v)(v - v_1)[S^{2-}] = 4\beta_2 K_{s0} t(v_2 - v)^2$$

which means that the square root of the function $(20 + v)(v - v_1)10 \exp (E_0 - E)/29.5$ gives $v = v_2 = 5.06$ ml, corresponding to $2(5.06 - 4.85)1.011/20 = 0.0212$ mmol l^{-1} of thiol (see Fig. 2).

Another feature of Fig. 1 is that after the titration of sulphide and thiols the main species in solution are $Hg(OH)_2$, HgOHRS and $Hg(RS)_2$. Since it has been shown that the solubility of $Hg(OH)_2$ is of the order of 2×10^{-4} M [11, 12] an excess of 2 ml of 1.011 mM mercury(II) chloride should not produce solid HgO.

TITRATION ACCORDING TO BOULÈGUE

Boulègue [1] titrated 50 ml of a synthetic solution containing 0.094 mM sulphide, 0.053 mM thiol, 0.18 mM thiosulphate and 0.26 mM sulphite with 10 mM mercury(II) chloride. After titration of the sulphide (v_1 = 50 × 0.094/10 = 0.47 ml) and the thiol ($v_2 = v_1 + 50 \times 0.053/20$ = 0.60 ml) the pH was lowered from 13 to 7. As a result the concentrations of $Hg(OH)_2$, HgOHRS and $Hg(RS)_2$ decreased due to the protonation of OH^- and RS^-, and reactions with the weaker bases $S_2O_3^{2-}$ and SO_3^{2-} are favoured. Figure 3 shows the results of a calculation of the species present during the titration. First the

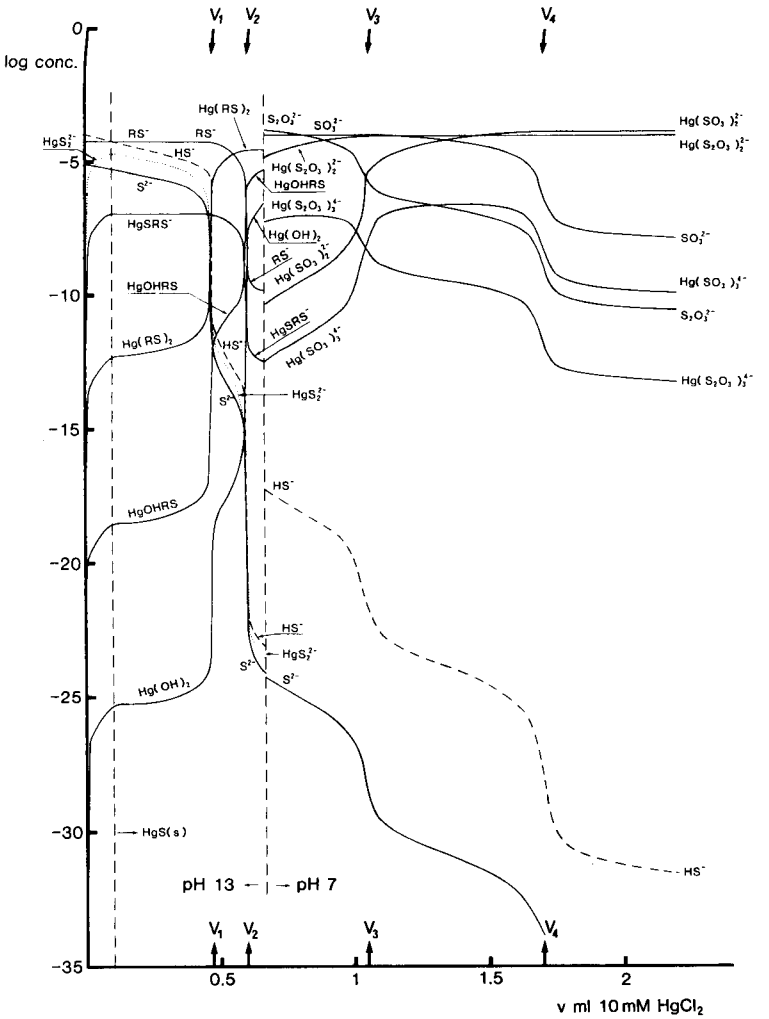


Fig. 3. Titration of 50 ml of a synthetic solution containing 0.094 mM sulphide, 0.053 mM thiol, 0.18 mM thiosulphate and 0.26 mM sulphite with 10 mM mercury(II) chloride. After titration of S^{2-} and RS^- the pH is lowered from 13 to 7.

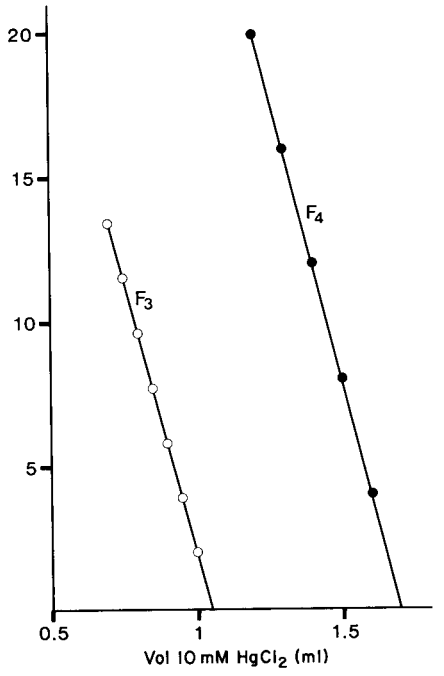


Fig. 4. Linear functions F_3 and F_4 suggested for the evaluation of the equivalence point for thiosulphate ($v_3 - v_2$ = 1.05 − 0.60 ml) and sulphite ($v_4 - v_3$ = 1.70 − 1.05 ml). The vertical axes are $F_3 = (50 + v)[S_2O_3^{2-}]$ or, when sulphide is measured, $F_3 = 10^{16}\sqrt{(50 + v)(v - v_2)[S^{2-}]}$ and $F_4 = (50 + v)[SO_3^{2-}]$ or, when sulphide is measured, $F_4 = 10^8\sqrt{(50 + v)(v - v_3) \times 1.51 \times [S^{2-}]}$. In practice a function proportional to the sulphide concentration is calculated from the potentiometric data.

thiosulphate is titrated, whereby mercury(II) forms mainly $Hg(S_2O_3)_2^{2-}$. Equations analogous to those for the reaction with thiols give (v_2 = 0.60 ml):

$$(50 + v)(v - v_2)[S^{2-}] = 4\beta_2 K_{s0} t(v_3 - v)^2$$

where β_2 is the stability constant for $Hg(S_2O_3)_2^{2-}$. The square root of the function $(50 + v)(v - v_2)10 \exp (E_0 - E)/29.5$ gives the equivalence point v_3 = 1.05 ml i.e. ($v_2 + 50 \times 0.18/20$). After the titration of thiosulphate the sulphite reacts with mercury(II) chloride forming $Hg(SO_3)_2^{2-}$ as the main product. This leads to the similar equation:

$$(50 + v)(v - v_3)(10^{7.18}10^{-7} + 1)[S^{2-}] = 4\beta_2 K_{s0} t(v_4 - v)^2$$

where the term $10^{7.18}10^{-7}$ accounts for the protonation of sulphite at pH 7 and β_2 is the formation constant of $Hg(SO_3)_2^{2-}$. Similarly, the square root of the function $(50 + v)(v - v_3)1.51 \times 10 \exp (E_0 - E)/29.5$ gives a straight line which intercepts the horizontal v axis at v_4 = 1.70 ml ($v_3 + 50 \times 0.26/20$). These results are shown in Fig. 4.

In the authors' experience it is not possible to titrate sulphite in sea water. Several halide complexes have ratios of $[HgCl_mBr_n^{2-m-n}]/[Hg^{2+}]$ on the order of 10^{13} to 10^{14} in sea water [9] while the ratio of $[Hg(SO_3)_2^{2-}]/[Hg^{2+}]$ is about 10^{14} for 10^{-5} M sulphite. Thus there will be considerable interference from halide complex formation.

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