The Development of Analytical Methods for

the Determination of Selenium

by

Kieran James McLaughlin

A thesis submitted for the Degree
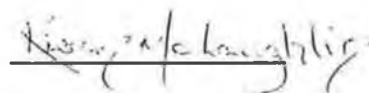
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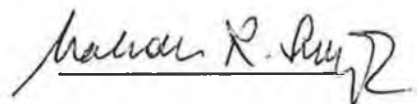Doctor of Philosophy

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Declaration

I hereby declare that the contents of this thesis, except where otherwise stated, are based entirely on my own work, which was carried out in the School of Chemical Sciences, Dublin City University, Dublin and in the Department of Chemistry, University of Oviedo, Oviedo, Spain.

Kieran McLaughlin

Malcolm R. Smyth

(supervisor)

To my mother and father

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Abstract

THE DEVELOPMENT OF ANALYTICAL METHODS FOR THE ANALYSIS OF SELENIUM

Kieran McLaughlin

Chapter 1 of this thesis describes the biological distribution and biochemical significance of selenium.

A review of the main methods used for the analysis of selenium in biological materials is given in Chapter 2.

In Chapter 3, the use of a gold fibre electrode in an electrochemical flow cell is described for the anodic stripping voltammetry of selenium. The manufacture of the flow electrodes and the subsequent optimisation of the analytical procedure are described. Chapter 4 is concerned with the cathodic stripping voltammetry of Se(IV) at mercury-coated carbon fibre electrodes. The optimisation of the mercury film formation conditions and the stripping voltammetric methodology at such electrodes are described.

The use of a flow injection hydride generation method of analysis and its involvement in an interlaboratory study are described in Chapter 5. The development of the system from an initial batch system is outlined.

A critical overview of the methods described in this thesis in relation to the commonly used methods is given in Chapter 6.

Chapter 1

The biological distribution and biochemical

significance of selenium

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1.1. INTRODUCTION

1.1.1. Historical Aspects

"Selenium was named after the moon, from the Greek
"selene", since it resembled, chemically, tellurium,
which had already been named after the earth (from
the Latin "tellus"). And so sodium selenite came to
be known as "moonstone", a word which conjures up a
certain mysticism or even madness. Professor Krehl
of Philadelphia called selenium the maddening mineral,
and we who are working in this field believe it is a
very apt description - maddening because of the
difficulty in estimating the nanogram amounts in
biological material, particularly in New Zealand
where concentrations are so low, and maddening
because, as Krehl explains, " selenium is perverse,
elusive and contradictory, and yet all the while vastly
intriguing " ".

Reproduced from the first Muriel Bell
Memorial Lecture given to the Nutrition
Society of New Zealand [1]

The element selenium was discovered in 1818 by Berzelius, and
further study of its chemistry led to many industrial uses for

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this element, which is almost as rare as gold. Many of these uses depend on the remarkable susceptibility of electrons in selenium to excitation by light, resulting in generation of an electric current. This has led to the use of selenium in photoelectric cells, light meters, rectifiers and xerographic copying machines. It is also used to decolourise the greenish tint of glass due to iron impurities, or, in excess, to create the ruby-red colour seen in warning signals and automobile tail lights.

The biological significance of selenium was not recognised until it was identified as the toxic substance causing lameness and death in livestock in the Dakotas and Wyoming [2]. Dr. Madison (1860) had earlier observed a number of toxicity signs, including hair loss in cavalry horses at Fort Randall in the old Nebraska Territory [2]. Lameness resulted from inflammation of the feet, followed by suppuration at the point where the hoof joins the skin and ultimate loss of the hoof. The consequent tenderness inhibited the search for food and water, and since no stored forage was available, death was at least partly attributable to starvation. Similar signs were described by Marco Polo in his travels in Western China near the borders of Turkestan and Tibet about the year 1295 [2]. Loss of hair and nails in humans, presumbably suffering from chronic selenosis, was first described in Colombia by Father Pedro Simon in 1560.

Early interest in selenium by nutritionists concerned its high concentration in certain range plants [2] and the consequent

toxicosis in animals that grazed on these plants. However, the discovery in 1957 that selenium was an essential nutrient [3] led to an entirely new era of research that continues to this day. Instead of a primary concern with toxicity, nutritionists turned their attention to the metabolic function of this element and the consequences of its deficiency.

More recently, the essential nature of selenium has become the centre of attention, and this element is now known to be required by animals and humans. Its role as an integral feature of the enzyme glutathione peroxidase has been established [4], and other possible functions are under active investigation.

1.2. Distribution of selenium

The selenium status of animals varies with the nature of the diet. The level of selenium in individual foods is highly variable; this variability stemming largely from soil differences in the areas in which the foods are grown. The selenium content of most soils lies between 0.1 and 2.0 ppm, but only a portion of this selenium in soils is available to the vegetation they support. With regard to their selenium content, soils can be classified into three categories, as described in the following sections.

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1.2.1. Toxic seleniferous soils

Soils that supply sufficient selenium to produce plants containing toxic amounts of selenium in their tissues are commonly referred to as "toxic seleniferous soils". These are normally alkaline in nature and occur in regions of low rainfall, with usually less than 8 cm total annual precipitation.

The presence of water-soluble selenium is an important characteristic of toxic soils [2]. Selenate has been identified as the main water-soluble form of selenium in soil that is translocated into vegetation containing toxic quantities of the element.

Selenium occurs in toxic amounts under humid conditions in certain parts of Limerick, Tipperary, and Meath [2]. The seleniferous soils lie in a poorly drained valley, and leaching of topographically higher rocks and soils has led to selenium enrichment of these soils. Extensive areas of seleniferous soils are found in South Dakota, Wyoming, Montana, Nebraska and Arizona in the U.S.A., and in areas of Alberta, Saskatchewan and Manitoba in Canada [2].

In 1937, researchers reported the selenium content of some foods produced on seleniferous farms in South Dakota, finding 0.16 to 1.27 ppm selenium in milk, 0.25 to 1.0 ppm in bread made from local flour, 0.25 to 9.14 ppm in eggs, and 1.17 to 8.0 ppm (dry basis) in meat [5]. Recent research [2] has shown that because

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many of the most seleniferous areas have been retired from farming, it is likely that there are very few locations today having the high concentration of selenium in foods that was found previously [5]. The United States and Canadian wheat crops are now produced in selenium-adequate areas and this results in moderately high average concentrations of the element in wheat-related foods in both countries.

1.2.2. Nontoxic seleniferous soils

Many soils of the world contain high levels of selenium, but low levels of water-soluble selenium, and consequently do not produce vegetation that has a toxic selenium level for animals. These soils are characterised by a zone of accumulated iron and aluminium compounds, and are developed under humid conditions. Hawaiian soils containing 6 to 15 ppm and Peurto Rican soils containing 1 to 10 ppm selenium do not produce seleniferous vegetation, whereas soils of Israel and South Dakota with lower selenium contents produce toxic plants [2]. The nontoxic seleniferous soils of Hawaii and Peurto Rico have an acid pH range (4.5 to 6.5) which, in the presence of ferric hydroxide, renders the selenium unavailable to plants.

1.2.3. Low-selenium soils

Most of the soils from the low-selenium areas of the United States contain less than 0.5 ppm selenium, and those of

eastern Canada contain less than 0.2 ppm selenium [6].
Low-selenium soils [7] appear to be responsible for selenium
deficiency disorders in livestock raised in certain regions of
Australia, New Zealand, Finland, Canada and the United States.

1.3. <u>Selenium in animal tissues and fluids</u>

Selenium occurs in all cells and tissues of the body in
concentrations that vary with the tissue and with the level of
selenium in the diet. The kidney, and in particular the kidney
cortex, is by far the organ that is highest in selenium
concentration, followed by the glandular tissues, particularly
the pancreas, the pituitary, and then the liver. The muscles,
bones, and blood are normally relatively low and adipose tissue
very low in selenium. Cardiac muscle is consistently higher in
selenium than skeletal muscle.

Few data on the selenium levels in normal human tissues, other
than blood, have appeared. Dickson and Tomlinson [8] examined
autopsy specimens of the liver, skin, and muscles of 10 adults
with the following results: liver, range 0.18-0.66, mean 0.44;
skin, range 0.12-0.62, mean 0.27; muscle, range 0.26-0.59, mean
0.37 ug Se/g of whole tissue. Analysis of a wider range of
tissues for two individuals, one an infant and the other an
adult, revealed the highest selenium levels in the kidney and
the thyroid and the lowest in the fat.

The concentration of selenium in the blood is so responsive to

changes in the selenium level of the diet that it is difficult to determine if significant differences, due to the species or age of the animal exist. The normal levels of selenium in human blood have been studied in several countries. Allaway and co-workers [3] examined samples from 210 male donors in 19 cities in the U.S.A. The selenium concentrations ranged from 0.10 to 0.34 ug/ml, with a mean of 0.21 ug/ml. The mean selenium concentration varied among different locations, with some evidence of a geographic pattern reflecting established regional differences in the selenium levels in crops.

In a study of whole blood selenium levels in children suffering from kwashiorker, Burk et al. [9] obtained mean levels of 0.08 and 0.11 ug/ml in two groups of affected children, compared with 0.14 and 0.22 ug/ml, respectively, in control children or recovered patients. The most likely explanation of the low blood selenium levels in children suffering from kwashiorker is that they are anaemic and hypoproteinemic and, therefore, circulate reduced levels of selenium-containing proteins.

1.3.1. Selenium metabolism

The extent to which selenium is absorbed from the gastro – intestinal tract, and its retention and distribution within the body, vary with the species, with the chemical form and amount of the element ingested.

Studies with ^{75}Se at physiological levels indicate that the

duodenum is the main site of absorption of selenium, and that there is no absorption from the rumen or abomasum of sheep, or the stomach of pigs [10].

Absorbed selenium is at first carried mainly in the plasma, apparently in association with the plasma proteins, from which it enters all the tissues, including the bones, the hair, and the erythrocytes [11]. The intracellular distribution of radio-labelled selenium varies with the tissue; in one study ^{75}Se was found to be uniformly distributed among the particulates and soluble fraction in the liver, whereas 75% of the activity in kidney cortex was found in the nuclear fraction [12].

It has long been accepted that selenium replaces sulphur in sulphur-containing compounds in the tissues and occurs predominately as selenocystine and selenomethionine in both protein-bound and non-protein bound forms. However, several researchers have produced new evidence [13] which challenges the whole concept of sulphur replacement by selenium. Their findings suggest that in the rabbit there is no pathway for the in vivo synthesis of selenocystine or selenomethionine from selenite and that the selenium-containing compounds associated with fractions containing cystine or methionine merely consist of selenite bound to the sulphur compounds.

Most of the selenium deposited in the tissues is highly labile. Following the transfer of animals from seleniferous to non seleniferous diets, or following the injection of stable or

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radioactive selenium [14], the retained selenium is lost from the tissues, at first rapidly and then more slowly. Selenium is excreted in the faeces, urine, and expired air; the amounts and proportions dependant upon the level and form of intake, the nature of the rest of the diet, and the species. Exhalation of selenium is an important route of excretion at high dietary intakes of the element, but much less so at low intakes.

Selenium is transmissible through the placenta to the foetus, whether supplied in inorganic or organic forms. The placenta, nevertheless, presents some form of a barrier to the transfer of selenium in inorganic forms. It has been shown that the concentration of ^{75}Se in the blood and most organs of the foetus, following injection of the ewe with ^{75}Se sodium selenite, is lower than in the mother [12]. Further work showed that, when ewes are injected with ^{75}Se-selenomethionine or ^{75}Se-selenocystine, the ^{75}Se concentration in the lambs is higher than when selenite is injected, and is nearly as high as in the mother [12].

1.4. Discovery of the essential nature of selenium

The discovery of the essential nature of selenium arose from the observation by Schwarz [15] that rats fed, a torula yeast diet, develop a fatal liver necrosis, which could be prevented by brewer's yeast, despite the absence of sufficient cystine or vitamin E to account for its protective function. This led Schwarz to postulate the presence of a third anti-liver necrosis factor, designated " Factor 3 ". In the course of procedures designed to identify the nature of Factor 3, a correlation between biopotency and selenium content was noticed, and supplements of selenite were shown to be protective against liver necrosis in the same manner as Factor 3 itself. Scott and his associates [16] had previously found that chicks fed torula yeast vitamin E-deficient diets grew poorly, revealed a high mortality, and developed a condition known as "exudative diathesis". Cooperative studies between these workers and Schwarz [17] disclosed a potency and distribution of the anti-exudative diathesis factors similar to that of Factor 3. Supplements of selenite were found to be effective in promoting the growth of the chicks and in preventing exudative diathesis, as they had for liver necrosis in rats.

Investigations in several parts of the world with farm animals subsequently disclosed a nutritional significance for selenium far beyond its relationship to the disorders that arise in rats and chicks fed specialised diets. Selenium therapy was found to

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be effective in the treatment of various myopathies, including white muscle disease in lambs, calves and foals [18] and hepatosis dietetica in pigs [19]. Selenium was also shown to promote growth, improve fertility, and reduce postnatal losses in sheep in certain areas.

1.5. Biochemical functions of selenium

Schwarz postulated that selenium functioned as an essential cofactor at specific sites of intermediary metabolism. Currently, the known biochemical functions of selenium are as an inherent component of the enzyme glutathione peroxidase, found in animals, and of several bacterial enzymes. The selenium deficiency signs observed in animals can be partially explained by a lack of glutathione peroxidase, but this does not eliminate the possibility of other roles for selenium in animals.

In 1954, Pinsent reported that selenium was necessary for the appearance of formate dehydrogenase activity in E.coli [20]. At about the same time that Rotruck et al. [21] reported that glutathione peroxidase in the rat was a selenoenzyme, the microbial enzymes formate dehydrogenase [22] and protein A of glycine reductase [23] were shown to be selenoenzymes. In glycine reductase and formate dehydrogenase the form of selenium has been shown to be selenocysteine.

Two other bacterial enzymes , nicotinic acid hydroxylase [24] and xanthine dehydrogenase [25], have also been reported to

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require the presence of selenium. A fifth possible selenoprotein, thiolase, has also been reported to contain selenium [26]. In animals, glutathione peroxidase is presently the only known selenoenzyme, and thus knowledge of its chemistry and biochemistry is an important part of our understanding of the biochemical role of selenium in animals.

1.6. Glutathione Peroxidase

Glutathione peroxidase was discovered in 1957 by Mills, who found that this enzyme in the presence of reduced glutathione would protect erythrocytes against H_2O_2-induced haemoglobin oxidation and haemolysis [27].

The enzyme has been purified from the tissues of cattle, humans, swine, sheep, and rats, and shown to be approximately an 80,000 dalton enzyme consisting of four apparently identical subunits [28]. Determination of the molecular weight of glutathione peroxidase by sedimentation equilibrium indicates that its molecular weight differs from species to species : 76,000 for rat liver, 95,000 for human erythrocytes and 83,800 for bovine erythrocytes.

Following the demonstration that the selenium in protein A of reduced microbial glycine reductase was selenocysteine [23], it was reported that the selenium in reduced glutathione peroxidase was also present as seleocysteine [29].

The amino acid composition of bovine erythrocyte glutathione

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peroxidase has been found to contain 178 amino acids and a tentative amino acid sequence has been published [29]. It has also been crystallised , and the three dimensional structure determined [29]. The subunits are nearly spherical, with a radius of 18.7 A ; the subunits are identical or at least very similar, with only one selenium atom per subunit. The glutathione peroxidase tetramer appears to be an almost flat, planar arrangement of two dimers. The selenium atoms are located on the surface of the enzyme, but it has not been established whether all four are catalytically active. The selenium atom appears as a "protrusion of the main chain density" , and it has been concluded [29] that a selenocysteine or a selenocystine derivative satisfactorily fits this density.

1.6.1. Function of glutathione peroxidase

The discovery that hydroperoxides were substrates for glutathione peroxidase provided an important clue to the biochemical function of this enzyme and thus of selenium. In cells, hydrogen peroxide, hydroperoxides, superoxide, various radicals including the hydroxyl radical, and possibly singlet oxygen are formed as products of necessary reactions in cells. Therefore, protective systems have evolved to contain and ultimately destroy these reactive species before they damage cells. These protective systems are compartmentalised and thus complement each other. As a lipid-soluble antioxidant, vitamin E

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scavenges free radicals and possibly singlet oxygen before they can attack cellular and intracellular membranes. Glutathione peroxidase destroys H_2O_2 and hydroperoxides in the cytosol and mitochondrial matrix space. Catalase degrades H_2O_2 in the peroxisome. Superoxide dismutase detoxifies superoxide in the cytosol and mitochondria before superoxide can react with H_2O_2 to form the hydroxy radical.

Decreases in tissue glutathione peroxidase activity and the development of selenium deficiency signs in animals are well correlated. In weaning rats fed a selenium-deficient diet, liver glutathione peroxidase activity falls to undetectable levels about the time liver necrosis develops. In chicks, a correlation between depressed plasma glutathione peroxidase and the developement of exudative diathesis has been reported. These diseases are prevented either by dietary selenium or vitamin E, suggesting that selenium and vitamin E have overlapping roles in the protection of cells.

1.7. Dietary levels consistent with good nutrition

Since no clear-cut pathological condition attributable to selenium deficiency alone has yet been observed in man, it is not possible to define a precise dietary requirement level for human beings. However, 0.1 - 0.2 mg selenium/kg diet is a nutritionally generous level for most species of animal [30]. If these animal data are extrapolated, a 70-kg man consuming 500g

of diet per day (dry basis), would need a daily intake of 50 - 100 ug selenium. The US National Research Council [30] has estimated that the safe and adequate range of the daily intake of selenium for adults is 50 - 200 ug, with correspondingly lower intakes for infants and children (Table 1.1). On this basis, the recommended intake for a 70-kg man would be equivalent to 0.7 - 2.8 ug/kg body weight per day. Any daily intake within the recommended range is considered adequate and safe, but the recommendations do not imply that intakes at the upper limit of the range are more desirable or beneficial than those at the lower limit.

Whilst no clear-cut pathological condition attributable to selenium deficiency alone has been demonstrated, evidence has been mounting to associate low selenium intake with several human diseases.

1.7.1. Keshan Disease

Results of research in China have suggested a relationship between low selenium status and the prevalence of Keshan disease, an endemic cardiomyopathy that primarily affects children [31]. Epidemiologically, the disease exhibits a regional distribution and occurs in a belt-like zone reaching from northeastern China to the southwestern part of the country. Cases of the disease have been recorded as early as 1907 in Heilongjiang Province of northeastern China. Since the etiology

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Table 1.1. Estimated safe and adequate range

of selenium intake

(reproduced from ref.30)

Group	Age (years)	Daily selenium intake (ug)
Infants	0 - 0.5	10 - 40
	0.5 - 1	20 - 60
Children	1 - 3	20 - 80
	4 - 6	30 - 120
	7+	50 - 200
Adults		50 - 200

of the disease was not known, it was named after the locality in which it was originally observed, i.e. Keshan County.

The criteria for diagnosing Keshan disease include acute or chronic cardiac insufficiency, heart enlargement, gallop rhythm, arrhythima, and ECG changes.

In the past, many different hypotheses were advanced in an attempt to explain the etiology of the disease. Recently, an hypothesis concerning Keshan disease etiology was proposed linking the disease to selenium deficiency after it was noted that severely endemic areas coincided with areas where the incidence of selenium deficiency diseases in farm animals was also high [31]. Since the initial proposal of the selenium-Keshan connection, much evidence has been gathered in support of this concept. For example , the average blood selenium content was 0.021 +/- 0.001 mg/litre for the affected areas and 0.095 +/- 0.088 mg/litre for the non-affected areas [32]. It was stated that an area could be considered to be unaffected wherever the selenium content of the grains was 0.04 mg/kg or more. The Chinese workers stated that the amount needed to prevent the disease was about 20 ug/day [32].

In some affected areas, there were highly localised pockets, so-called "safety islands", that were free from the disease. Apparently, these islands were protected because of the higher selenium content of their crops in the immediate vicinty. The average selenium contents of rice and soybeans in one such island were 0.020 and 0.025 mg/kg respectively, whereas the

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values for the corresponding crops in a nearby affected area were 0.0078 and 0.0057 mg/kg [31].

These relationships between selenium and Keshan disease led the Chinese workers to conduct a randomised intervention trial to test the possible prophylactic effect of selenium against this condition in the population at risk (i.e. children 1 - 9 years old). In a scientific study in 1974 [31], 4510 children took sodium selenite and 3985 children took the placebo (Table 1.2). The treated children took 0.5 mg sodium selenite per week if 1 - 5 years old, or 1.0 mg per week if 6 - 9 years old. The morbidity rate due to Keshan disease was 1.35% in the placebo group (54 cases out of 3985 children) but only 0.22% in the treated group (10 cases out of 4510 children). Since a significant difference was also shown in the 1975 study (0.95% morbidity rate in the placebo group compared with 0.1% in those treated) the placebo groups were abolished in 1976 and 1977. As a result, the case rate dropped to 0.034% and 0% in these two years, respectively.

Since 1976, more extensive intervention trials with sodium selenite have been carried out in five counties in the same area of Sichaun Province [12]. All children, 1 to 12 years of age, in some of the most severely affected communes, were treated with selenium as previously described, while untreated children in nearby communes served as controls. The incidence rate of Keshan disease in the selenium treated children was lower in each year of the five year period than among the untreated children

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Table 1.2.   Effect of selenium on keshan disease

in children

(reproduced from ref. 31)

Treatment	Year	No. of cases	OUTCOME OF ALIVE CASES			Death
			Latent	Improve	Chronic	
Placebo	1974	54	16	9	2	27
	1975	52	13	10	3	26
Sodium	1974	10	9	0	1	0
Selenite	1975	7	6	0	0	1

Table 1.3. Keshan disease incidence rate in selenium treated
             and untreated children
             (reproduced from ref.32)

TREATED CHILDREN

Year	Number of subjects	Number of cases	Incidence (per 1000)
1976	45,515	8	0.17
1977	67,754	15	0.22
1978	65,953	10	0.15
1979	69,910	33	0.47
1980	74,740	22	0.29
1981	323,872	88	0.27

UNTREATED CHILDREN

1976	243,649	448	2.00
1977	222,944	350	1.57
1978	220,599	373	1.69
1979	223,280	300	1.34
1980	197,096	202	1.07
1981	1,197568	1713	1.55

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(Table 1.3)

Selenium intervention has proved to be very effective in the prophylaxis of Keshan disease and it is very likely that selenium insufficiency plays an important role in its etiology. Nevertheless, the Chinese workers recognised that there were certain epidemiological characteristics of the disease, which suggested that additional etiological factors were involved, and at present, research is being carried out to elucidate these. Perhaps the best way to account for all the characteristics of the disease is to assume that the disease has a multifactorial etiology, and that a combination of several factors, of which selenium insufficiency is one, may be involved.

1.7.2. Kashin-Beck disease

Kashin-Beck disease is an endemic osteoathropathy that occurs in eastern Siberia and in certain parts of China, which is characterised as a chronic, disabling, and degenerative osteoarthrosis that mainly involves children [33].
Although the etiology of the disease has not been fully established, present work shows that selenium deficiency may be one of the main causes. This concept has arisen because, firstly, in China most of the endemic areas are located in the same low-selenium zone as Keshan disease. Residents in these areas have low-selenium status characterised by low blood and hair selenium levels, low blood glutathione peroxidase activity,

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and low urinary excretion. A survey carried out in one of the most heavily affected provinces in China, Heilongjiang province, showed that the average hair-selenium levels in 151 children in endemic areas (0.096 +/- 0.026 mg/kg) was significantly lower than that of the 235 children in the non-endemic areas (0.223 +/- 0.083 mg/kg) [34]. Secondly, sodium selenite has been reported to have both therapeutic and prophylactic effects on this disease. In a survey in Shaanxi province in China, 325 cases of Kashin-Beck disease were randomly divided into a treated and control group. In the treated group, 3-10 year old children were given 1 mg/week of sodium selenite and 11-13 year olds were given 2 mg/week, whilst those in the control group were given a placebo. After one year, X-ray examination of the metaphyseal changes of fingers showed that 81.9% of the cases in the treated group had improved, 18.1% showed no change, and none were getting worse. In the control group, however, only 39.6% of the cases had improved, 30% were getting worse, and 41.5% showed no change.

In another study, children of 1-5 and 6-10 years of age in an endemic area of Gansu province in China were supplemented with 0.5 and 1.0 mg sodium selenite respectively per week, over a period of six years. X-ray examination showed that the incidence of Kashin-Beck disease declined from 42% to 4% after the selenium intervention.

However, as with Keshan disease, the involvement of other factors is suspected, and research is being carried out to

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determine their role in the etiology of the disease.

1.7.3. Cancer

In the 1960's, the relationship between selenium levels in
human blood and cancer death rates in various regions of the USA
was first pointed out [35].

In one study, it was reported that blood selenium levels in
patients with cancer of the colon, pancreas, stomach, and in
Hodgkin's disease and liver metastases were statistically
significantly lower than those in normal controls. However, of
29 patients with rectal cancers, 6 had lower selenium levels
than controls, and 23 had normal levels. Similarly, normal
levels were observed in patients with breast cancer and in
patients with other types of carcinoma [36].

In a later study [37] , it was reported that there was no
difference in serum selenium levels between 110 cancer patients
and controls. However, patients with the lowest serum selenium
levels had shorter survival rates, higher incidence of multiple
primary malignancies, higher rate of recurrence of the primary
lesion, and were more likely to have dissemination of cancer
than those with the highest selenium levels. In a further study
by the same researchers, of 59 patients with primary malignant
reticuloendothelial tumours and controls, no difference in serum
selenium levels between the two groups was found [38].

However, in a 1984 study [39], it was found that 44 patients

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diagnosed as having gynaecological cancer had lower serum selenium concentrations than 56 control subjects. In a second study by the same research group [40], it was reported that patients with ovarian cancer had significantly lower serum selenium concentrations than matched controls.

Whilst it would seem, from the research carried out in this area, that low selenium intake increases the risk of cancer, the differences in the effects of selenium according to age, race, sex, and smoking status, need to be examined further. Furthermore, the possibility, that the selenium measured in the blood is not truly the protective factor, but is merely an indicator of some other compound(s) or nutrient(s) that are directly involved in the relationship, cannot be ruled out.

1.7.4. Selenium and heart disease

The leading cause of heart disease death is myocardial infarction, which is the death of heart tissue because of the lack of blood in a region of the heart. This lack of blood is usually caused by a blood clot, i.e. thrombus, in a coronary artery. In recent years, a lot of attention has been focussed on the development of arterial plaques or atherosclerosis. These plaques, which are normally thought of as cholesterol deposits, originate as mutant muscle cells in the wall of the artery. The calcification of the arterial plaques produces hardening of the arteries. In this condition, known as arteriosclerosis, the heart takes on a white, chalk-like appearance. Animal nutritionists have noted that animals living in selenium deficient areas developed such calcification of their hearts; the disease was later named white-muscle disease. It has also been noted that selenium deficiencies cause nutritional muscular dystrophy and a degeneration of skeletal muscle called Zenker's disease. A similar degeneration of the Purkinje fibres which cause the heartbeat, has been observed in selenium deficiency. In fact, the hearts of most selenium deficient animals will collapse when surgically removed, while hearts from adequately nourished animals will hold their shape.

A recent case control study from Finland suggested a possible association between the serum selenium level and the risk of

death from acute coronary heart disease, as well as the risk of fatal and non-fatal myocardial infarction [41]. The case control pairs were derived from 11,000 persons residing in eastern Finland, an area with a very high incidence of death from cardiovascular disease. The cases were middle-aged persons who had died of coronary heart disease, other cardiovascular diseases or suffered a non-fatal myocardial infarction over a seven year follow-up period. Attempts were made to control for potential confounding factors by using controls matched for six major coronary heart disease risk factors: age, sex, serum-cholesterol, diastolic blood pressure, smoking, and history of angina pectoris. However, the cases had slightly higher blood pressure than the controls.

The mean serum selenium levels were 51.8 and 55.3 ug/litre for cases and controls respectively. A serum selenium level of less than 45 ug/litre was associated with an increased risk of coronary and cardiovascular death and myocardial infarction.

Although few necropsies were performed, the authors felt it was unlikely that the excess cardiovascular mortality observed in their subjects was due to Keshan disease.

The authors cautioned that the apparent association between low serum selenium levels and cardiovascular risk might only be a marker for other dietary factors more directly related to increased coronary heart disease. They also emphasised that, even if their results truly reflected a casual relationship between low selenium intake and increased ischaemic heart

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disease, most such disease is still due to the other well known risk factors of elevated cholesterol levels, high blood pressure, and smoking. Moreover, it was pointed out that any association between low serum selenium levels and ischaemic heart disease is likely to be of significance only for populations in areas where the dietary intake of selenium is very low.

In the USA, an inverse correlation was reported between the plasma selenium level and the severity of coronary athero-sclerosis [42]. On the other hand, neither Ellis et al. [43] nor Robinson et al. [44] were able to demonstrate any correlation between the traditional risk factors for cardiovascular disease and blood selenium levels or glutathione peroxidase activity.

In addition, the results of a WHO research programme showed no difference in tissue selenium concentrations between patients who died with, or without, myocardial infarction.

1.8. Effects of high selenium intake on man

When it became apparent that selenium was the toxic factor in plants that caused alkali disease in livestock raised in seleniferous areas, public health personnel became interested in the possible hazards for human health in such regions, since seleniferous grains or vegetables grown on high selenium soil could enter the human food chain.

In the 1930's, Smith et al. [4] surveyed a rural population living on farms or ranches known to have a history of alkali disease. Their survey investigated the health status of 111 families and also determined the actual comsumption of locally grown foods. However, no specific sympton could be definitely attributed to selenium poisoning in man. Nevertheless, it was noted that the incidence of vague symptons of ill health and symptoms suggesting damage to the liver, kidneys, skin and joints was rather high. Apart from the more vague symptons of anorexia, indigestion, general pallor, and malnutrition, the following more pronounced disease states were observed: bad teeth, yellow discoloration of the skin, skin eruptions, chronic arthritis, diseased nails, and subcutaneous oedema.

In 1976, Jaffe carried out a field study in Venezuela and compared 111 children living in a seleniferous area, Villa Bruzual, with 50 living in Caracas [28]. Symptoms of dermatitis, loose hair and nails were reported as more frequent among the children in the seleniferous area than in those living in

Caracas ; however no quantitative information was given.

Due to the nutritionally beneficial effects of selenium in animals, some investigators have deliberately given inorganic or organic forms of the element to people with the aim of producing some desirable health benefit. Westermarck [28] administered selenium, as selenite, in oral doses of 0.05 mg/kg body weight per day, for more than a year, to patients with neuronal ceroid lipofuscinosis (NCL) and did not observe any toxic manifestations. On the contrary, it was felt that some NCL patients showed at least a transitory improvement in their condition.

Ingestion of superpotent selenium tablets, meant to be consumed as a "health food" supplement, resulted in 12 cases of human selenium toxicity in the USA in 1984 [28]. Each tablet contained 27-31 mg selenium by analysis; about 182 times more than the amount stated on the label. Based on the limited information available, the symptoms reported in these cases as most common were nausea and vomiting, nail changes, hair loss, fatigue and irritability.

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Chapter 2

Analytical methods for the determination

of selenium in biological matrices

2.1. Introduction

The increased awareness in recent years of the biological importance of selenium has given rise to a variety of techniques for its analysis.

The selenium concentration in a sample is a major consideration in deciding which technique to use, but the availability of special equipment, demands for reliability, presence of interfering substances and the time and cost requirements must also be taken into account.

2.2. Standard reference materials

For the development of accurate and precise techniques of analysis for a particular analyte, it is essential that materials are available with assigned concentrations of the analyte of interest for validation of the analytical methods. In the case of selenium, the first such reference material was a powdered kale preparation [1], for which a concensus value for selenium was established by means of an interlaboratory cooperative study.

Subsequently, the US National Bureau of Standards (NBS) issued several reference materials, among which were orchard leaves, bovine liver, wheat flour and rice flour with certified levels of selenium. In addition, fish flesh, human hair and lypholised animal blood reference materials, with certified levels of

selenium, are available from the International Atomic Energy Agency (IAEA).

In a recent interlaboratory cooperative study [1], conducted under the auspices of the IUPAC Sub-committee on Selenium, of the Commission on Toxicology, to establish the total selenium concentrations in two human blood serum pools, atomic absorption spectrometry (AAS), spectrofluorimetry and neutron activation analysis (NAA), were the most commonly used analytical techniques amongst the 27 laboratories involved in this worldwide investigation.

2.3. Atomic absorption spectrometry

The two main approaches taken in the atomic absorption spectrometric determination of selenium are:

    (1) graphite furnace AAS;

    (2) hydride generation AAS.

2.3.1. Graphite furnace AAS

The direct determination of selenium in biological materials by graphite furnace atomic absorption spectrometry [2-11] is challenging for a number of reasons.

Firstly, samples can be difficult to dispense reproducibly into the graphite furnace, and therefore it is often necessary to dilute the sample and add a wetting agent, or surfactant such as

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Triton X-100. The use of a surfactant allows better results to be obtained, probably because the sample spreads out more evenly in the furnace providing better contact with the graphite surface, resulting in more effective ashing.

In the development of graphite furnace methods, all steps in the temperature programme must be carefully optimised. Reproducible drying of the sample material can be difficult to obtain, but ramping can be used to produce good results.

Ideally, the ash step should remove the bulk of the sample matrix without loss of the analyte, and therefore careful optimisation of this step is critical for the overall accuracy of the technique.

The volatilisation of the analyte during the atomisation step should produce a clean, easily measured atomisation signal free from interferences. When samples such as whole blood are being analysed continuously, a carbonaceous residue may build up on the graphite tube or on the platform, affecting dispensing characteristics and the precision and accuracy of the analysis. Therefore, proper choice of ramp and atomisation temperature parameters are also important for the efficient removal of the sample material from the furnace.

2.3.1.1. Matrix modifiers for selenium determination

The inorganic reactions of selenium in the graphite furnace are complex. Many compounds of selenium, such as selenium dioxide,

selenium chloride, and atomic or diatomic selenium, are very volatile and can be lost in the early stages of the temperature programme. Selenium reacts with many metals, and the formation of a more stable selenide species has been used to minimise volatility problems, thereby greatly improving graphite furnace analyses.

The thermal stabilities of several metal selenides [2] are shown in Table 2.1. In this study of the effect of various matrix modifiers upon the ash temperature, it was noted that the sensitivity and possible ash temperature were influenced by the concentration of the modifier, whether the metal was added as a chloride or nitrate salt, and by the condition of the graphite tube.

Despite the fact that matrix modifiers have been widely used to produce a more accurate determination of volatile elements in graphite furnace AAS, very little evidence has been presented to try and explain the mechanisms by which enhancement of the signal occurs.

Nickel has been one of the most widespreadly used matrix modifiers for the determination of selenium. It has been suggested [3] that the signal enhancement resulting from addition of nickel is due to the formation of a high boiling selenide, thus permitting a higher ash temperature to be used. In addition, the reduction of SeO_2 by nickel oxide, formed by the decomposition of selenite or selenate to a less volatile form, is another possibility.

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Table 2.1.   Thermal stabilities of various

metal selenides.

(reproduced from ref.2)

Modifier (1000mg/l)	Maximum ash temp. (^{O}C)	Relative Sensitivity
Nickel	700 – 900	1.00
Copper	800 – 1000	1.00
Cobalt	800 – 1000	1.09
Zinc	500 – 700	1.13
Palladium	900 – 1100	0.70
Rhodium	1000 – 1200	1.05
Silver	500 – 700	0.58
Molybdenum	1200 – 1400	0.24

In recent years, the use of palladium as a matrix modifier has become more widespread. Voth-Beach and Shrader [2] have reported that when a reducing agent was added with selenium, the results were significantly improved. It has also been reported [3] that, under carefully controlled temperature conditions, a doubling of the selenium signal over that of a simple Se(IV) solution could be obtained, if albumin and palladium were added for matrix modification.

It was proposed that, under the temperature conditions in this study, albumin was converted into graphite. This "new graphite surface" was thought to be better able to physically absorb the selenium species and this, in conjunction with retainment of selenium by the palladium, resulted in a higher sensitivity for the determination of selenium.

Electron microscopic studies showed that the palladium and selenium were physically associated with each other in a 1:1 mole ratio on the surface of the graphite tube.

2.3.1.2. Spectral interferences

In an ideal situation, the only absorbing species within the optical beam during the measurement period in furnace AAS should be the analyte atoms. However, in practice, the situation is rarely, if ever, as simple as this, since other species may be produced during the sample atomisation process. Should these species be capable of absorption at the analyte wavelength, and

if this absorption overlaps in time with the absorption of the analyte, the resulting absorbance measurement will be higher than the given atom population.

This background interference poses a serious problem for accurate determination of analytes at trace levels and, in a bid to overcome this, background correction techniques have been developed.

It has been reported that calcium orthophosphate and calcium hydrogen orthophosphate caused a negative interference at the 196 nm line when the deuterium lamp was used for background correction [10]. This interference was not present when the Zeeman background corrector was used. Saeed and Thomassen have also reported [11] the calcium orthophosphate interference for the determination of selenium at 196 nm, suggesting that it was caused by absorption of background radiation by molecules of PO. The addition of nickel has been shown to remove this interference [11], even with deuterium background correction; the correction is more complete however with a Zeeman correction system.

The absorption of background radiation near the 196 nm line by iron is the cause of serious interference for the determination of selenium using deuterium background correction. As a result, Zeeman background correction has been recommended for use in the determination of selenium in biological matrices containing iron, by graphite furnace AAS.

2.3.2. Hydride generation AAS

Since first being described by Holak [12], hydride generation atomic absorption spectrometry has gained widespread acceptance as a sensitive, accurate and easily operated method of determining selenium in a wide variety of matrices [13,14,15,16] The generation of the analyte of interest as a volatile hydride allows both the preconcentration of the analyte and separation of the analyte from the matrix. This is of particular importance in those situations where the analyte is present at trace levels in a very complex matrix.

The majority of applications of hydride generation AAS for the determination of selenium have involved the use of batch type generators, similar to that described in section 5.2.1. In this case, hydrides are formed after the rapid addition of reductant to a constant sample volume, with the hydride being subsequently swept into an atomisation cell, normally an externally heated quartz tube, where atomisation occurs.

Delves has reported on the use of such a system for the determination of selenium in a variety of biological matrices, such as blood [13] and heart tissue [14]. Dawczynski et al. [15] have successfully applied such an approach to the determination of selenium in 200 ul of whole blood. The analogous determination in a graphite furnace requires Zeeman background correction.

The commercial production of hydride generation systems has, to

a large extent, been developed along the lines of continuous flow systems. These systems not only allow the methodology to be greatly simplified, but allows the overall precision of the technique to be improved. The use of such a system was recently reported for the determination of selenium in fish [16].

The combination of flow injection and hydride generation opens the possibility of carrying out the determination of selenium in small sample volumes. The use of such a system has been reported for the determination of selenium in geological materials [17], water [18] and in biological reference materials [19,20].

Use of hydride generation techniques in conjunction with externally heated quartz atomisation cells permits the determination of selenium at the ng/ml level. However, the use of trapping techniques have allowed much lower detection limits to be achieved [21,22]. Tolg [21] has described a system, incorporating a liquid nitrogen trap, which allowed the determination of selenium down to 6 pg/ml. Such methods have, however, not been widespreadly used because of the degree of operator attention required for their operation.

2.4. Spectrofluorimetry

Spectrofluorimetric methods of analysis have gained widespread acceptance for the determination of selenium in biological materials [23 - 29], and about half the laboratories involved in its analysis in blood make use of the technique [1].

The fluoresence procedure is usually based on the reaction of tetravalent selenium with an aromatic diamine to form a fluorescent complex. The aromatic diamines, 3,3'-diamino - benzidine (DAB) and 2,3-diaminonapthalene (DAN) have been the most successful, with DAN being the most commonly used because of its greater fluorescent sensitivity [30].

The piazselenol formed in the reaction between selenium and the aromatic diamine is usually extracted into an organic phase, where its fluoresence is measured, using excitation and emission wavelengths of 369 and 518 nm respectively [31].

Care must be exercised in ascertaining the purity of the DAN reagent, because it undergoes rapid decomposition in light to give fluorescent products, which must be removed before the reagent is of any analytical value. Purification is normally achieved by dissolving the reagent in 0.1 M HCl, heating in a water bath to $50^{\circ}C$ for 40 minutes, and then extracting the solution three times with cyclohexane.

The complexation reaction of DAN with Se(IV) is pH-dependent, with a pH of 1.8 generally being considered as being optimal.

The success of the spectrofluorimetric procedure depends upon

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selenium being in the +4 state. Therefore, digestion of the sample material is necessary, as is the case in hydride generation AAS. Haddad and Smythe [30] have reported that, in the fluorimetric determination of selenium with DAN, the digestion of the matrix and the conversion of selenium into its +4 oxidation state are the two critical steps in the complete procedure.

The reaction should be carried out under mild reducing conditions to prevent the interference of oxidising agents. Hydroxylamine is the most commonly used reducing agent and is normally added with EDTA to produce a combined masking-reducing solution [30].

Fluorescence detection has also been used for the determination of selenium by high performance liquid chromatography [32,33]. Among the advantages reported for this approach are enhanced selectivity and sensitivity, and the individual components can be resolved as distinct peaks on the chromatogram, whereas in the classical DAN-Se procedure in cuvettes, there can be uncertainty about the relatively large contribution of background components to the fluorescence signal [33].

2.5. Neutron activation analysis

Neutron activation analysis (NAA) entails irradiation of the sample to produce radioactive selenium isotopes, the concentrations of which are measured by monitoring γ-radiation of specific energies. Thermal neutron activation is the most commonly used form of activation. It produces several radio - nuclides, of which ^{81}Se (half-life 18.6 min), ^{77}Se (half-life 17.5 sec), and especially ^{75}Se (half-life 120 days) have been the most useful for analysis.

The instrumental version, in conjunction with γ-ray spectrometry, involves no chemical operations and is one of the very few techniques not requiring sample decomposition. It is widely accepted as being an excellent method for the determination of Se in biological materials [1]. With serum samples, little error occurs from effects due to γ-ray self absorption. Should matrix activation lead to interferences, resort can be made to the radiochemical separation version [1].

In NAA, carrier selenium is added following activation to provide for better recovery of the ^{75}Se. Usually, a cooling period of several weeks precedes the separation to allow for the decay of short half-lived radionuclides.

In the recent interlaboratory cooperative study, carried out under the auspices of IUPAC [1], six laboratories used NAA techniques. Of these, four laboratories measured the γ- ray emmission of ^{75}Se after a 1-2 month cooling period, whilst the

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other two relied upon counting the 17 second half-life of ^{77}Se. Very good agreement was obtained for these laboratories and, also with other laboratories using atomic absorption spectrometric and fluorimetric techniques.

The use of neutron activation analysis for the determination of selenium is limited by the fact that very few laboratories are equipped with the highly specialised equipment necessary. Where it can be carried out, it has provided excellent results in a number of important biomedical studies [34-37].

2.6. Electrochemical techniques

Electrochemical techniques have been applied to the trace determination of a wide variety of metals. The analysis of selenium by electrochemical methods can be achieved by:

    (1) cathodic stripping voltammetry;

    (2) anodic stripping voltammetry.

2.6.1. Cathodic stripping voltammetry

The cathodic stripping voltammetric determination of selenium has been traditionally carried out at a hanging mercury drop electrode [38-44].

The basis of this approach depends upon the formation of a sparingly soluble deposit on the surface of the mercury electrode by the judicious selection of electrolyte and

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deposition potential. The ability to concentrate the selenium in this form on the electrode means that the technique is extremely sensitive. The sensitivity is governed, to a large extent, by the length of time allowed for the deposition of the analyte; a value of 1-3 minutes is typical.

The use of differential pulse cathodic stripping voltammetry has been, by far, the most common approach employed in electro - chemical studies [38-44]. The use of square wave cathodic stripping voltammetry has been reported [45] for the determination of selenium in rainwater samples.

The use of the standard addition method is normally required in analytical procedures involving cathodic stripping voltammetric techniques to overcome matrix effects. Bond et al. have reported on the stripping voltammetric determination of selenium in biological materials by direct calibration [40]. In this method, separation of the Se(IV), prior to voltammetric analysis, was achieved with the use of an anion exchange resin. The use of adsorptive stripping techniques has been reported to allow detection limits which are several orders of magnitude lower than those normally attainable by direct cathodic stripping voltammetric techniques [46-49]. The most common approach has involved the adsorption of the 3,3'-diamino-benzidine piazselenol complex on the mercury electrode, followed by a differential pulse cathodic stripping scan. Interferences normally encountered in previous stripping voltammetric procedures [38,39] were overcome by extraction of the

piazselenol into an organic solvent and back-extraction into an aqueous solution [46-48].

Van den Berg et al. have reported [49] on an adsorptive cathodic stripping voltammetric approach involving the adsorption of a $Cu(I)_2Se$ complex species on a hanging mercury drop electrode, followed by a differential cathodic stripping scan. The method was applied to the determination of estuarine seawater samples and allowed a detection limit of 0.01 nM to be obtained when a deposition time of 15 minutes was used.

Hua et al. have reported [50] on the use of constant current stripping analysis at mercury-coated carbon fibre electrodes for the determination of Se(IV) in milk powder. The method described represented the first application of such electrodes for the voltammetric determination of selenium.

2.6.2. Anodic stripping voltammetry

In comparison to cathodic stripping voltammetry, anodic stripping voltammetry has not been as commonly applied to the determination of selenium. The use of gold electrodes has been recommended for use in this mode, either in the form of a gold disc electrode [40,51], or a gold film electrode deposited in situ on a glassy carbon electrode [52,53].

2.7. X-ray fluorescence spectroscopy

The use of X-ray fluorescence spectroscopy has been very limited for the analysis of selenium because of its limited sensitivity, and therefore a preconcentration step is necessary [54-57]. The reproducibility and sensitivity of the method depends on the preconcentration procedure used. This approach does, however, allow the analysis of selenium without destruction of the sample.

2.8. Gas chromatography

Gas chromatography has not been widely used in the determination of selenium. The reaction of selenium with 4-chloro- or 4-nitro-o-phenyldiamine has been used for the gas chromatographic determination of selenium in sea water [58] and plant tissues [59]. Use has also been made of the reaction of selenium with DAN for the determination of selenium by gas chromatographic methods [60].

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Chapter 3

Anodic stripping voltammetry of selenium

in a gold fibre electrochemical cell

3.1. INTRODUCTION

Flow analysis represents a broad and diverse field ranging from continuous monitoring through laboratory flow analysis, (e.g. continuous flow and flow injection analysis), to chromatography. Electrochemical methods are well suited to flow measurements in view of their sensitivity, controllable selectivity, good precision and accuracy, simplicity and ease of signal handling and automation.

In recent years, the use of microelectrodes for electrochemical detection purposes in flowing streams has received increased attention, particularly in relation to chromatographic and capillary electrophoretic methods of analysis [1,2,3].

3.1.1. Flow sensors using microelectrodes

The aim of the development of flow sensors using micro-electrodes is the construction of very inexpensive, and thus disposable, electrodes which have a small dead volume and exhibit a negligible internal resistance potential drop.

In the work of Huiliang and Hua [4], several different flow cells, as shown in Figure 3.1, were investigated. The structure of the fibre flow electrode used in all the flow cell designs is shown in Figure 3.2. These flow fibre electrodes were prepared by first rinsing polyvinyl tubing (outer diameter 2 mm, inner diameter 0.5 mm) with acidified ethanol. An injection needle

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2.5 mm

Carbon fibre

Ag-epoxy glue

PVC tube
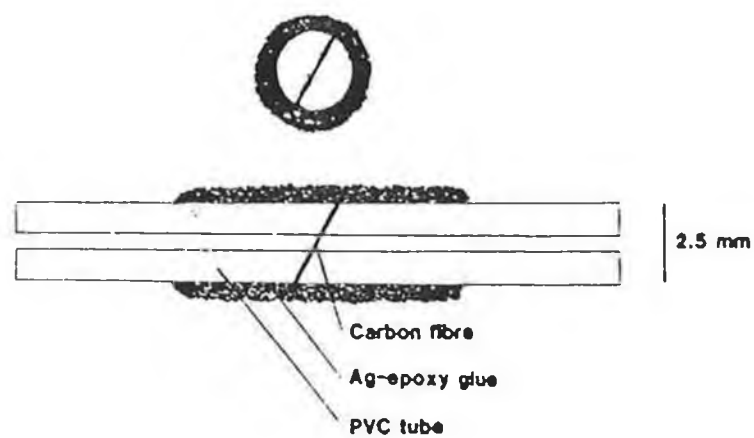
Figure 3.1. Structure of a typical carbon fibre flow
electrode (reproduced from ref. 4)

a

Pt tube   Carbon fibre   Pt tube   Thin layer cell
           working   counter   Calomel reference

b

Pt tube   Carbon fibre   Pt tube   Ag/AgCl tube
           working   counter   reference

c

Pt tube   Carbon fibre   Ag/AgCl tube
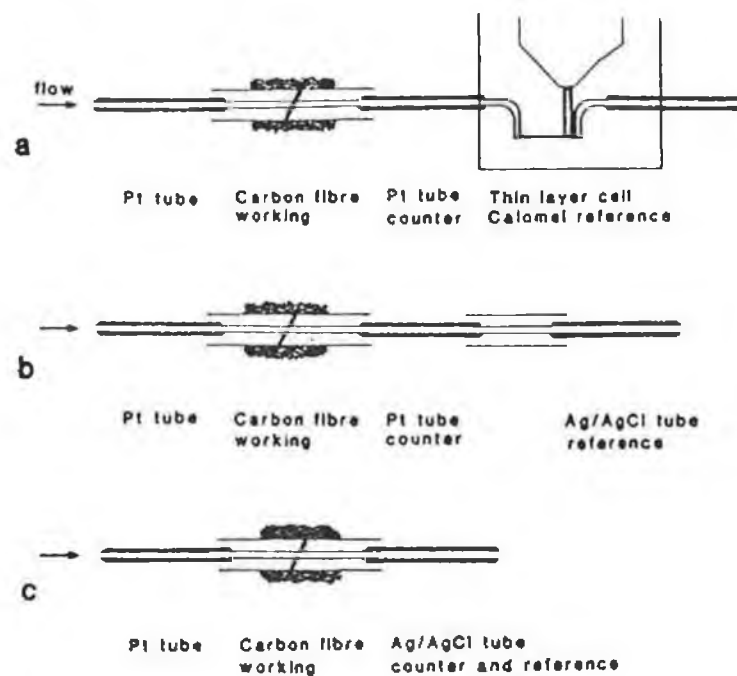   working   counter and reference

Figure 3.2.   Schematic representation of three flow cell

designs (reproduced from ref.4)

(outer diameter 0.4 mm, inner diameter 0.2 mm) was then inserted through the centre of a 25 mm piece of the tubing, at an angle of approximately 30° to the length of the tube. A fibre was then inserted, with the aid of a microscope, into the injection needle which was subsequently removed from the tubing, thus leaving the fibre behind. The PVC surface in the vicinity of the fibre-tube junction was then covered with silver epoxy, which not only permitted electrical contact with the ends of the fibre, but prevented the tube from being bent during handling.

Of one hundred electrodes tested [4], forty electrodes did not yield any response at all. This was attributed to failure in the manufacturing process, the most common failure being fracture of the fibre in the 0.5 mm flow channel of the polyvinyl chloride tube.

The sixty electrodes yielded very similar responses, the relative standard deviations from fibre to fibre being in the range of 20 to 30% [4]. In addition, the fibre electrodes were more sensitive to electrical noise than macroelectrodes. The three different flow cells in Figure 3.1. were compared with respect to the signal to noise ratio in comparison to a thin layer glassy carbon cell. It was found that the noise level of the fibre electrode, when calomel was used as a reference was approximately five times higher than the noise level of the thin layer glassy carbon cell. The cell arrangement in Figure 3.1.b., yielded noise levels similar to that of the thin layer cell, whereas in Figure 3.1.c., where the Ag/AgCl tube was used both

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as a counter and reference electrode, the noise level was approximately five times lower than the thin layer cell.

From the point of view of noise level, the use of a Ag/AgCl tube as both counter and reference electrode is obviously highly advantageous. This cell arrangement does, however, have several disadvantages, the most serious being that the potential of the reference electrode is dependent on the chloride concentration of the solution being sucked through the tube. Furthermore, if the solutions used contain high concentrations of, e.g., bromide or iodide, the reference electrode is transformed into an Ag/AgBr or an Ag/AgI electrode.

One distinct advantage of fibre electrodes is the small dead volume in the flow cell designed to incorporate them. This small dead volume is of particular importance when it is necessary to replace a solution completely by the next solution.

Hua et al. [5] have reported on the automated determination of total arsenic in sea water by flow constant current stripping analysis. During this analysis, it was found that extensive and frequent cleaning of the electrode was necessary to obtain reproducible readings, as the surface of the electrode was readily poisoned by elemental arsenic. In both determinations [5,6], rapid stripping analysis was possible and excellent agreement with certified reference materials was obtained.

In comparison to carbon fibre electrodes, the use of gold fibre electrodes has been limited. Huiliang et al. [6] described the simultaneous determination of mercury (II), copper (II) and

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bismuth (III) in urine by flow constant-current stripping analysis with a gold fibre electrode.

3.2. EXPERIMENTAL

3.2.1. Apparatus

All experiments were carried out using an EG&G Princeton Applied Research (PAR) Model 264A potentiostat, in conjunction with a PL-3 X-Y recorder (JJ Instruments). A Minipuls 3 peristaltic pump was used for the delivery of all solutions.

A Ag/AgCl reference electrode was manufactured to fit into one of the arms of the flow cell, shown in Figure 3.3. For this, a 2 cm length of PVC tubing was washed, following which, one end of the tube was tightly packed with filter paper, which had previously been allowed to soak in a saturated potassium chloride solution. The tube was then filled to within 5 mm of the top with 1 M potassium chloride, and into this was inserted a 3 cm length of silver wire. This had previously been chloridised in 1 M hydrochloric acid by connecting the silver wire and a platinum wire to the anode and cathode of a 9 V battery for five minutes, until the surface of the silver had become darkened. After insertion of the silver wire into the electrode body, the top of the tube was sealed, and the entire assembly fitted into the flow cell.

A stainless steel tube was fitted into another of the arms of the flow cell and served as an auxillary electrode throughout the investigation.
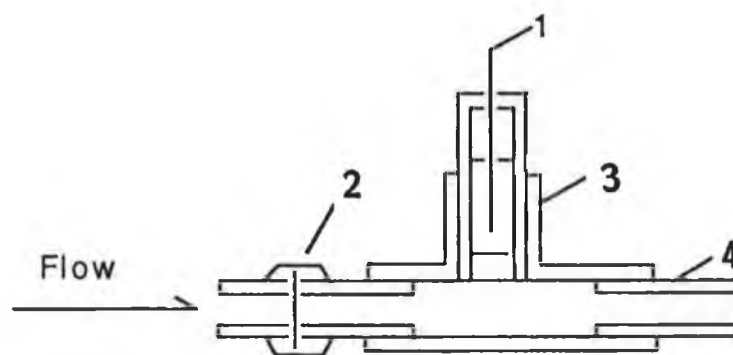
Figure 3.3.   Structure of flow cell. (1) reference
electrode (2) working electrode (3) flow
cell (4) auxillary electrode

3.2.2. Reagents

All chemicals used were of analytical grade. All solutions were prepared in deionised water, obtained by passing distilled water through a Millipore Milli-Q water purification system.

All glassware was washed in a 1% solution of Teepol, rinsed with distilled water, soaked in a 2 M nitric acid bath and finally rinsed with deionised water prior to use.

Gold fibre of 25 um diameter and 99.99% purity was obtained from Goodfellow (Cambridge,UK).

3.2.3. Working electrode and flow cell fabrication

The gold fibre working electrode was fabricated according to the procedure reported by Hua et al. [5]. As shown in Figure 3.4, a hyperdermic syringe needle was passed through the walls of a PVC tube, perpendicularly to the flow direction of the tube. After doing this, it is necessary to ensure that the internal bore of the needle is not blocked with PVC. A single gold fibre was then inserted into the internal bore, after which the needle was withdrawn from the tube leaving the gold fibre behind in place.

The junction between the fibre and the tube was heat-sealed by careful application of a soldering iron. This step is of critical importance to the overall fabrication process. A considerable degree of care must be taken to ensure that the junction is completely sealed, as failure to do so may result
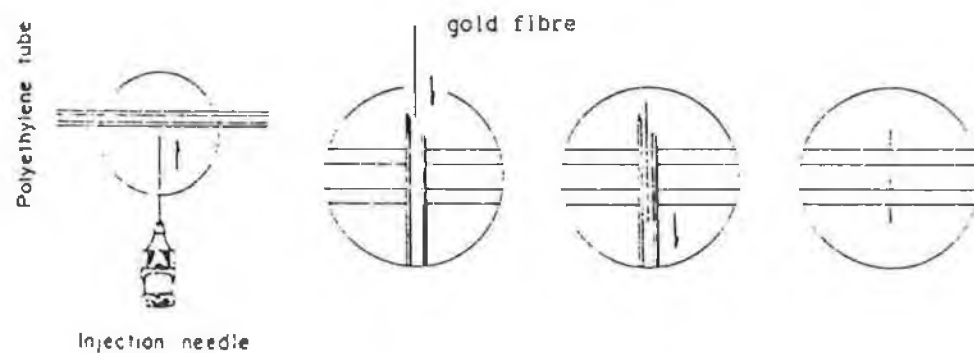
Figure 3.4.   Schematic representation of gold fibre flow electrode fabrication procedure

in leaks in the system, or, when the epoxy is applied during the final fabrication step, some may polymerise on the electrode surface thus inactivating the electrode. In practice, it was found that the best way to ensure a complete seal was to allow water to flow through the electrode assembly; in this way, any faults can be easily located where droplets of water leak out of the seal, and can therefore be readily corrected.

Electrical contact with the fibre was made by means of a copper wire, after which the complete junction was covered with epoxy. This method of working electrode fabrication is simple, requires no specialised apparatus, and has a success rate of manufacture of greater than 95%.

The flow cell, as shown in Figure 3.3, was manufactured from Teflon and has an internal volume of approximately 0.9 ml. The working electrode was simply fitted into the appropriate arm of the cell without any specific fixing procedure. In this way, the working electrode could be easily removed and replaced, and when in operation no leakages occurred. In addition, different working electrodes, e.g. carbon fibre, can be readily incorporated into the cell [5].

The Ag/AgCl reference electrode and stainless steel auxillary electrode were fitted into the appropriate arms of the cell and, as with the working electrode, could be easily removed and replaced.

3.2.4. Procedure

In the voltammetric stripping procedure, 0.1 M perchloric acid was first passed through the flow cell at a flow rate of 0.3 ml/min. A potential of -1.00 V was then applied to the electrode for 20 seconds followed by an anodic scan at 20 mV/s. Subsequent to this scan, the standard solution was allowed to enter the flow cell at a rate of 0.3 ml/min, after which deposition of Se(IV) was carried out at -1.00 V for 20 seconds followed by the anodic scan at 20 mV/s. When the stripping signal for Se(IV) had been obtained, the cell was washed with 0.1 M $HClO_4$ and the background scan repeated, as described above.

In this manner, between 6-10 stripping measurements and the associated background response could be performed, before it was found necessary to clean the electrode according to the procedure of Hua et al. [5]. Following this cleaning procedure, the electrode could almost invariably be used as before. It was possible to use the same electrode for two to three days.

3.2.5. Electrode cleaning procedure

When gold electrodes are used, a cleaning procedure is required to remove the oxides which foul the electrode surface. Normally, this involves the use of aqua regia and the application of a suitable potential to remove the oxide film. This is usually followed by a replating step, in which a new surface of gold is

deposited on the electrode surface fron a gold containing electrolyte, and by judicious selection of a deposition potential.

In the procedure reported by Hua et al. [5], this basic format is followed. The procedure involves firstly, the washing of the electrode with 99% (v/v) ethanol for 10 seconds at -0.05 V, followed by a wash in a solution, consisting of 6 M nitric acid and 2 M sulphuric acid for ten seconds at +2.00 V. Finally, a potential of +0.90 V was applied for two seconds in 5 M hydrochloric acid. After this, an electrode pretreatment solution, consisting of 50 mg/l gold(III) in 1 M hydrochloric acid was allowed into the cell. A potential of -0.10 V was applied for 1 second, after which the potential was increased to 0.10 V for 20 seconds and to 0.40 V for 1 second. Finally, a potential of 0.10 V was applied for 30 seconds.

3.3. RESULTS AND DISCUSSION

3.3.1. Initial Considerations

Anodic stripping voltammetry of selenium(IV) is normally performed using a solid electrode such as gold, with 0.1 M perchloric acid often being recommended as the electrolyte of choice [7-10].

Andrews and Johnson [8] have reported that the electrodeposition of Se(IV) at a gold electrode in 0.1 M perchloric acid produces selenium in three distinct states of activity, and three anodic stripping peaks are observed for large quantities of deposited selenium.

Multiple peaks have been reported for the deposition and stripping of many metals at solid electrodes, including lead from a gold electrode [9] and bismuth from a platinum electrode [10]. These multiple peaks are indicative of multiple states of activity for the deposit resulting from various states of interaction with the electrode material.

The stripping of very small quantities of selenium in 0.1 M perchloric acid following deposition at a low flux has been reported [8] to yield a single anodic peak at 0.80 V vs SCE. This was concluded to result from the oxidation of a quantity of selenium, probably not exceeding the equivalent of a monolayer which is strongly adsorbed by the electrode surface; this state is normally referred to as "adsorbed selenium".

Following depostion at high fluxes of Se(IV), two anodic peaks at 0.63 V and 1.15 V vs SCE, in addition to that corresponding to the adsorbed selenium, were reported to occur [8].

The anodic stripping peak at 0.63 V was concluded to result from the oxidation of bulk deposited selenium. The stripping peak for the adsorbed selenium occurs at a potential more positive than that for bulk selenium because of the stabilisation by one-dimensional interaction with the gold electrode surface by adsorption.

The peak occuring at 1.15 V was reported to result from the stripping of selenium from an intermetallic gold-selenium compound formed following diffusion of selenium into the gold electrode from the bulk deposit. Such a diffusional process is promoted by the large selenium activity gradient at the gold-selenium interface produced by the deposition of bulk selenium. The stoichiometry of the gold-selenium alloy, resulting from this diffusional transport of selenium into the gold electrode, is, however, unknown.

The application of anodic stripping voltammetry for the determination of trace Se(IV) is possible in 0.1 M perchloric acid, if the total deposit on the gold electrode surface does not exceed the equivalent of one monolayer [8].

As 0.1 M perchloric acid has been the recommended electrolyte for previous studies [7,8] involving the anodic stripping voltammetry of Se(IV) at gold electrodes, it was therefore chosen for use throughout this investigation.

3.3.2. Influence of deposition potential

The influence of the deposition potential on the anodic stripping voltammetric response of Se(IV) at the gold fibre electrode was investigated using 0.1 M $HClO_4$ electrolyte, a 5ng/ml Se(IV) standard, a deposition time of 20 seconds, and by varying the deposition potential between -0.10 V and -1.30 V. The resulting relationship between the stripping peak current and the deposition potential is shown in Figure 3.5. From this, it can be seen that there is an increase in the sensitivity of the selenium stripping peak with increasingly negative deposition potential. The resulting stripping voltammetric peak for selenium, as shown in Figure 3.6, occurs at a potential of +0.80 V vs Ag/AgCl.

In previous work using anodic stripping voltammetry for the determination of Se(IV), the recommended deposition potentials lay between +0.15 V and -0.30 V [7,8]. It was reported that the use of such potentials allowed only the formation of adsorbed selenium on the gold surface,which, in turn, resulted in a single, sensitive stripping response.

From Figure 3.5, it can be seen that the sensitivity of the selenium stripping response increases as the deposition potential becomes more negative. The optimum deposition potential lies in the range -1.00 V to -1.30 V. Therefore, there is a considerable difference between the deposition potentials reported for conventionally sized electrodes, e.g. gold disc
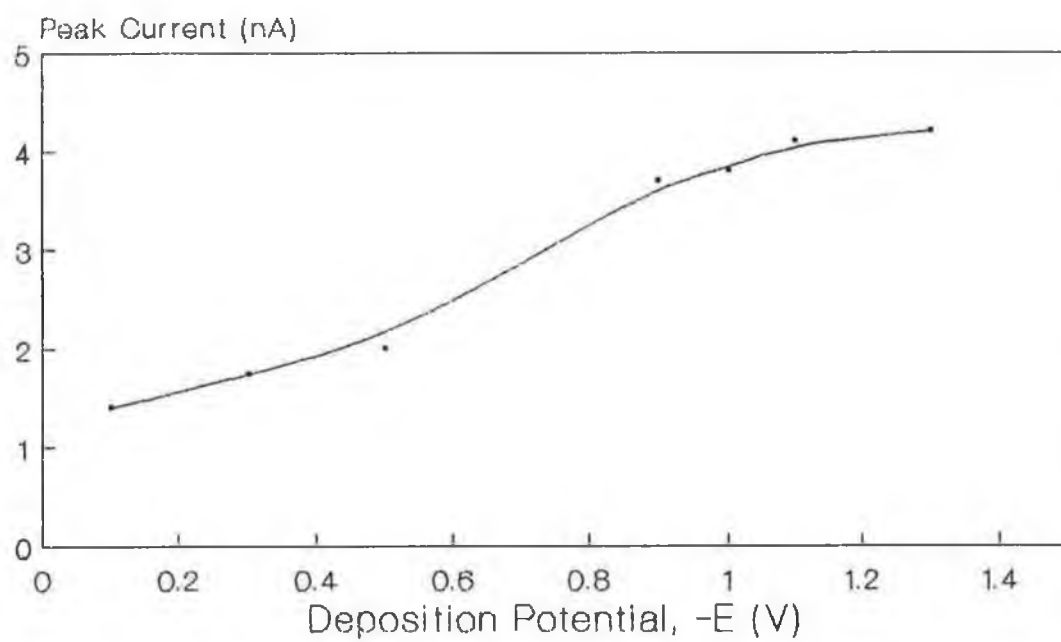
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Figure 3.5.   Influence of deposition potential on

stripping peak current

5 ng/ml
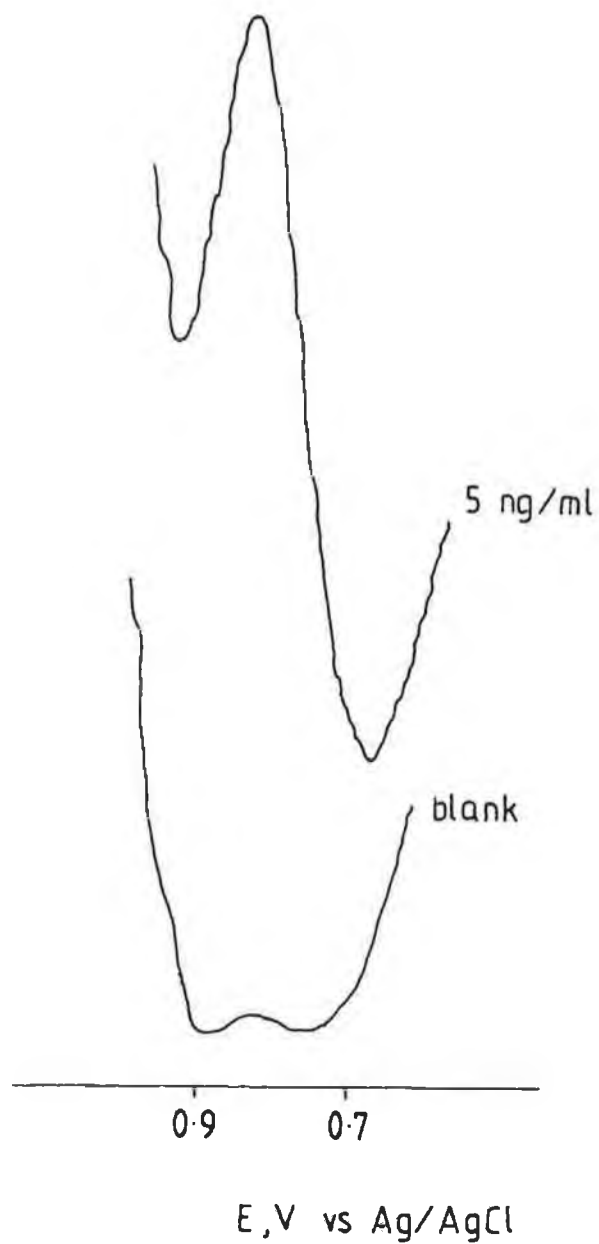
blank

0·9   0·7

E,V vs Ag/AgCl

Figure 3.6.   Typical anodic stripping voltammogram for
Se(IV) at a gold fibre electrode

electrode, and the fibre electrode used in the present investigation. The reason behind such a difference lies in the unique diffusional mass transport characteristics of such small diameter electrodes.

Wang et al. [11] have shown that at mercury-coated carbon fibre electrodes, significant deposition of metal ions occurred during the anodic stripping scan period from the deposition potential to the peak potential. As a result of this so-called "continued plating" effect, considerable differences between the anodic stripping voltammetric behaviour were observed between conventional electrodes and microelectrodes.

In effect, deposition of Se(IV) on the gold fibre electrode surface is not limited by the constraints encountered with macroelectrodes, where deposition is at a defined optimum potential. In this study, the deposition process is effectively the cumulative deposition over a range of potentials. The extent of this range is the critical factor in determining the ultimate sensitivity, and therefore, in choosing an "optimum" deposition potential, one is defining the optimum potential range over which deposition of the analyte gives the most sensitive stripping response.

In this investigation, a potential of -1.00 V was chosen for use throughout the evaluation of the stripping voltammetry of Se(IV) at the gold electrode surface.

3.3.3. Effect of scan rate

The relationship between the scan rate and the stripping response was investigated using a 5 ng/ml solution of Se(IV), a deposition potential and time of −1.00 V and 20 seconds respectively, and by varying the scan rate between 2 mV/s and 20 mV/s.

As shown in Figure 3.7, there is a linear relationship between scan rate and the stripping response over the range investigated. There is a significant non-zero intercept of 8 nA which is indicative of the continuous plating of Se(IV) as the potential is scanned from −1.00 V.

A scan rate of 20 mV/s was chosen for use throughout this investigation, as this allowed a good compromise to be obtained between the sensitivity of the stripping peak and the shape of the peak.
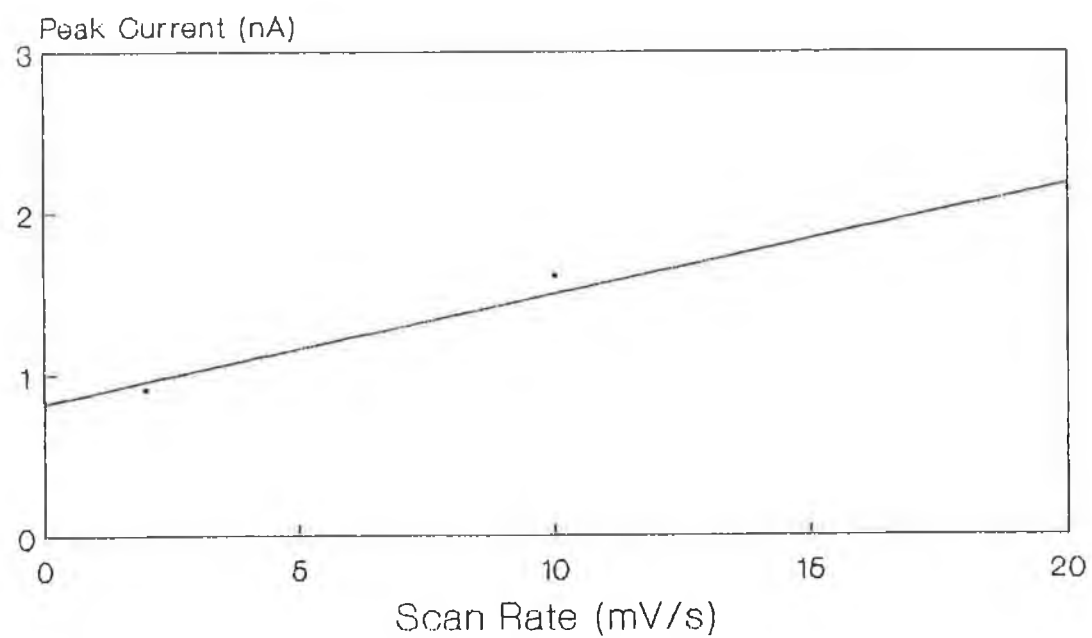
Figure 3.7. Effect of scan rate on stripping response

3.3.4. Influence of pulse height

The influence of the pulse height was investigated by evaluating the sensitivity of the stripping response at pulse height values between 20 mV and 100 mV, using a 5 ng/ml solution of Se(IV), a scan rate of 20 mV/s, and a deposition time and potential of 20 seconds and -1.00 V respectively.

There was an increase in the sensitivity of the response with increasing pulse height. However, at high pulse height values the symmetry of the stripping peak deteriorated and, therefore, as a compromise between sensitivity and peak definition, a value of 50 mV was selected.

3.3.5. Effect of drop time

The effect of the drop time upon the stripping peak current was investigated using a 5 ng/ml Se(IV) solution, a scan rate of 20 mV/s, a deposition time and potential of 20 seconds and -1.00 V respectively, and by varying the drop time between 0.2 seconds and 1 second.

As a drop time of 0.5 seconds allowed optimal sensitivity, it was chosen for further use in this investigation.

3.3.6. Deposition time dependence of stripping peak current

The relationship between the deposition time period and the selenium stripping peak current was investigated using a deposition potential of -1.00 V, a scan rate of 20 mV/s, solutions containing 0.5 ng/ml and 2 ng/ml Se(IV), and by varying the accumulation time period between zero and 35 seconds.

As shown in Figure 3.8, there are significant non-zero intercepts in the plots of deposition time vs stripping peak current. This is in contrast to the behaviour exhibited by conventional sized electrodes, where, at low concentrations, the relationship between deposition time and the stripping response is a linear one with a zero intercept.

In the case of a zero second deposition time, significant accumulation of Se(IV) on the gold electrode surface occurred during the scan period as a result of the non-linear diffusional mass transport characteristics of these electrodes. Similar behaviour has also been observed for the anodic stripping voltammetric determination of lead [12] and for the cathodic stripping voltammetric determination of Se(IV) at mercury-coated carbon fibre electrodes [13]. This has important consequences for the methodology of the stripping procedure, as it opens the oppurtunity of substantially reducing or eliminating the accumulation time period. A deposition time of 20 seconds was used in this study as this allowed optimal sensitivity, and
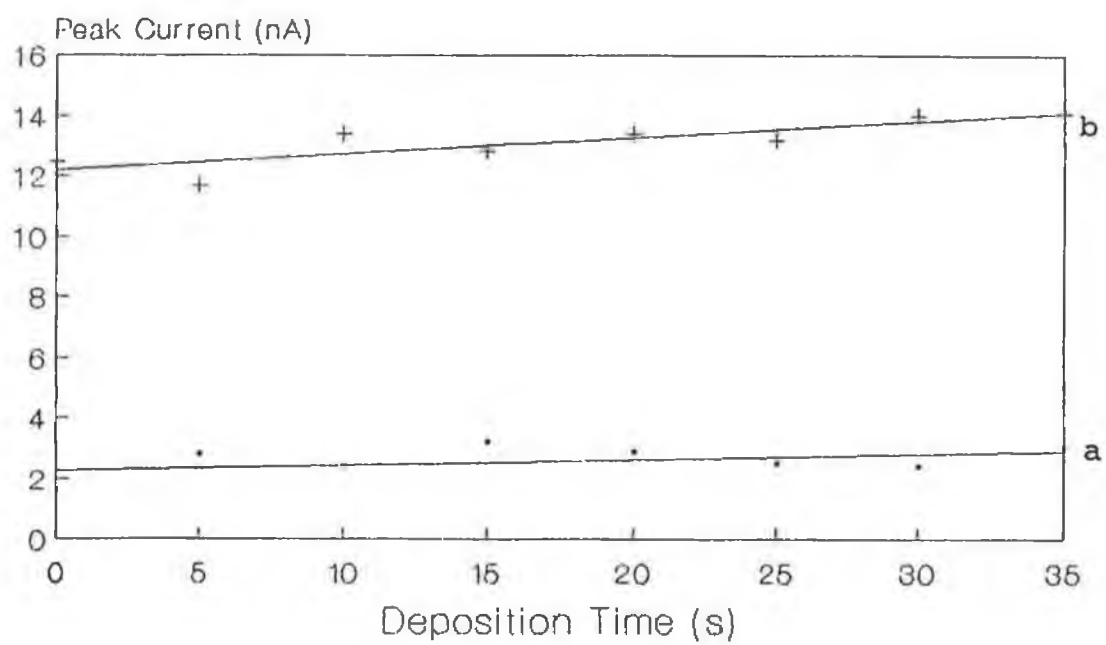
Figure 3.8.   Deposition time dependence of stripping

peak current; (a) 0.5 ng/ml Se(IV)

(b) 2 ng/ml Se(IV)

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under these conditions a linear calibration curve was recorded between 0 - 15 ng/ml, as shown in Figure 3.9., with a relative standard deviation of 6% at 5 ng/ml.

One of the major drawbacks in using gold electrodes is that the surface of the electrode can become very easily contaminated. In response to this, cleaning procedures have been developed to overcome the situation. In the conventional approach to electrochemical measurements this complicates the analytical methodology. In the present system, the use of a flowing stream simplifies the procedure considerably. Nevertheless, a significant stripping response for Se(IV) was obtained in the blank during the course of this investigation. The extent of this blank contribution could be reduced by employment of the cleaning procedure previously described in section 3.2.5., but never completely eliminated. The size of the blank response also varied with the age of the electrode. Therefore, in determining the detection limit the nature of the blank response had to be considered. It was decided that the detection limit would be that concentration that could be definitely distinguished from the blank response. A concentration of 0.5 ng/ml, corresponding to three times the average blank response, was obtained for the detection limit.

The ease with which a gold electrode surface becomes contaminated and the subsequent problems this creates in the analytical procedure need to be given serious consideration when deciding upon the use of these electrodes.
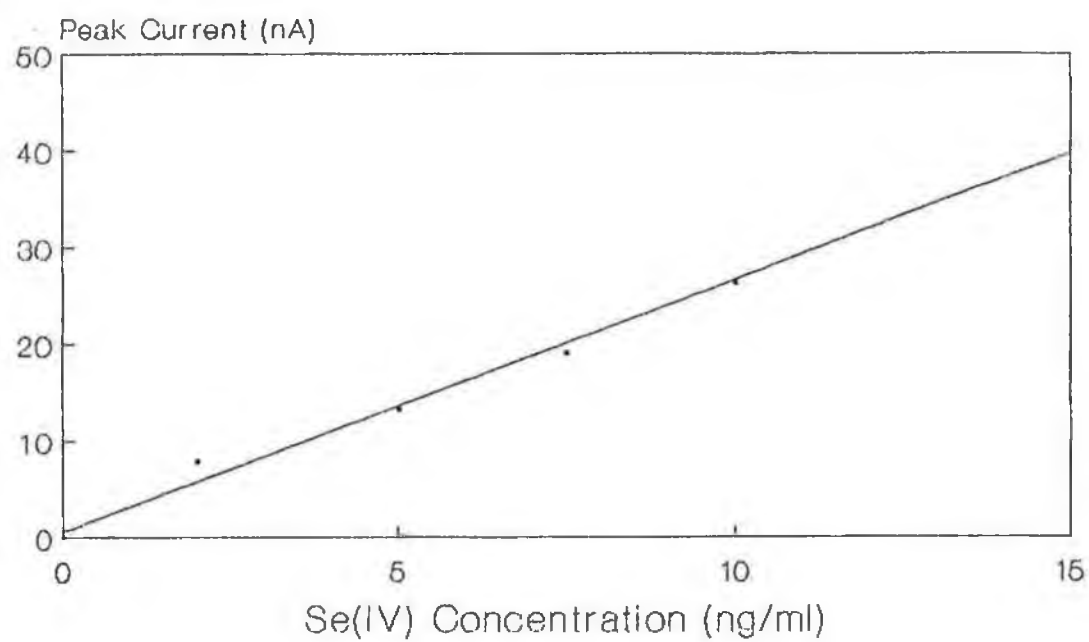
Figure 3.9.   Plot of Se(IV) concentration vs stripping
peak current showing linear range

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3.4. CONCLUSIONS

In this investigation, a gold fibre electrode has been used for the first time for the anodic stripping voltammetry of Se(IV). In addition, the use of such a working electrode provides the opportunity to develop an electrochemical flow cell with a very small dead volume.

The fabrication procedure for both the working electrode and the flow cell are extremely simple, requiring the minimum of specialised manufacturing apparatus and operator technique. Moreover, the ease and success rate of the working electrode manufacture means that a large number of electrodes can be made in a relatively short period of time. Whilst this study has confined itself to the use of gold fibres, the working electrode material can be readily changed to incorporate other electrode materials, e.g. carbon fibres [14] or platinum wires. The flow cell can also be adapted to carry out electrochemical measurements in conjunction with other analytical techniques, e.g. high performance liquid chromatography [14].

All of these factors are particularly important, in situations where the demands placed upon an electrochemical detection system include ease and low cost of fabrication and maintenance, flexibility for use in a variety of situations, and, where the turnover of electrodes is high, a cost effective approach for producing sufficient electrodes to meet the demand.

The particular properties exhibited by electrodes of small

diameters, means that the voltammetric behaviour of analytes such as Se(IV) differs from that expected at coventionally sized electrodes. This difference stems largely from the unique mass transport characteristics of such small electrodes.

The enhanced diffusional mass transport behaviour at the gold fibre electrode, means that a considerable degree of accumulation of Se(IV) occurs during the scanning period from the deposition potential to the peak potential. This has important consequences upon parameters such as the deposition potential, scan rate and the deposition time.

At conventionally sized electrodes, deposition of the analyte of interest is normally carried out at an experimentally defined potential, which allows for the most sensitive stripping response. However, at the gold fibre electrode used in this investigation, deposition of Se(IV) occurs over a range of potentials, so that, rather than a particular optimal deposition potential governing the final sensitivity of the stripping response, it is the size of the deposition potential range which is the critical factor.

Similarly, the mass transport characteristics have an influential effect upon the differences observed for the deposition time dependence of the stripping response at the fibre electrode. Normally, there is a linear relationship between the deposition time and the stripping peak current for low concentrations of analyte. In the present investigation, however, substantial accumulation of Se(IV) occurred during the

scan period, as a result of non-linear diffusional mass transport of the metal ions on to the electrode surface. This was evidenced in the non-zero intercepts obtained for the plots of deposition time against stripping peak current.

This finding opens the possibility of modifying the voltammetric procedure normally carried out with macroelectrodes to suit the particular properties of the fibre electrode. The deposition time period, which is the most time consuming step in the voltammetric procedure, can be substantially reduced or eliminated altogether. In the present investigation, a deposition time of 20 seconds was selected as this allowed a linear relationship between Se(IV) concentration and stripping response over the range 0-15 ng/ml to be obtained.

In this investigation, it was possible to determine Se(IV) levels down to 0.5 ng/ml. This is comparable with values previously reported in the literature of anodic stripping voltammetry at a gold disc electrode [7] and a gold plated glassy carbon electrode [15].

Whilst cathodic stripping voltammetry at a hanging mercury drop electrode [7], or at mercury coated carbon fibre electrodes [13] allows the determination of Se(IV) down to sub-ng/ml levels, anodic stripping voltammetry at a gold fibre electrode does provide the required sensitivity to allow the determination of Se(IV) in the majority of matrices normally studied.

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Chapter 4

Cathodic stripping voltammetry of selenium

at mercury-coated carbon fibre electrodes

4.1.<u>INTRODUCTION</u>

Microelectrodes are normally defined as electrodes with characteristic diameters smaller than about 20 um, and have grown in popularity in recent years due mainly to their advantageous mass transport properties and reduced capacitative currents.

The size of a microelectrode has been redefined over time. In electroanalytical applications, a microelectrode that has an area less than 0.1 cm^2 is considered to be a standard, not small, size. The bulk of the literature citations on microelectrodes reflect the usefulness of electrodes with diameters less than 10 um. The recent emphasis among electrochemists interested in the electroanalytical, theoretical, and fundamental physical properties of electrodes with at least one dimension less than 1um has given rise to the designation "ultramicroelectrode".

The first major research programmes involving microelectrodes were carried out by Fleischmann and co-workers at the University of Southampton in the late 1960's [1]. Reducing the area of an electrode has three major consequences:

(i) mass transport rates to and from the electrode are increased.

(ii) the double layer capacitance is reduced due to the decrease in surface area.

(iii) ohmic losses are reduced due to the diminished current.

As a consequence of the reduced capacitative charging currents and increased mass transport rates, microelectrodes exhibit

excellent signal-to-noise characteristics. In addition, there are dramatic changes to "conventional" electrochemical responses when very small electrodes are substituted. In this respect, the increased rates of mass transport to and from microelectrodes has important consequences upon their properties.

4.1.1. Effects of Mass Transport

In Figure 4.1 a cyclic voltammogram is shown for the oxidation of ferrocene in acetonitrile at a microelectrode using a relatively fast scan rate of 10 V/s. As the potential is scanned in the positive direction from 0.0 V the half-wave potential for ferrocene is approached and the current begins to increase. The current reaches a maximum after which it returns to the baseline. This situation arises because the rate of electrolysis of the compound greatly exceeds the rate at which it can diffuse to the electrode surface, resulting in a depletion of the species at the surface with a consequent current decrease. At this scan rate the majority of diffusion of the compound is perpendicular to the electrode surface as is shown in Figure 4.2a. On the reverse scan, the electrolysis products are reduced resulting in a current flowing in the opposite direction.

If the cyclic voltammogram is repeated, but this time at a slower scan rate, the response as shown in Figure 4.1b is obtained. As in the previous case, the current increases as the half-wave potential is approached. However, as the potential

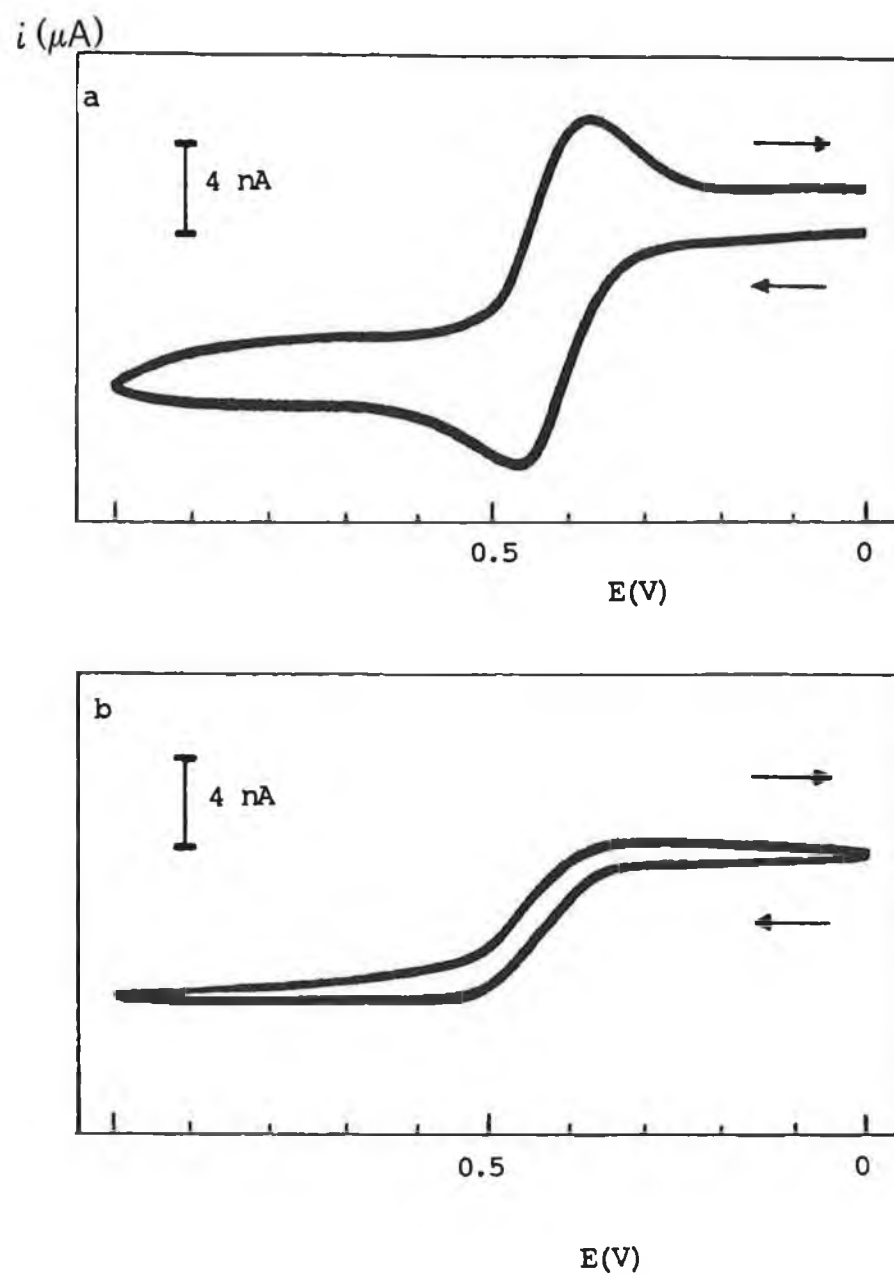Figure 4.1. Cyclic voltammogram of ferrocene (1.0 mM) in aceto-
nitrile with 0.1 M tetra-n-butyl- ammonium
perchlorate at a gold microdisk electrode
(r=6.5 um). (a) 10 V/s scan rate. (b) 0.1 V/s scan
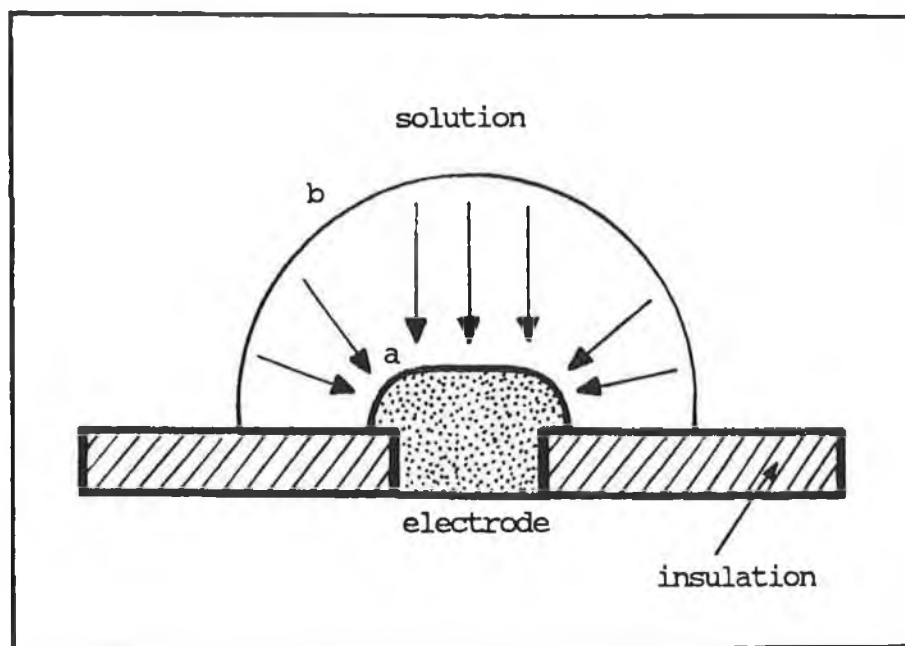rate. (reproduced from ref.2)

Figure 4.2.   Diffusion profile of electrolysed species

at a microvoltammetric electrode.

(a) Profile at 10 V/s scan rate.

(b) Profile at 0.1 V/s scan rate.

(reproduced from ref.2)

becomes more positive the current is essentially steady-state. This is due to the fact that the rate of diffusion of the compound to the electrode surface is now approximately equal to the rate of electrolysis, with the result that there is no depletion of the species at the surface and, hence, no consequent decrease in current. At these slower scan rates, the contribution of radial diffusion to the edges of the surface of the microelectrode, in addition to that perpendicular to the surface, assumes much greater importance, as is shown in Figure 4.2b. At an electrode of larger size, this type of contribution is difficult to obtain because very slow scan rates must be employed for radial diffusion to have a significant effect on the current. These steady-state contributions to the current, colloquially referred to as" edge effects", have long been recognised by electrochemists. In 1941, Laitinen and Kolthoff [2] worked with disc electrodes of approximately 3 mm diameter and obtained the responses shown in Figure 4.3. For each point on these curves, the current was monitored at a fixed potential for two or three minutes. This was because the ratio of molecules diffusing perpendicular to the electrode relative to those diffusing in at the edges is much greater at larger sized electrodes and therefore the steady-state response is not observed for a longer period of time. The steady-state response thus obtained is similar to the response at microelectrodes, except that such measurements are greatly simplified at the latter. The shape of these microelectrodes is not confined to a disc format, as an
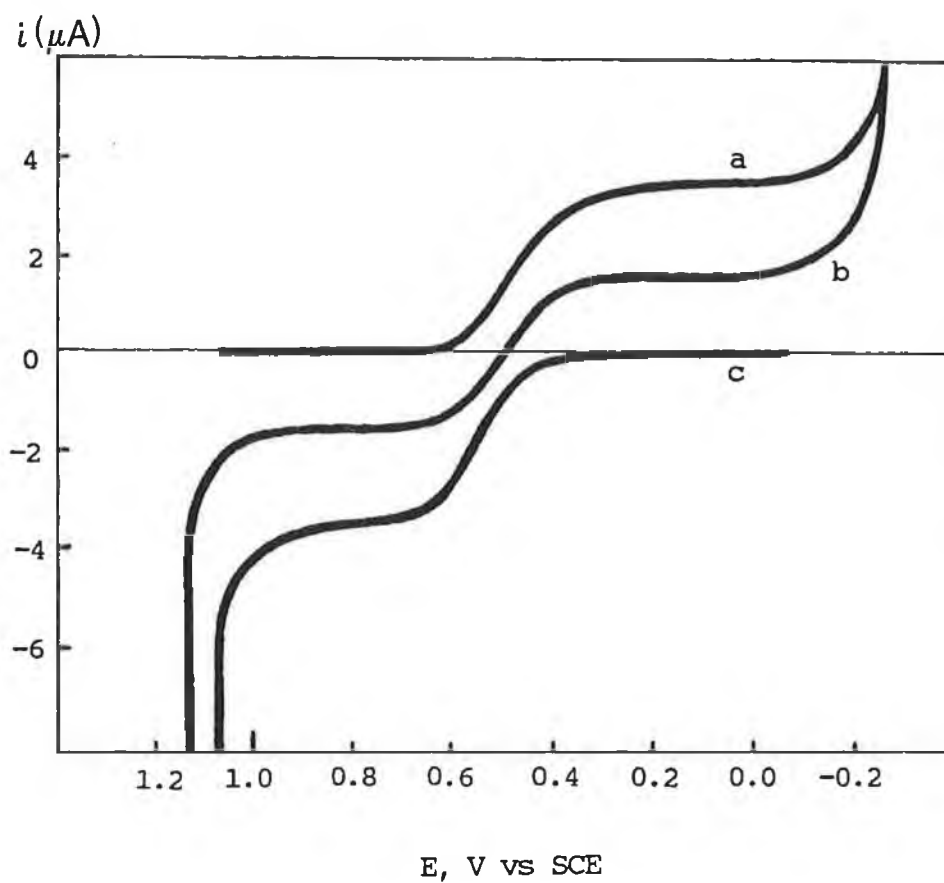
Figure 4.3.   Electrolysis of ferrous chloride and

ferric chloride in 0.5 N HCl.

(a) 0.002 M $FeCl_3$.

(b) 0.001 M $FeCl_2$, 0.001 M $FeCl_3$.

(c) 0.002 M $FeCl_2$.

(reproduced from ref.2)

electrode of virtually any geometry of small enough size will give a similar response.

In addition to these mass transport characteristics, micro-electrodes also exhibit reduced double layer capacity and small current generation during voltammetry. The current required to charge the double layer is small relative to those measured at electrodes of conventional size since the double layer capacity is proportional to the electrode area. Although the absolute value of the current from the electrolysis of solution components is similarly diminished, the magnitude is sufficient for measurement with modern instrumentation.

4.1.2. Voltammetry in high resistance solutions

The very small currents which occur at microelectrodes should facilitate their use in solutions of high resistance. This reduced iR effect is of importance for measurements in:

    (i) nonpolar solvents in the presence of appropriate
        supporting electrolytes.

    (ii) polar solvents and mixtures of polar and nonpolar
        solvents in the absence of purposely added supporting
        electrolytes.

Bond et al. [3] have shown that voltammograms for the oxidation of ferrocene in acetonitrile can be obtained in the absence of supporting electrolyte with electrodes of 0.5 um radius.

4.1.3. Voltammetry at fast scan rates

The application of very high scan rates are possible at microelectrodes because of the low time constant of the electrochemical cell and minimal distortion from iR drop with respect to results obtained at conventionally sized electrodes. Practical cyclic voltammetry with electrodes of conventional size is restricted to an upper limit of 100 V/s because of instrumental and iR limitations and is essentially the lower limit at which microelectrodes can be used. Both the faradaic current at high scan rates and the charging current, which tends to dominate the electrode response at very high scan rates, are proportional to the electrode area. Therefore, as the electrode radius is decreased, these contributions to iR drop are reduced proportionally, and voltammograms can be obtained on a microsecond time scale without the need for iR compensation. However, there is a practical limit on the scan rate which can be used since, in cyclic voltammetry, the ratio of charging current to the faradaic current increases with the square root of the scan rate. Howell et al. [4] have demonstrated the use of scan rates of up to 100, 000 V/s for cyclic voltammograms of the reduction of anthracene in acetonitrile. Instrumental distortion was negligible up to 20, 000 V/s, and at this scan rate distortion due to the iR drop was small.

4.1.4. <u>In vivo analysis</u>

The first electrode for <u>in vivo</u> electrochemical studies was developed by Adams <u>et al</u>. [5]. This was a carbon paste microelectrode which was used to detect the presence of ascorbate, dopamine, 3,4-dihydroxyphenylacetic acid, norepinephrine, and the indoleamines serotonin and 5-hydroxyindoleacetic acid, in brain tissue.

Gonon <u>et al</u>. [6] used carbon fibre microelectrodes for the detection of ascorbate and the catecholamines. There was some confusion over whether the dopamine peak could also be attributed to the dopamine metabolite 3,4-dihydroxyphenylacetic acid (DOPAC). However, electrochemical pretreatment of the carbon fibre was found to allow selective detection of DOPAC.

The carbon fibres were treated with two consecutive 20 second periods of electrical pulse. The results showed the detection of ascorbic acid and DOPAC in the first and second signals respectively on the voltammogram.

The choice of carbon fibre electrodes for <u>in vivo</u> studies has been due in no small way to their small size. However, the electrochemical and surface characteristics of carbon fibres have proven to be useful, and as so little of the electroactive material is consumed, measurements with these electrodes are essentially non-destructive. Nerve terminals have diameters in the 1 um range and therefore microelectrodes are large with respect to these cells. Wightman has reported that these

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electrodes appear to be essentially non-perturbational in brain tissue since the measured current does not change significantly with repetitive potential pulses [2]. In addition, as the electrode must be implanted in the brain and monitored over a specific time period, normally several hours, the opportunity for any electrode renewal is non-existent and, therfore, the constancy of the electrode response is of fundamental importance in their use for in-vivo studies. In this respect, pulsed waveforms have been shown to be best for maintaining electrode stability [2].

The remarkable changes in voltammograms that can be brought about by electrochemical pretreatment of the carbon fibre have also been of great benefit for in-vivo analysis. A new carbon fibre, the HTA-7, is reported to allow selective and sensitive detection of dopamine and DOPAC and permits both these compounds to be determined individually [7]. The electrochemical renewal of the electrode surface before each measurement means that reproducible results can be obtained.

4.1.6. Stripping voltammetry at microelectrodes

Stripping voltammetric techniques with hanging mercury drop (HMDE) or mercury film electrodes (MFE) are recognised as some of the most sensitive techniques for trace metal analysis. The mercury film electrode is preferred for ultratrace determinations since its larger area-to-volume ratio permits superior sensitivity and selectivity. The major drawbacks of such techniques are the length of time for sample deoxygenation and preconcentration of the analyte, requirement for quite large sample volume, and the relatively poor precision.

The attributes which have made microelectrodes attractive for in vivo studies also make them amenable for the developement of stripping procedures otherwise impossible at the hanging mercury drop and thin film mercury electrodes.

The steady state diffusional characteristics exhibited by microelectrodes means that preconcentration of the analyte on the mercury can be accomplished without forced convection. In addition, since stirring of the solution is not required, stripping voltammetry can be carried out in sample volumes of the order of 5-ul.

Following the initial use of carbon fibre microelectrodes for in vivo studies, several reports appeared emphasising their use as a surface for mercury deposition and subsequent stripping analysis. Golas and Osteryoung attempted to indicate the limitations of carbon fibres for mercury deposition and found

that the number of active sites at which mercury could be reduced depended strongly on the potential [8]. The film thickness and most reproducible film formation increased with increasing negative potential. In addition, on exposure to air whilst transferring the mercury-coated microelectrode from one solution to another, the mercury film size decreased. The most probable causes for this could have been air oxidation of the mercury or simply mechanical removal of the film as it is taken out of solution, as mercury films on carbon are unstable.

Wang et al [9] reported on the differential pulse anodic stripping voltammetry (DPASV) of bismuth, cadmium, and lead and found that the voltammetric behaviour differed considerably from that expected at conventially sized electrodes. These differences arose in main from the steady-state diffusional mass transport characteristics of the microelectrodes. Consequently, because of the enhanced diffusional flux, significant plating of the metals occurred during the period of time required for the scan. The low ohmic drop at these electrodes means that the traditional use of high concentrations of electroyte in DPASV applications can be circumvented. This is of importance in speciation studies, since the addition of supporting electrolyte can cause errors due to contamination and disturbance of natural ionic equilibria [10]. Applications of stripping voltammetry using conventially sized electrodes, without the addition of supporting electrolyte, have been confined to marine samples. Wang and Zadeii [10] showed that the application of microelectrodes to the study of metal

speciation in water samples allowed accurate speciation data to be obtained.

Wehmeyer and Wightman [11] reported on the anodic stripping voltammetry of lead and cadmium at a mercury coated platinum disc microelectrode. They found that ASV at this electrode was superior to convential ASV techniques at mercury drop or film electrodes in terms of the reproducibility for trace measurements. This was attributed to radial diffusion and the use of quiescent solutions which eliminated the need for forced convection, and thus associated errors, typical at convential sized electrodes.

Whilst the bulk of research concerning mercury coated microelectrodes has involved the use of carbon fibres, there have been reports on the use of other electrode materials. Platinum has tended to be the alternative material of choice as it possesses some advantages over carbon fibres. The poor adhesion of mercury to carbon surfaces means that mercury deposits on carbon fibre microelectrodes tend to be less stable than their platinum counterparts. In addition, carbon fibres are more fragile, thus requiring more careful handling. However, platinum is reported to have the disadvantage of strongly adherent surface oxide films, low hydrogen overpotentials and finite solubility in mercury, all resulting in non-reproducible mercury deposits and stripping peaks. Boon-Tat Tay et al. [12] reported on the use of such electrodes for the anodic stripping voltammetric determination of zinc, cadmium, lead and copper in a flow

injection system and found that the microelectrode was hydrodynamically stable and allowed reproducible measurements at trace levels.

Baranski and Quon [13] used a potentiometric stripping technique for the determination of cadmium, lead and copper at mercury-coated carbon fibre electrodes. They reported that the elimination of convective transport decreased random errors associated with stripping analyses. In addition, in order to take full advantage of the applicability of microelectrodes for analyses in small sample volumes, a specially designed cell was fabricated. In this cell, a 7-um diameter carbon fibre was used as the working electrode and a 50-um diameter gold wire as a reference electrode. A 5-ul sample volume was placed on top of the working electrode, the reference electrode immersed in the sample and the cell was flushed with argon. Since the cell volume is so small the time for deaeration is considerably reduced. The likelihood of electrode contamination with organics is also reduced as the electrode is only in contact with a very small sample volume.

Wong and Ewing [14] have recently reported on the use of mercury-coated ultrasmall carbon-ring electrodes for anodic stripping voltammetry of lead. These electrodes have a total structural diameter of appproximately 1-um and, to date, represent the smallest diameter used for anodic stripping voltammetry. Anodic stripping voltammetry was carried out in the absence of supporting electrolyte as is shown in Figure 4.4. In
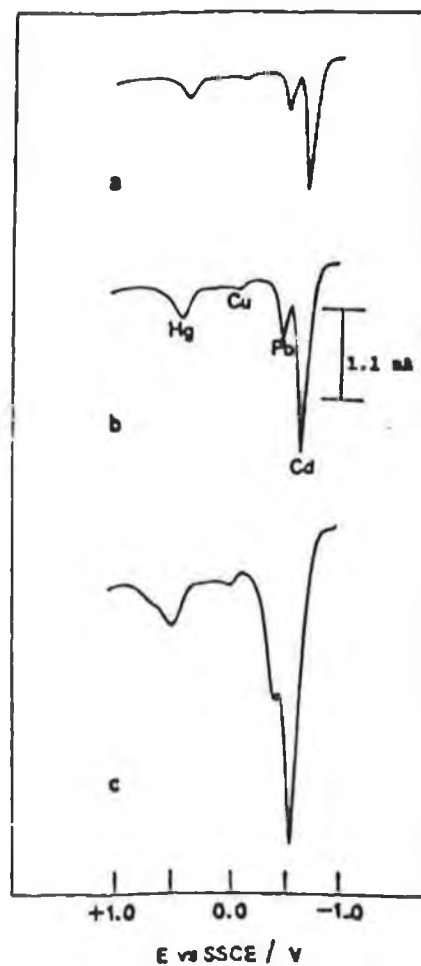
Figure 4.4. Anodic stripping voltammetry of lead at mercury-
coated carbon ring electrodes.

(a) 1×10^{-6} M Hg(I) in water.

(b) 1×10^{-6} M Hg(I) and 2×10^{-7} M
lead in water.

(c) 1×10^{-6} M Hg(I) and 2×10^{-7} M
lead in 0.1 M KNO$_3$.

(reproduced from ref.14)

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Figure 4.4a a stripping voltammogram is shown at a mercury-coated carbon-ring electrode in doubly distilled containing 1 x 10^{-6}M mercury(I). The stripping peaks at 0.0 V and +0.4 V are due to the oxidation of an apparent copper impurity and mercury respectively. Figures 4.4b and 4.4c show stripping voltammograms at the same electrode in doubly distilled water, containing 1 x 10^{-6}M mercury(I) and 2 x 10^{-7}M lead, and in 0.1M potassium nitrate, containing 1 x 10^{-6}M mercury(I) and 2 x 10^{-7}M lead, respectively. In both cases, stripping peaks are observed at -0.44, -0.66, and +0.34 V corresponding to the oxidation of lead, copper and mercury respectively. The mercury stripping peak in the presence of the 0.1M KNO_3 (pH 3) is greater than in the absence of the added supporting electrolyte, a state of affairs probably attributable to the more favourable kinetics of the oxidation of mercury in acidic media. In contrast, the stripping peak for lead is considerably greater in the absence of the supporting electrolyte. Wong and Ewing speculated that this was as a result of the less favourable conditions for mercury oxidation, hence allowing greater coverage of lead on the electrode surface. Irrespective of the true reasons underlying this, these results clearly illustrate the unique potential of microelectrodes for carrying out stripping voltammetric determinations without the presence of supporting electrolyte.

4.7. Microelectrode Fabrication

Gonon et al. [6] were the first to use carbon fibres for microvoltammetric electrodes. Their electrodes had approximately 500 um of the fibre protruding from the glass capillary.

In the scheme proposed by Schulze and Frenzel, the carbon fibre was inserted into a glass disposable pipet and sealed with a resin. The disadvantages of this method were the relatively long time needed for hardening of the resin and that the proportions of hardener to resin had to be controlled precisely. If this latter condition was not fulfilled, adhesion of the resin to the fibre was lost and the seal leaked.

Golas and Osteryoung [8] investigated several alternatives for microelectrode manufacture. For one type of electrode, a carbon fibre was inserted into the capillary tip of a soft glass pipet which had been cut and narrowed at the tip by heating. Then the tip was put into the flame of a small burner. This had a special glass tube head fixed onto the the standard burner with a cork washer, as is shown in Figure 4.5. The tip must be in the narrow wall of flame which surrounds the edge of the tube so that the fibre is inside where it is cool. Then it is held carefully until the tip collapses after which the electrode is taken out quickly. The outside part of the fibre should not be longer than 1.5 mm. If longer, the fibre begins to wave in the flame and usually burns. The inside of the glass part of the electrode was filled with mercury to enable the electrical connection with silver or
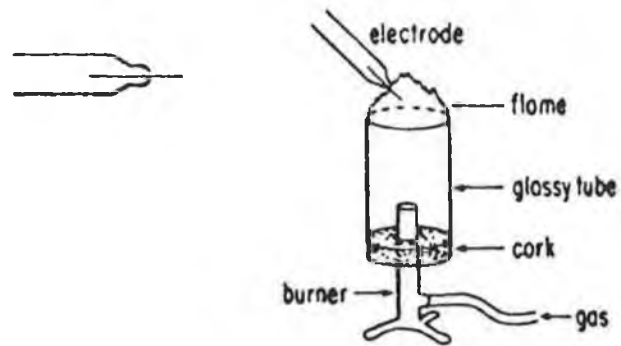
Figure 4.5. Schematic representation of microelectrode

fabrication process

(reproduced from ref.8)

copper wire. Before use each fibre was examined under a microscope to ensure that no damage had occurred during the fabrication process.

Hua et al. have reported on the use of carbon fibre flow cells for the constant stripping determination of selenium. These were fabricated by passing a carbon fibre through a PVC tube and sealing the joints with epoxy resin. Electrical contact was made via copper wire. The success rate reported for the manufacture of such electrodes was of the order of 95%.

4.2. EXPERIMENTAL

4.2.1. Apparatus

All experiments were performed using a Metrohm Polarecord E-506 potentiostat in conjunction with a Metrohm VA-663 voltammetric stand. A 10-ml electrochemical cell, which admitted the working electrode, reference electrode (Ag/AgCl), platinum wire auxillary electrode and nitrogen delivery tube through its Plexiglass cover, was used throughout the investigation.

A Metrohm E-612 VA-scanner and a Metrohm E611 VA-detector were used in the study of the cyclic voltammetric behaviour of ferrocene in acetonitrile.

Purging of electrolyte solutions prior to voltammetry was carried out using nitrogen for 10 minutes and, after deaeration, a blanket of nitrogen was kept over the solutions.

4.2.2. Reagents and Materials

All chemicals used were of analytical grade. Stock solutions of 1 ppm Se(IV) and $1x10^{-2}M$ Hg(II) were prepared, from SeO_2 (Merck) and $HgCl_2$ (Merck) respectively, in doubly distilled water, which was obtained by passing distilled water through a Millipore Milli-Q water purification system, The required standards were prepared daily by dilution of the appropriate

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stock solution.

The epoxy resin, ERL-4206 (TAAB, Berkshire, England), used for microelectrode manufacture was prepared as recommended by Spurr [16].

All glassware was washed for four days in 4 M nitric acid, rinsed and soaked in doubly distilled water until use.

4.2.3. Microelectrode fabrication

Carbon fibre microelectrodes were prepared from carbon fibres (Donnay, Belgium) having a nominal diameter of 7.5 um. The fibres were immersed in 10% nitric acid, rinsed with distilled water, soaked in acetone, rinsed with water and finally dried in an oven at 70°C.

A carbon fibre was inserted into the tip of a 100 ul micro-pipette to a distance of approximately 2 cm. A small drop of the sealing resin was carefully applied onto the tip of the micropipette. Capillary action pulls the resin inside the tip to a distance of 1-2 mm, after which the assembly was placed in an oven and cured at 70°C for eight hours to ensure complete polymerisation of the resin.

Each fibre was then examined under a microscope to ensure that there were no droplets of resin present on the portion of the fibre protruding from the micropipette tip. In the event that there was resin present on the fibre, that particular microelectrode was discarded.

Each microelectrode was back-filled with mercury into which a metal rod (60% Cu, 40% Zn) was inserted to make electrical contact. Finally, more resin was placed over the mercury and a second polymerisation at 70°C was carried out. After this had been completed, a micropipette segment was used as a guide for the metal rod to give the final microelectrode assembly structure shown in Figure.4.6. The final success rate of microelectrode manufacture was in the region of 95% [17].

As shown in Figure 4.6., an optional protective sheath can be used to prevent damage to the electrode. This was made by simply placing a micropipette tip, in which openings had been made, over the carbon fibre. During the course of this investigation it was found unnecessary to include such protection in the final assembly.

The resin used in the preparation of these electrodes, once polymerised, is compatible with the vast majority of organic solvents and with inorganic acids and alkalis at high concentrations [17].

Before use, all the microelectrodes were examined under a microscope to ensure that the electrode surface was devoid of any resin or contaminating particulate matter.

Note

The epoxy resin, ERL-4206, used in the fabrication of the microelectrodes is moderately toxic, causing dermatitis. It is
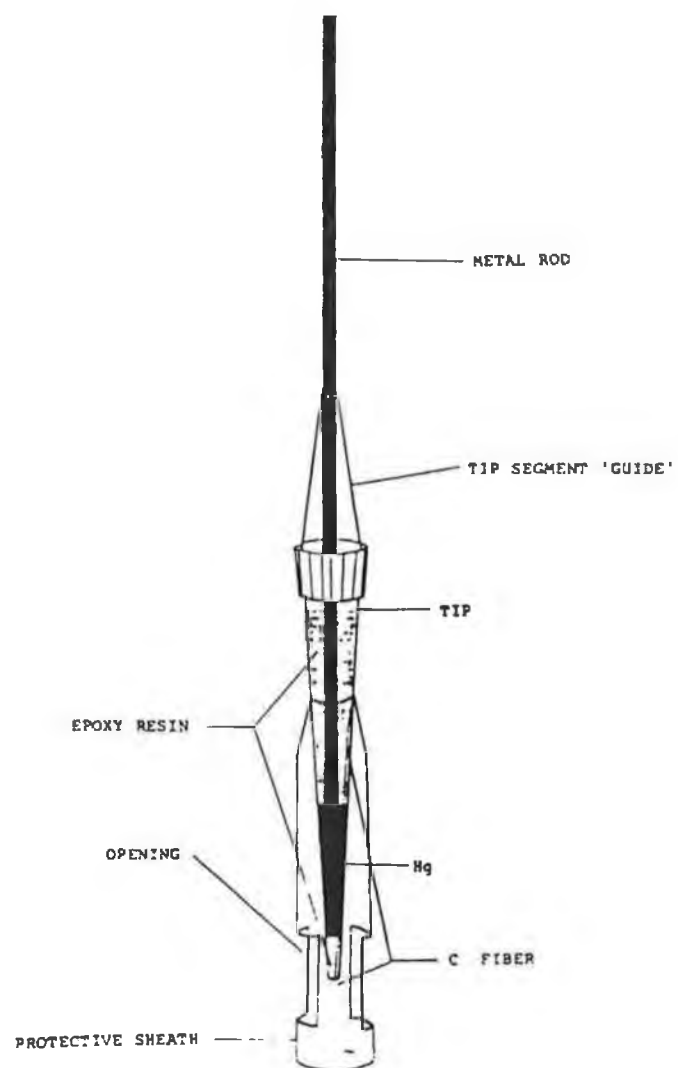
Figure 4.6. Structure of final microelectrode assembly

recommended that areas of the skin contacted by the resin or its mixture with other materials should be washed with soap and water. The resin should only be used in areas of adequate ventilation and repeated contact with the liquid component should be avoided.

4.2.4. Characterisation of microelectrode behaviour

In order to ensure that the microelectrodes manufactured as previously described exhibited the properties associated with them, the cyclic voltammetric behaviour of ferrocene in acetonitrile was determined.

The cyclic voltammogram of ferrocene in acetonitrile shown in Figure 4.7. shows that the electrodes exhibit the steady-state properties associated with microelectrodes.

Figure 4.7.   Cyclic voltammetry of ferrocene in

acetonitrile

(a) 50 mV/s

(b)   5 mV/s

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4.3. RESULTS AND DISCUSSION

4.3.1. Initial considerations

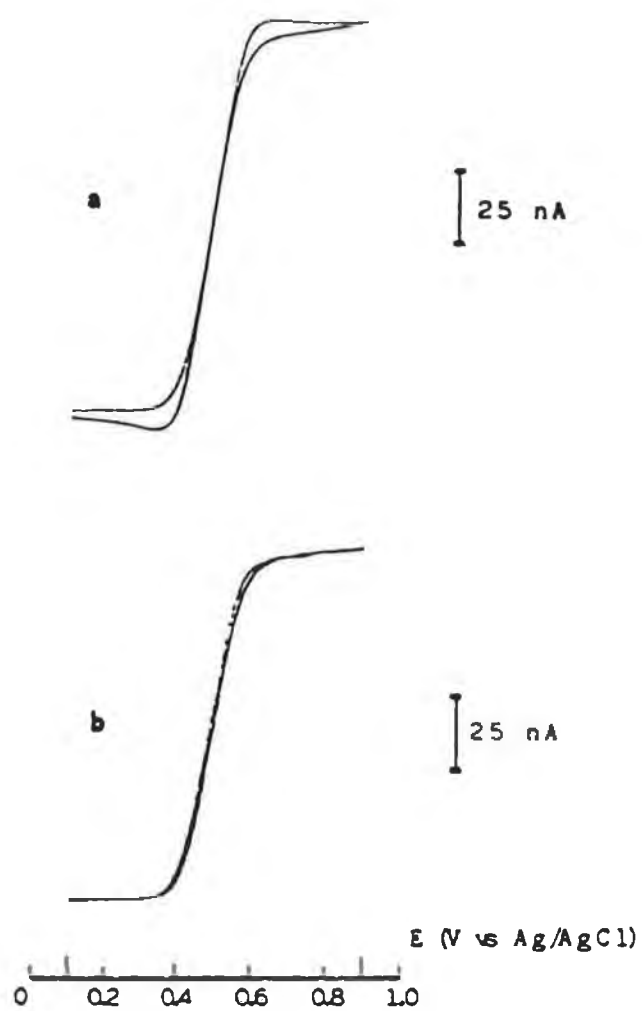For the cathodic stripping voltammetric determination of selenium, the composition of the supporting electrolyte plays an important role in determining the form in which selenium is absorbed onto the mercury electrode surface.

Bond et al. [18] have reported that acidic electrolytes such as hydrochloric, hydrobromic, sulphuric, perchloric, and nitric acids gave much better sensitivity for selenium than neutral or basic electrolytes, a finding consistent with the literature.

4.3.2. Influence of supporting electolyte

As hydrochloric acid has been recommended as the electrolyte of choice for the cathodic stripping voltammetric determination of selenium [18,19], it was used as the electrolyte in this study.

It has been postulated that the cathodic stripping voltammetric determination of selenium in acidic solution involves:

(i) a deposition step involving formation of mercuric selenide:

$$Hg + H_2SeO_3 + 4H^+ \longrightarrow HgSe(Hg) + 3H_2O \qquad [4.1]$$
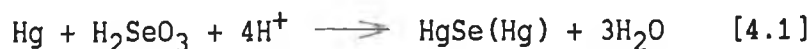
which is subsequently followed by:

(ii) a stripping step involving reduction of mercuric selenide:

$$HgSe(Hg) + 2H^+ + 2e^- \longrightarrow H_2Se + Hg \qquad [4.2]$$

The stripping process postulated above has been suggested by other workers [18,19]. A plot of peak potential, E_p, against $-\log_{10}[H^+]$ for 5 ng/ml Se(IV) in hydrochloric acid solutions gave rise to a relationship the value of whose slope, 62 mV, is consistent with a two-electron, two-prot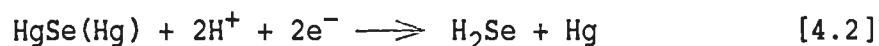on reduction process. The relatively low sensitivity obtained in alkaline solutions, as observed by Bond et al. [18], may be indicative of the hydrogen ion dependence of the electrode processes shown above. Adeljou et al. [20] have reported that the low sensitivity at pH values greater than 5 may indicate that some of the selenium exists in other forms that may be electrochemically inactive.

The relationship between the stripping peak current for Se(IV) and the hydrochloric acid supporting electrolyte concentration is shown in Figure 4.8. There is an almost linear increase of the stripping peak current with increasing acid concentration. This finding is in contrast with the data reported in the literature at a hanging mercury drop electrode, where it was normally found that the sensitivity of the selenium stripping
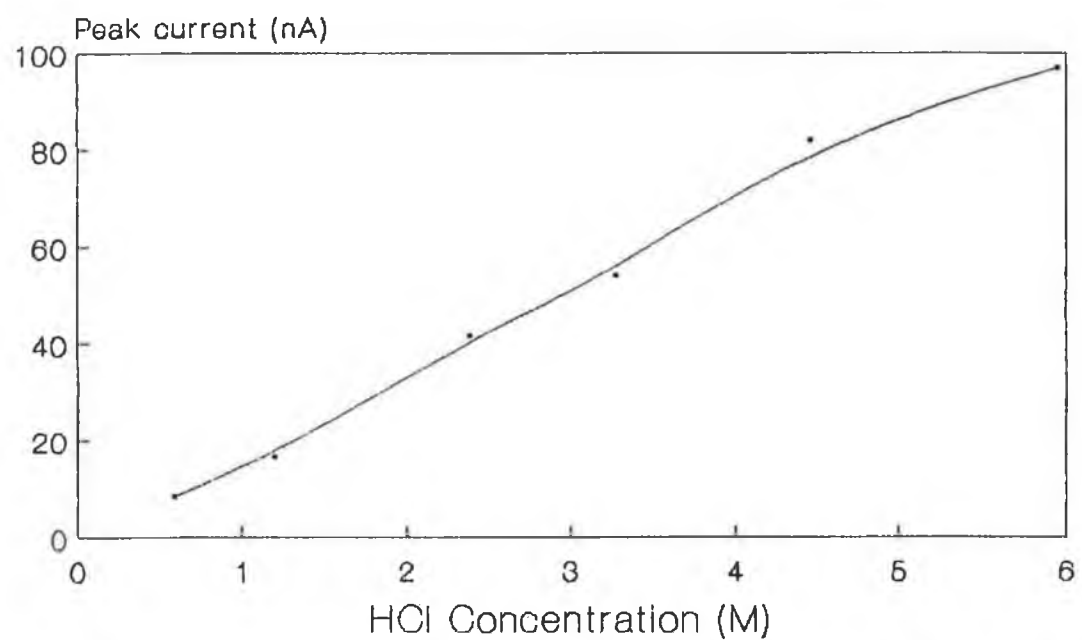
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Figure 4.8. Relationship between stripping peak
current and HCl supporting electrolyte
concentration

response was at its maximum in 0.1-0.3 M HCl, after which the sensitivity decreased with increasing acid concentration. However, the relationship shown in Figure 4.8. is consistent with the findings of the only other study of the cathodic stripping voltammetric behaviour of selenium(IV) at a mercury coated carbon fibre microelectrode [15]. In their work, Hua et al. [15] reported a linear increase of the selenium response with increasing acid concentration in the range 3-6 M HCl.

The increase in sensitivity of the stripping response is in accordance with the hydrogen ion dependence of the reduction of the mercuric selenide during the stripping step, as shown in equation 4.2.

In the work of Hua et al. [15], the carbon fibre electrode was part of a flow system and consisted of a carbon fibre fixed perpendicular to the tubular axis of a PVC tube. They reported that hydrochloric acid concentrations above 6 M could not be investigated because of the rapid deterioration of the carbon fibre sensor, probably caused by reactions between the PVC tube and the strong acid.

Since the microelectrode construction in the present investigation was shown to be resistant to acids at high concentrations [17], the choice of supporting electrolyte concentration used throughout this investigation was governed, not by structural considerations, but by the conditions normally required in any sample decomposition procedure required for selenium determination.

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In the determination of selenium, the use of 4-6 M HCl is recommended [21,22] to reduce selenium to its electroactive Se(IV) state subsequent to any sample decomposition procedure. Recent studies [18,19] involving the cathodic stripping voltammetric determination of Se(IV) at a hanging mercury drop electrode recommend a 0.1-0.3 M HCl electrolyte solution. Therefore, in order to carry out a cathodic stripping voltammetric analysis of selenium(IV) in such a sample digest, some degree of dilution to obtain the required 0.1-0.3 M hydrochloric acid electrolyte concentration is necessary. In this investigation, a 5 M hydrochloric acid concentration was chosen as this would approximate the final hydrochloric acid concentration in the recommended digestion procedures [18,19]. This would also have important consequences in any procedure with such electrodes involving a sample decomposition step. The levels of selenium present in biological materials is normally at trace levels and therefore it is of great importance to minimise sample handling. In contrast to the reported conditions for cathodic stripping voltammetric determinations at the hanging mercury drop electrode, the use of 5 M HCL as the supporting electrolyte in this study would eliminate any requirement for dilution of the final sample digest to a concentration optimal for voltammetric investigations, as is the case with macro-electrodes.

4.3.3. Influence of deposition potential

The conventional approach for the cathodic stripping voltammetric determination of selenium in acidic solution is based on the reduction of the electroactive Se(IV) to Se^{2-}, according to equation 4.1, by use of a deposition potential, E_d, between +0.05 V and -0.05 V vs. SCE [23]. However, as the determination of selenium at these deposition potentials is quite susceptible to interferences from other metal ions, the use of negative deposition potentials in the region -0.2 V to -0.4 V vs Ag/AgCl has been recommended [18] to reduce their effects. The effect of the deposition potential on the stripping response was investigated by varying the deposition potential between -0.2 V and -0.32 V.

The upper limit of the deposition potential range was governed by the behaviour of the mercury film. In the 5 M hydrochloric acid electrolyte, the mercury film begins to oxidise at potentials greater than -0.15 V, as shown in Figure 4.9.

Florence et al. [24] have reported that in chloride containing electrolytic media, care must be exercised in selecting the potentials to be applied to a mercury electrode. If the potential applied is too positive, then calomel formation can occur on the mercury surface. This may have quite adverse effects on the performance of the electrode and normally results in extremely irreproducible results. In the case of a hanging mercury drop electrode, this can be very easily circumvented by

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Peak current (nA)

-0.5   -0.1
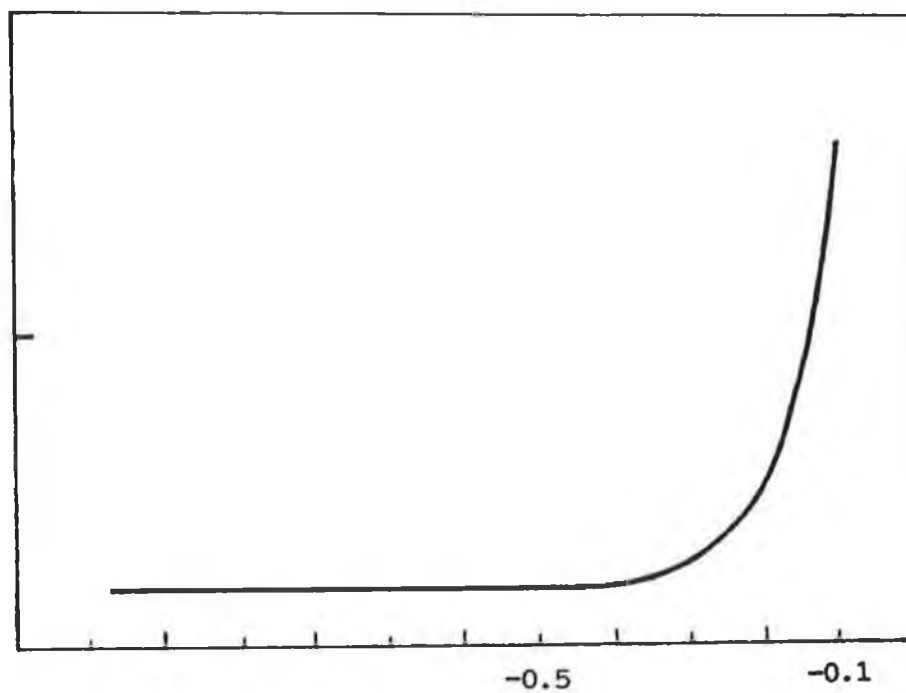
E, V vs Ag/AgCl

Figure 4.9. Oxidation of mercury film in 5 M HCl

supporting electrolyte

forming a new drop for each analysis. However, in the particular case of thin film mercury electrodes, first reported in the early 1970's by Florence and his co-workers, such an approach is not as applicable. The use of thin mercury films as electrodes involves the in-situ deposition of mercury onto a glassy carbon surface. This surface could then be used for stripping voltammetric determinations of various metal ions [24]. Florence reported that the optimal conditions for the use of such a film for voltammetric purposes involved the use of a single film for 7-10 successive stripping scans. The reproducibility exhibited in such a procedure was excellent. However, in the case where calomel formed on the surface of the film, such reproducibility was not possible and the mercury film had to be removed and a new film formed, thus complicating the analytical procedure.

As the carbon fibre surface used in the present investigation is structurally similar to the glassy carbon surface used in the formation of thin film mercury electrodes, similar care must be exercised in control of the potentials applied to the mercury electrode.

The relationship between the deposition potential, E_d, and the stripping response, as shown in Figure 4.10., shows a decrease in the sensitivity of the response as the potential is varied from -0.2 V to -0.32 V. A potential of -0.2 V was selected for deposition of selenium(IV) throughout this study.

In previous work involving cathodic stripping voltammetry at hanging mercury drop electrodes, Van den Berg et al.[25] and
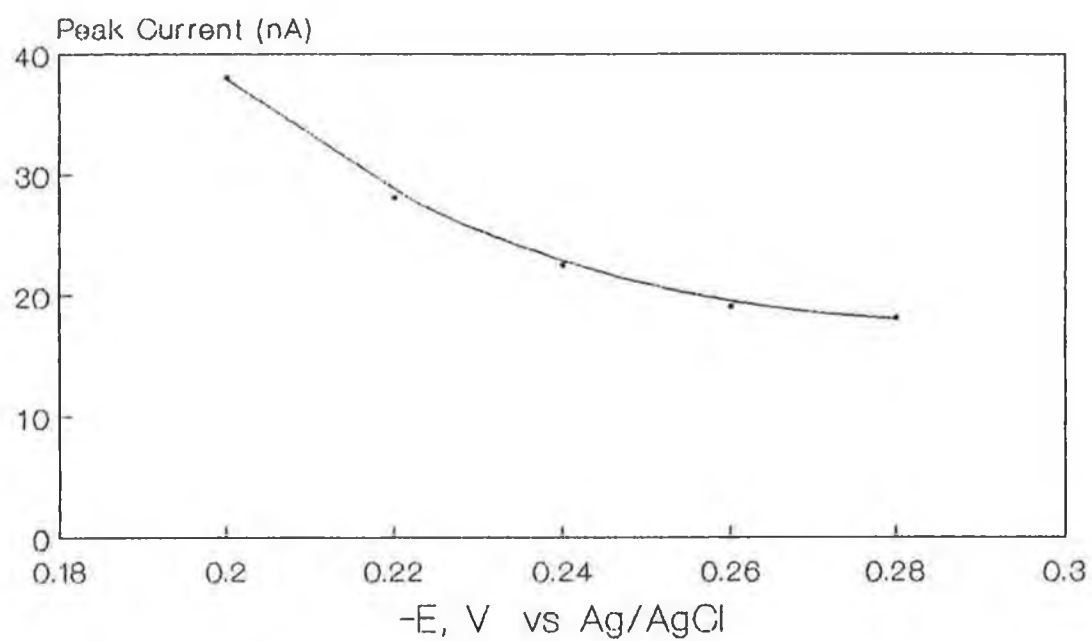
Figure 4.10. Relationship between deposition potential and the stripping response

Bond et al. [18] have reported the use of −0.4 V and −0.3 V respectively for the deposition of selenium(IV). The finding that a potential of −0.2 V in this study gave the most sensitive stripping response may be due in part to the high rates of mass transport exhibited by micro- electrodes and is not entirely, as is the case with the hanging mercury drop electrode, governed by a specific deposition potential at which the formation of mercuric selenide is optimal.

Wang et al. [10] have reported that during the anodic stripping voltammetric determination of cadmium at mercury coated carbon fibre electrodes, substantial plating of cadmium occurred during the scanning period from the deposition potential to the peak potential. This continued plating effect became more pronounced for larger differences between the deposition and stripping potentials and was attributed to the nonlinear diffusional mass transport characteristics of microelectrodes. This was also shown to be the case by Schroeder [7] who reported that the results of stripping experiments at micromercury drops showed that very high concentrations of metal ions were obtained in the drop simply during the scan time period.

The concentration of the metal in the amalgam, C_a, can be written as;

$$C_a = \frac{i_L \left(t_d + \dfrac{[E_d - E_p]}{v} \right)}{nFV} \qquad [4.3]$$

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where t_d and E_d are the deposition time and potential respectively, E_p is the peak potential, v is the scan rate, and V is the volume of the mercury electrode [9]. The limiting current for the deposition of the metal, i_L, may depend upon whether the microelectrode is a hemisphere, microcylinder or a microband.

The term $(E_d - E_p)/v$ in equation 4.3 represents the scanning period during which plating occurs. Deposition of selenium(IV) at a hanging mercury drop electrode is carried out in stirred solutions, whereas the stripping scan is performed in quiescent solutions. Therefore, since the deposition process is controlled by convective transport, plating of the metal can occur only during the deposition step and not during the scan period. Consequently, the optimal potential for deposition will be that potential where mercuric selenide is most favourably formed. In contrast, deposition of metal ions on mercury coated carbon fibre electrodes is by nonlinear diffusional mass transport and can therefore be carried out in quiescent solution. Since the formation of the mercuric selenide can occur over the potential range from +0.05 V to -0.4 V, the optimal potential for deposition is now additionally governed by the scan period. Therefore, in Figure 4.10., it can be seen that as the difference between the peak potential, -0.36V, and the deposition potential increases, the stripping peak current increases.

The deposition of selenium(IV) is therefore dependent on the

inter-relationship of parameters such as the deposition potential, E_d, the deposition time, t_d, the scan rate, v, and the pulse width.

4.3.4. Effect of scan rate

The effect of the scan rate upon the stripping peak current was examined using a 50 ng/ml Se(IV) solution in 5 M hydrochloric acid electrolyte, a -0.2 V deposition potential and a series of scan rates in the range 5 to 30 mV/s.

The resulting relationship, shown in Figure 4.11., exhibits a linear increase of peak current with scan rate, with a correlation coefficient of 0.999 and an intercept of 6.6 nA.

The linear dependence on scan rate indicates that a diffusion-controlled rate determining step is present in the stripping process at the mercury coated carbon fibre electrode. The relatively large intercept obtained can be attributed to the continued deposition of selenium(IV) as the potential is scanned from the deposition potential to the peak potential, while the solution remains quiescent during both the deposition and stripping steps. This continued plating of metal ions during the scan period has also been reported for the determination of lead by means of anodic stripping voltammetry at mercury films deposited on ultrasmall carbon-ring electrodes [14].

Despite the fact that a scan rate of 30 mV/s allowed greater sensitivity, a scan rate of 10 mV/s was used throughout the
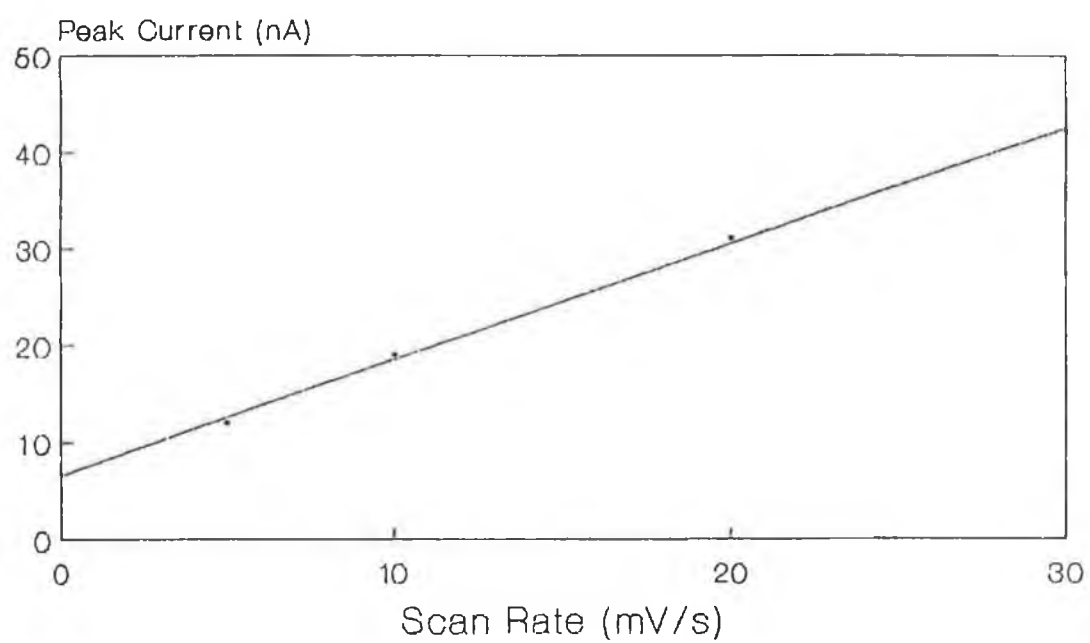
125

Figure 4.11.   Influence of scan rate on the cathodic

stripping response

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investigation as this allowed well defined peaks to be obtained.

4.3.5. Influence of pulse width

The influence of the pulse width upon the stripping peak current
was determined using a 50 ng/ml Se(IV) solution in 5 M hydro-
chloric acid electrolyte, a deposition potential of -0.2 V, a
scan rate of 10 mV/s and varying the pulse width between 10 mV
and 80 mV.

A plot of pulse width versus peak current, shown in Figure
4.12., results in a curved relationship, with an almost linear
increase of peak current with pulse width up to 60 mV after
which there is no significant increase in the stripping
response. A similar relationship was reported by Wang et al.
[10], during an evaluation of the usefulness of differential
pulse anodic stripping voltammetry at mercury coated carbon
fibre electrodes for the determination of lead and cadmium.

A pulse width of 60 mV was selected for use in this study as it
allowed the best sensitiviy to be achieved, whilst still
retaining the peak definition.

During differential pulse stripping voltammetry, it is an
accepted fact that some of the metal stripped from the electrode
during the pulse is replated onto the electrode in the waiting
period between pulses. Due to the greatly increased rates of
mass transport at microelectrodes, such replating of the metal
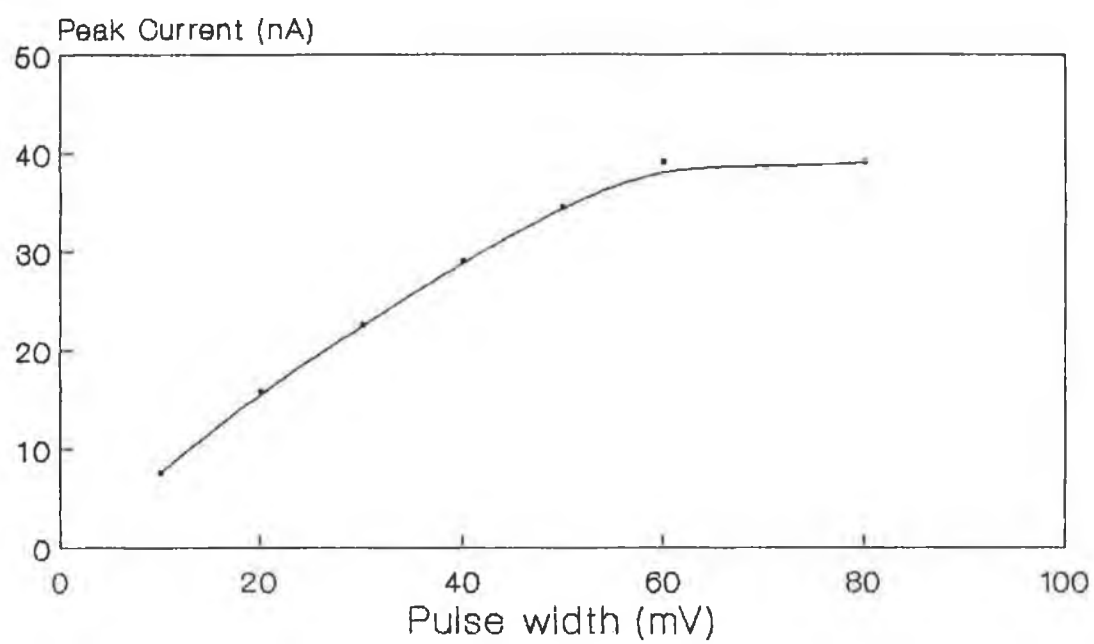between pulses is substantially magnified [10].

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Figure 4.12. Effect of pulse width on stripping

peak current

4.3.6. Formation of mercury film

In the early 1970's Florence reported [24] on the use of thin mercury films for the anodic stripping voltammetric determination of various metals. In the initial stages of this work it was found that the reproducibility of the results was inferior to that at the hanging mercury drop electrode. This irreproducibility was attributed almost entirely to the preliminary step of depositing mercury on platinum and nickel. There was considerable difficulty in preparing mercury films of consistent and uniform thickness and even more difficulty in preserving them in an active state for any length of time. In addition, platinum and nickel have strongly adherent surface oxide films, low hydrogen overpotentials, and a finite solubility in mercury.

In an effort to overcome these problems, glassy carbon was used as a substrate for film formation. It was found that by adding mercuric nitrate to the sample solution and depositing at a potential where both the mercury and metals could be deposited simultaneously, a very thin film, of the order of 0.001-0.01 um thickness, could be formed from which the metals could be stripped anodically. This approach offered excellent sensitivity and superior reproducibility to other anodic stripping methods. The use of carbon fibres as a substrate for the formation of thin mercury films was a logical extension of this approach in order to allow the particular properties of microelectrodes to

benefit voltammetric investigations at mercury electrodes.

Golas and Osteryoung [26] have reported that the number of active sites at which mercury(II) can be reduced depends strongly on the potential used for film formation, with an increase in the apparent film thickness being observed with increasing negative potential.

4.3.6.1. Effect of mercury plating concentration

The reduction of mercury(II), shown in Figure 4.13., was studied by carrying out cyclic voltammetry of 1×10^{-4}M Hg(II) in 5 M hydrochloric acid electrolyte at a scan rate of 100 mV/s. There was no significant degree of Hg(II) reduction until the potential reached approximately -0.8 V, after which the degree of reduction became much more significant. During the reverse scan the degree of oxidation of the deposited mercury was not significant until -0.05 V, where the mercury was rapidly oxidised to give a correspondingly sharp peak in the cyclic voltammogram. It was noted at this stage, that if multiple cyclic voltammograms were carried out, the degree of noise on each successive voltammogram increased. This was attributed to the formation of calomel on the carbon fibre surface at the peak potential of the mercury oxidation peak. This is in accordance with the findings of Florence [24], as mentioned previously in section 4.3.3.

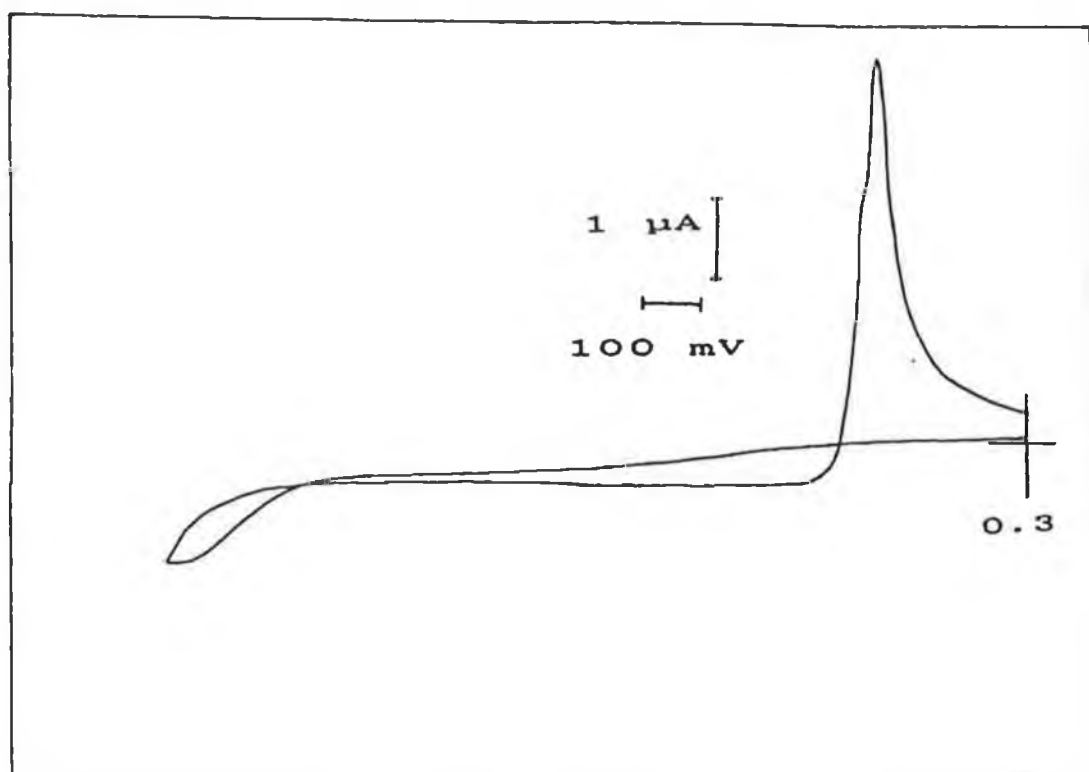In this work, the selenium stripping response obtained on

Figure 4.13. Cyclic voltammetry of mercury (II) in

5 M HCl supporting electrolyte

(scan rate 100 mV/s)

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mercury films formed at negative potentials, e.g. -1.2 V, was superior to that obtained on films formed at more positive potentials, e.g. -0.2 V. On the basis of this qualitative observation, mercury film formation was performed by applying a potential of -1.2 V in 5 M hydrochloric acid electrolyte containing Hg(II). Although deposition appears to be diffusion-controlled at each potential [8], the number of active sites where mercury can be reduced depends strongly on the applied potential. Despite the fact that the term "film" is widely used in the literature to describe mercury deposits on carbon surfaces, the deposit is in fact an array of droplets; each droplet being formed by the reduction of mercury at a particular active site. It seems likely that at the more positive potentials, mercury is deposited only on the most active sites of the carbon fibre, whereas at the more negative potentials the number of active sites and hence the number of mercury droplets is greater. Consequently, a much greater surface coverage by mercury of the carbon fibre occurs leading in turn to a superior stripping response.

For the in situ formation of the mercury film, mercury was added to the electrolytic medium in the form of mercury(II) chloride. The relationship between the mercury plating concentration and the selenium stripping response, shown in Figure 4.14., was investigated in 5 M hydrochloric acid containing 5 ng/ml Se(IV) and variable concentrations of Hg(II), by performing a mercury deposition step at -1.2 V for 120 seconds, a selenium deposition
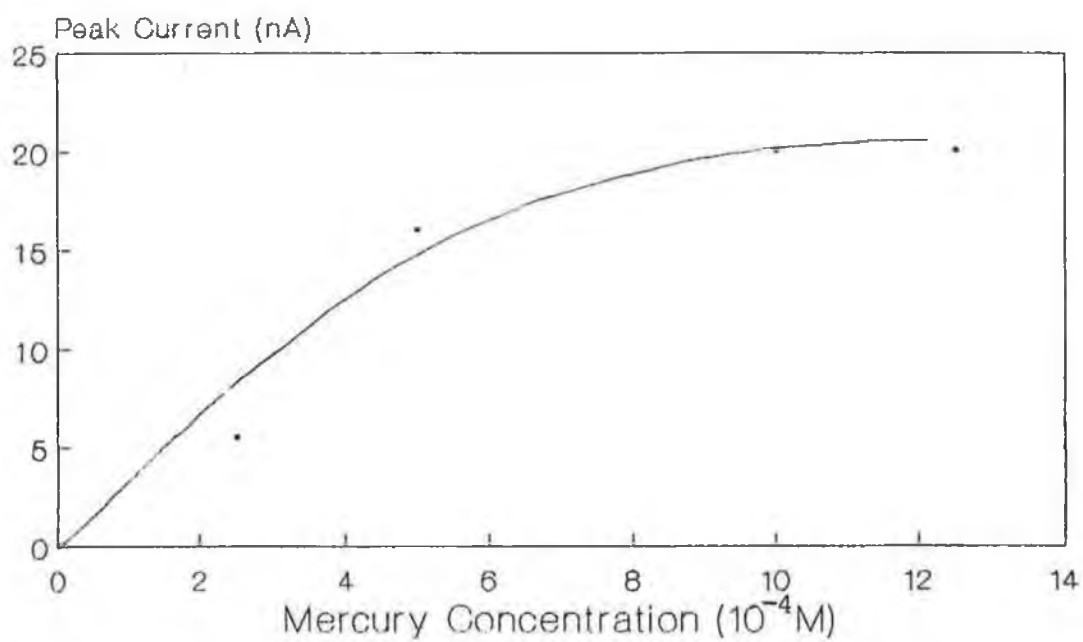
Figure 4.14.   Relationship between mercury plating
              concentration and selenium stripping
              response

step at −0.2 V, followed by a cathodic stripping scan. The stripping peak current increases with the mercury concentration up to a value of 1×10^{-4}M, above which there was no significant increase. The plateau in the plot would most likely correspond to the situation on the carbon fibre surface where mercury had been deposited on all the available active sites. A mercury plating concentration of 1×10^{-4}M was chosen for all further work.

4.3.6.2. Influence of mercury plating time

The influence of the mercury plating time upon the stripping response was investigated using the same conditions as above with a mercury plating concentration of 1×10^{-4}M. The plating time was varied over the range 0 − 420 seconds and the resulting relationship with the selenium stripping peak current is shown in Figure 4.15. There was a significant increase in the sensitivity of the response with plating time up to approximately 120 seconds after which there was no further improvement in the signal. As before, the plateau region in the relationship is probably indicative of the fact that all available sites for mercury droplet formation had been occupied. It is, however, interesting to note that the reproducibility of successive selenium stripping measurements for plating times in excess of 200 seconds was less than that for lower plating times. A possible explanation for this behaviour may lie in the
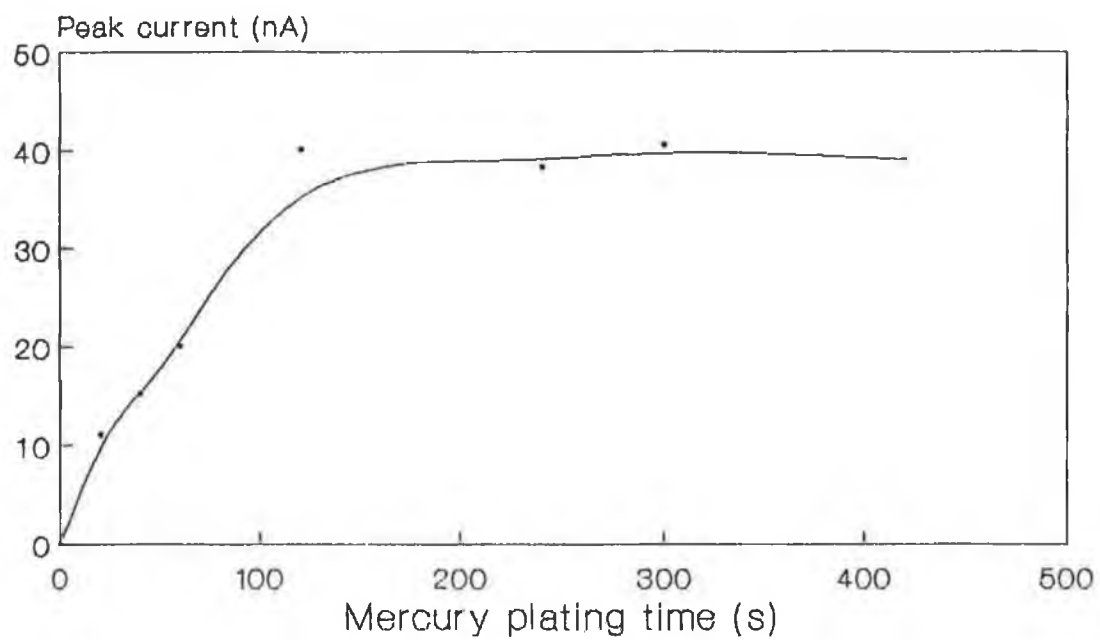
134

Figure 4.15.   Influence of mercury plating time on

selenium stripping response

nature of the film on the carbon fibre surface. The droplet array of mercury on carbon surfaces has been very well documented [24,26]. In this case, as the plating time increases, the number of active sites which become involved in droplet formation increases up to approximately 120 seconds. After this, further deposition of mercury will occur on previously formed droplets, therefore resulting in an increase in the droplet size; in effect, the film thickness increases. However, if plating continues for longer time periods, e.g. in excess of 200 seconds, then the probability that the droplets will become too large and consequently fall off the carbon fibre surface increases. In this situation, it would prove impossible to maintain a reproducible mercury film, and consequently the reproducibilty of the selenium stripping response would be adversely affected. As previously mentioned, the difficulties in obtaining reproducible results during the initial stages of the development of thin mercury film electrodes on glassy carbon was due in the main to an inability to maintain a reproducible film coverage. Golas and Osteryoung [26] have also pointed out that at higher deposition times, and hence higher surface coverages, the reproducibilty of stripping peaks for mercury were worse than for lower surface coverages.

In this investigation, a mercury deposition time of 150 seconds was chosen for all further work.

4.3.7. Influence of deposition time

The influence of the deposition time upon the stripping peak
response was investigated using a deposition potential of -0.2
V, a scan rate of 10 mV/s and 1, 2, and 5 ng/ml Se(IV) solutions
in 5 M hydrochloric acid electrolyte. The resulting relationship
is shown in Figure 4.16.

The accountable electrolysis time in stripping analysis includes
the deposition time and the equilibration period. However, in
the case of microelectrodes, at the slow scan rates normal in
differential pulse stripping measurements, there is no distinct
"borderline" between the deposition and stripping steps, thus
obviating the need for an equilibration period between the two
[9]. Therefore, in the case of mercury coated microelectrodes,
the accountable electrolysis time consists solely of the
deposition time period.

From the relationship shown in Figure 4.16., it can be seen that
there are quite significant non-zero intercepts, even at very
low concentrations. This is in contrast to the behaviour which
is normally observed at the hanging mercury drop electrode,
where at low concentrations in the region of 2 to 10 ng/ml the
observed peak current is directly proportional to the
electrolysis time.

This behaviour at the microelectrodes can be attributed to the
deposition of metal ions during the scan period from the
deposition potential to the stripping peak potential, due to the

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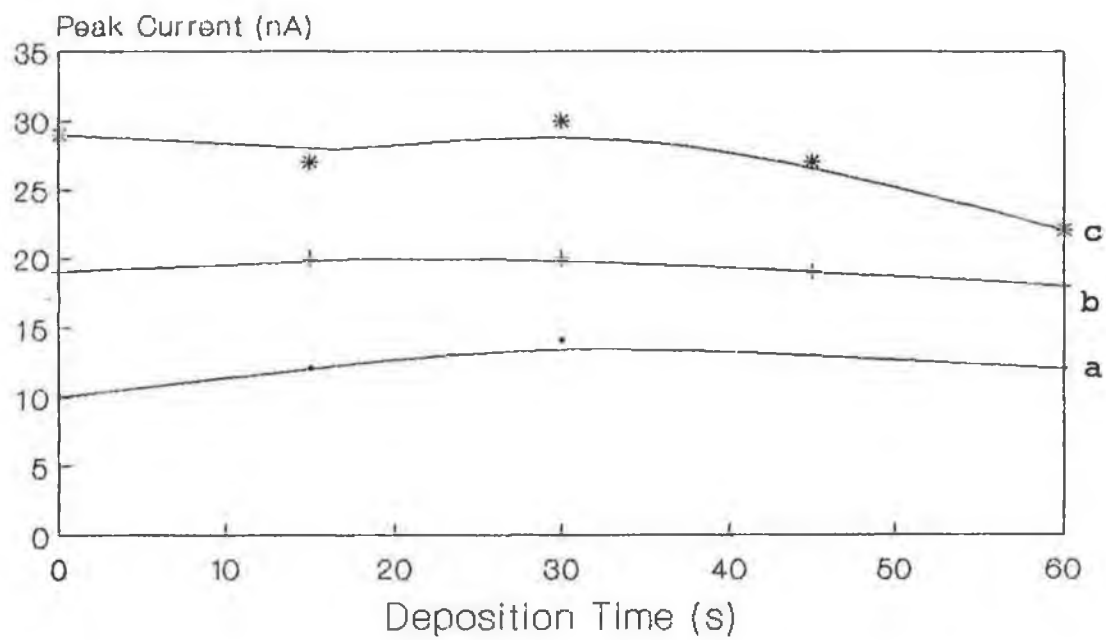
Figure 4.16.   Deposition time dependence of selenium

stripping peak current

(a) 1 ng/ml Se(IV)

(b) 2 ng/ml Se(IV)

(c) 5 ng/ml Se(IV)

nonlinear diffusional mass transport characteristics exhibited by these electrodes.

This "continued plating" effect, as previously mentioned in relation to the deposition potential and scan rate, has important consequences in the development of a stripping voltammetric procedure at these electrodes. Firstly, the ability to carry out deposition in a quiescent solution eliminates any potential sources of error which may otherwise arise under the convective transport conditions normally required for the deposition process at macro-electrodes. In addition, as there is significant deposition of selenium without any specific deposition time period, the necessity to control a time parameter can be eliminated, and as a consequence any associated errors.

As the deposition time enhancement of the selenium stripping signal was confined to short time values, no specific deposition time period was employed in this investigation. This not only circumvents any of the possible errors mentioned above, but also speeds up and simplifies the overall stripping voltammetric procedure.

4.3.8. Investigation of mercury film formation conditions

The nature of the mercury film on the carbon fibre electrode surface is of fundamental importance to the sensitivity and reproducibility of the overall stripping voltammetric procedure. As previously mentioned in section 4.3.6, the concentration of mercury and the mercury deposition time used are of critical importance in ensuring that the correct degree of surface coverage is achieved.

However, the approach taken in film formation has also a major bearing upon the analytical performance of the technique. The formation of mercury films on carbon surfaces can be achieved by two means; either by predeposition or in situ deposition of mercury.

4.3.8.1 Predeposition of mercury film.

Predeposition of a mercury film on the carbon fibre surface was achieved using 0.1 M sodium acetate, pH 4.5, containing 1 x 10^{-4} M Hg(II) and applying a potential of -1.0 V for 120 seconds, subsequent to which the mercury coated electrode was removed from the electrolyte and transferred to the 5 M hydrochloric acid electrolyte in which the stripping voltammetry was performed. The results obtained however were extremely irreproducible and there was quite considerable variation in the sensitivity exhibited by successive predeposited films. Baranski

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has reported [27] on the use of carbon fibre electrodes with predeposited mercury films for the anodic stripping voltammetric analysis of lead. It was found that good reproducibility could be achieved in some measurements, whilst in others a significant drop in electrode sensitivity between replicate measurements was observed. Similarly, Batley and Florence have reported [28] that pre-formed mercury films on glassy carbon gave results of inferior precision to those obtained using in situ deposited films when applied to the anodic stripping voltammetry of cadmium and lead. They concluded that there was no advantage in incorporating in the analytical method the additional step of pre-forming a mercury film on the glassy carbon surface and recommended the use of in situ formed films.

The problems associated with predeposition of the mercury film stem largely from the fact that carbon is not wetted by mercury and consequently mercury films on carbon surfaces are unstable. Golas and Osteryoung [26] have reported that when a carbon fibre electrode with a deposit of mercury on it was exposed to air for longer than 2 minutes, the stripping peak obtained when the mercury was removed electrochemically decreased significantly, In a typical experiment, the peak height decreased to 27% of its initial value after exposure to air for 6 minutes, and to 8% after 2 hours. The air oxidation of mercury to form mercury oxides is strongly favoured thermodynamically. Although clean, dry mercury metal undergoes this reaction very slowly, the freshly deposited mercury dispersed on the fibre may be oxidised

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more rapidly. It is also possible that the mercury film may be partially stripped mechanically as the electrode is taken out of and put into solution [27].

Due to the disruption of the integrity of the mercury film during the transfer process by either of the above or a combination of the above reasons, the predeposition of mercury films does not represent a reproducible approach to stripping voltammetric measurements.

In an effort to circumvent the aforementioned disruption of the mercury film, in situ deposition was therefore examined.

4.3.8.2. In situ formation of mercury film

Th basis of the in situ formation of mercury film involved the deposition of mercury on to the carbon fibre surface at a potential of -1.2 V for 150 seconds in the 5 M hydrochloric acid electrolyte containing 1×10^{-4} M Hg(II), immediately after which the stripping voltammetric procedure was carried out in the same media. Several variations upon this approach were evaluated in order to identify a method which allowed the sensitive and reproducible determination of Se(IV).

The first approach involved the formation of the mercury film using the conditions mentioned above. Subsequent to film formation, 50 ng/ml Se(IV) was added to the electrolyte and a cathodic scan carried out from -0.2 V to -0.5 V at a scan rate of 10 mV/s with zero seconds deposition time. Immediately after

the cathodic stripping scan, the electrode was removed from the film formation/stripping electrolyte and was rinsed with distilled water to ensure all traces of hydrochloric acid were removed from the electrode prior to being transferred to 0.1 M sodium acetate, pH 4.5, in which the mercury film was removed anodically.

Having completed this, the "clean" electrode was removed from the electrolyte, washed in distilled water and then placed in a new film formation/stripping media in which the procedure for mercury film deposition and stripping voltammetric measurements were performed as before.

This procedure of film formation, stripping determination and film removal was repeated for each stripping measurement and is analogous to the formation of a new drop in procedures involving the hanging mercury drop electrode. However, considering that the electrolytic media must be deaerated each time, coupled with the need to change media for film removal and the associated care in washing the electrode during the transfer process, means that this approach is extremely time consuming. In addition, although the carbon fibres are in fact quite rugged despite their small dimensions, there is the risk of breaking the fibre by accidentally touching it with the wash bottle or against any of the electrochemical apparatus.

Despite this, however, the method proved to be quite reproducible with a typical RSD of 7% (n=5) at the 50 ng/ml Se(IV) level. This is an indication that the mercury film was

uniform in nature for each analysis, as the possibility of any mechanical stripping or air oxidation of the mercury deposit had now been eliminated.

In an effort to reduce the time required for the overall analysis process, a variation upon the above procedure was examined. This involved, as above, the _in situ_ formation of the mercury film followed by the stripping voltammetry of selenium. However, in contrast to the first procedure, mercury film removal was carried out in the stripping medium and not, as before, in a separate solution specific for the removal step; in effect this represented an "_in situ_" film removal step. This therefore circumvented the need for deaeration of the electrolytic media prior to each analysis and also any risk of damaging the fibre during a washing/transferring process.

The first stripping scan obtained showed excellent sensitivity for 50 ng/ml Se(IV) and was similar to the results of the previous procedure. However, after removal of the film and the formation of a new one, the subsequent stripping signal showed a considerable degree of baseline noise. As the procedure was repeated, the signal deteriorated and the results became irreproducible.

This finding is in accordance with those of Wojciechowski _et al_. [29] who reported on the use of square wave anodic stripping voltammetric measurements at mercury coated glassy carbon electrodes. In the reported procedure, _in situ_ formation of the film was carried out in 0.1 M hydrochloric acid containing 1 mM

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Hg(II). After film formation and the subsequent anodic stripping scan, the mercury film was removed "in situ" by applying a potential of +0.4 V. However, stripping voltammograms recorded after this step were distorted by frequent and irreproducible current spikes. This behaviour was attributed to the presence of calomel on the electrode surface, which was formed during the film removal step, and was not completely reduced to mercury during the next film formation step. The removal of calomel from a glassy carbon surface can be easily achieved by simply wiping the surface with a damp tissue. However, in the case of carbon fibre electrodes this cannot be considered because of their fragile nature. This therefore meant that once calomel had formed on the carbon fibre surface, the electrode in effect became redundant.

In an attempt to combine the reproducibility of the first procedure with the reduced time and simpler experimental protocol of the above method, another approach, based on the work of Florence et al. [24] with mercury coated glassy carbon electrodes, was taken.

In this approach, in situ film formation was carried out as before, for 150 seconds at -1.2 V in 5 M hydrochloric acid containing 1 x 10^{-4} M Hg(II), after which a stripping voltammetric scan was performed. After completion of the scan, the electrolyte was stirred briefly and allowed to quiesce for 10 seconds after which another stripping voltammetric scan was carried out. This procedure could be performed for up to seven

to 10 successive scans with a typical RSD of 2.7% for 50 ng/ml Se(IV) (n=7). The sensitivity of the stripping scan began to deteriorate slightly after seven to ten scans after which it deteriorated significantly. The mercury film was then removed by placing the electrode in 0.1 M sodium acetate, pH 4.5, and applying a potential of +0.3 V for 20 seconds.

In this approach, the same mercury film was used for a number of successive scans, which is similar to the approach adopted by Florence et al. [24] in their work involving thin mercury films on glassy carbon electrodes. The absence of a mercury film removal/formation step between each scan means that the stripping voltammetric procedure is speeded up and simplified considerably.

However, as explained in section 4.3.6.2., the amount of mercury deposited on the fibre can affect the droplet size and consequently the reproducibility of the Se(IV) stripping measurements. According to the cyclic voltammetric behaviour of Hg(II) in 5 M hydrochloric acid, as shown in Figure 4.13., the degree of mercury reduction is relatively small in the potential region from −0.2 V to −0.5 V in comparision to that at more negative potentials; if the current flow, as a result of the reduction process, is compared, then the degree of mercury reduction in the region of −0.2 V to −0.5 V is approximately 7% of that at −1.2 V. Consequently, despite the fact that some degree of mercury deposition will occur, the thickness of the mercury film should remain relatively constant during a number

of successive scans from -0.2 V to -0.5 V. Further evidence for this was provided when the simultaneous deposition of mercury and Se(IV) was attempted at -0.2 V. In the resulting cathodic scan, no stripping signal for Se(IV) was recorded, showing that the degree of film formation at such potentials was not sufficient to enable a significant quantity of Se(IV) to be deposited in the form of mercuric selenide.

The deterioration in the stripping signal after 7 - 10 scans may be due to the fact that the film becomes disrupted with the application of an increasing number deposition/stripping cycles.

By adopting this approach, a linear working range from 0 - 3 ng/ml could be obtained, as shown in Figures 4.17 and 4.18, with a detection limit of 0.11 ng/ml and a precision, expressed as relative standard deviation, of 4.4% for 5 ng/ml Se(IV) (n=9).

Typically, as mentioned above, between 7 - 10 scans could be performed on a single mercury film before film removal proved necessary and normally up to forty mercury films could be formed on a carbon fibre before a decrease in the sensitivity of the measurements necessitated a change. This decrease in the sensitivity is probably due to the number of active sites on the electrode surface being reduced. In the case of glassy carbon electrodes, such a situation is overcome by carrying out electrochemical pretreatment of the surface to regenerate the active sites. This however adds another step to the analytical procedure. Since carbon fibres are very inexpensive, it is much more practical to omit such a step by replacing the electrode.
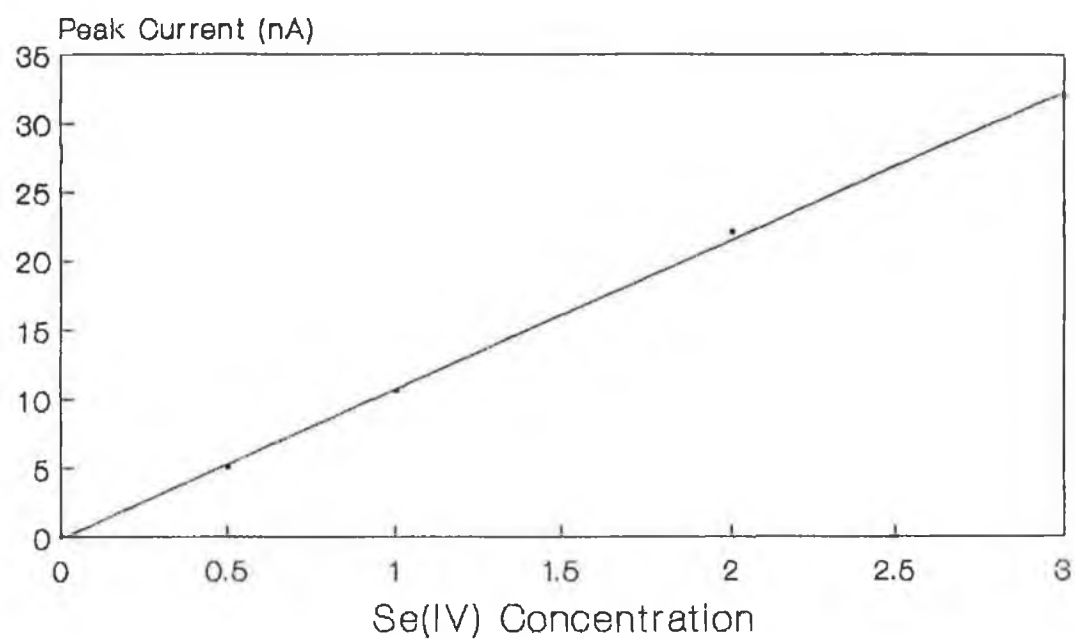
147

Figure 4.17.   Relationship between Se(IV) concentration
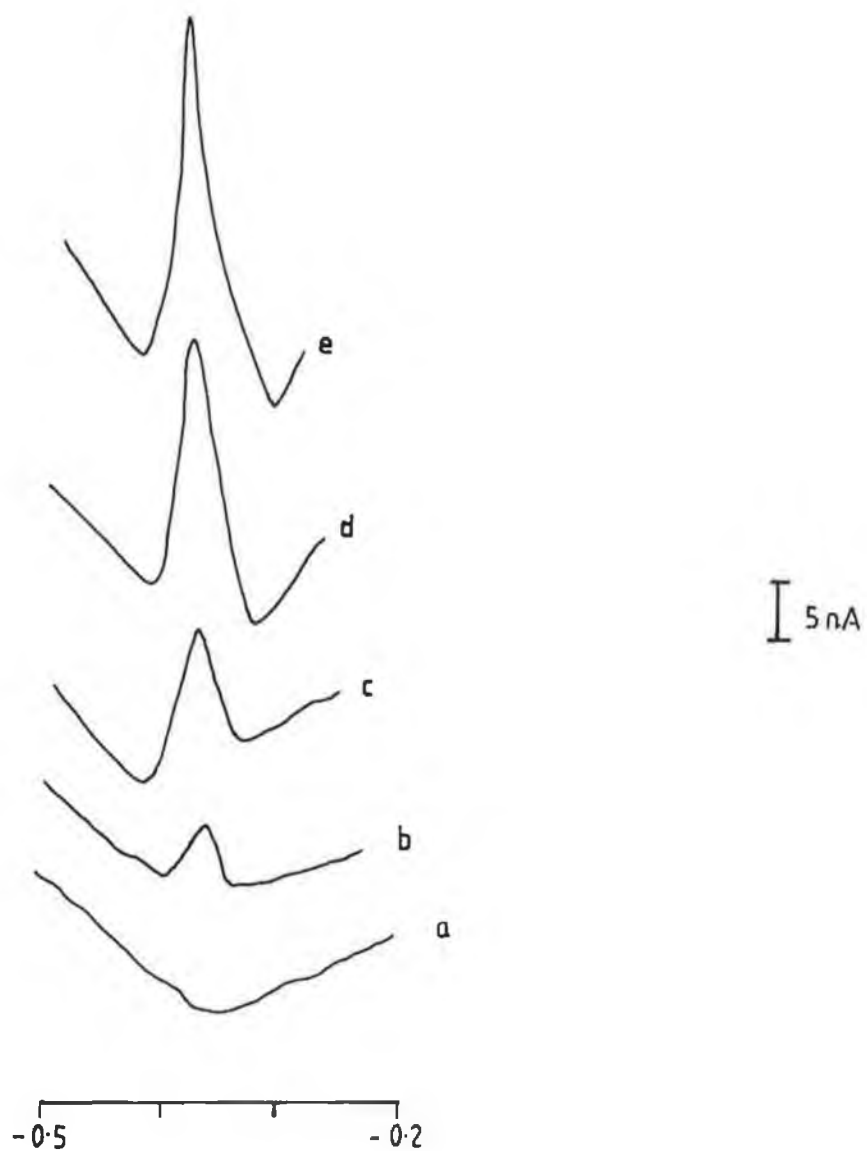and the stripping response over linear
range

Figure 4.18. Typical cathodic stripping voltammograms
for Se(IV) concentrations in the linear
range (a) blank (b) 0.5 ng/ml (c) 1 ng/ml
(d) 2 ng/ml (e) 3 ng/ml Se(IV)

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4.3.9. CONCLUSION

Cathodic stripping voltammetry on a mercury electrode is now a well established and accepted method for the sensitive and reproducible analysis of Se(IV). The vast majority of work reported upon this technique deals almost exclusively with the hanging mercury drop electrode, with the approach of Hua et al. [15] involving mercury-coated carbon fibre electrodes being the only example of a new variation upon the theme.

The use of carbon fibres as substrates for the deposition of mercury and consequently for voltammetric measurements has been widely recognised. The particular properties of these electrodes offer the possibility of extending the scope of stripping voltammetric techniques.

Normally, the cathodic stripping voltammetry of Se(IV) at a hanging mercury drop electrode involves a deposition time period in stirred solution, followed by a cathodic stripping scan in quiescent solution. However, in the case of mercury coated carbon fibres, the greatly increased diffusional mass transport properties, characteristic of such electrodes, results in a behaviour which differs from that expected at the conventional macroelectrode.

These diffusional mass transport characteristics permit the use of quiescent solutions throughout the complete voltammetric procedure, thus eliminating any potential errors which may occur because of the difficulties involved in achieving reproducible

convective transport conditions; the reproducibility of the convective transport process is recognised as a major source of error in stripping voltammetric techniques. In addition, the equilibration step may be eliminated with use of micro-electrodes, thus allowing the speeding up of the procedure whilst obviating the need for a time controlled step and consequently any associated errors.

The interrelationship of parameters such as the deposition potential, deposition time and the scan rate is governed to a great degree by the mass transport properties. At a macro-electrode, the stripping procedure is dependent upon convective transport conditions during the deposition process and quiescent conditions during the stripping process; in effect, accumulation of metal ions is confined to a specific time period and potential whilst the solution is stirred, after which no further deposition is possible. However, as the mass transport characteristics of microelectrodes allow deposition to be carried out in quiescent solution, accumulation of metal ions is not confined by the above potential and time constraints. In the case of Se(IV), deposition of the metal ions occurs over the complete range of potentials between -0.2 V and -0.32 V. Whilst the use of a deposition potential of -0.2 V allows an optimum sensitivity for the stripping response, it is in fact the cumulative deposition of the analyte of interest over this range of potentials. It is therefore probably more correct to refer to the deposition potential as the deposition potential range and

-0.2 V as the initial potential.

This deposition process over a range of potentials, or the so-called "continuous plating" effect, has a marked influence upon one of the most critical parameters in stripping voltammetric procedures, i.e. the deposition time. At macro-electrodes it is the length of this time period which has a direct effect upon the ultimate sensitivity of the technique. Normally, accumulation times up to five minutes are recommended for ultratrace determinations. However, at microelectrodes the significant plating of analyte which occurs simply during the scan period means that a stripping signal can be obtained at zero seconds deposition time for extremely low levels of Se(IV), a situation which would be impossible at a hanging mercury drop electrode. The elimination of a specific deposition time period simplifies the overall procedure and also circumvents any errors which may result from the associated time keeping.

The detection limit of stripping methods can only be discussed in relative terms since it is inversely proportional to the deposition time period. The detection limit of 0.12 ng/ml, with a zero second deposition time, reported here compares with previously reported detection limits of 0.1 ng/ml [20] and 0.25 ng/ml [18], at deposition times of 210 seconds and 60 seconds respectively. Van den Berg et al. [25] have reported a detection limit of 0.004 ng/ml, with a 180 second deposition time, for an adsorptive cathodic stripping voltammetric procedure involving the adsorption of a $Cu(I)_2Se$ complex on the mercury electrode

surface. This detection limit is the lowest reported for the voltammetric determination of Se(IV) and is of obvious advantage in the determination of Se(IV) in those matrices where it is present in extremely low quantities; e.g. sea-water, in which Se(IV) may be present in sub ppb levels.

In the vast majority of biological matrices, Se(IV) is normally present at levels greater than 1-2 ng/ml and as such any of the reported voltammetric techniques [18,20,31] have found application. In the case of blood plasma and serum, the volume of sample may be quite limited; particularly so for young children. In these instances, Zeeman graphite furnace atomic absorption spectroscopy has found widespread use. This has been due in large to the fact that microlitre sample volumes are required for analysis. In order for electrochemical techniques to provide this same capability, a detection system requiring small sample volumes is important. Such a system, involving carbon fibre electrodes, has been reported by Baranski and Quon [13]. As previously stated in section 4.1.5., a sample volume of the order of 5-ul is required in this system. This is comparble with the volumes used in the atomic absorption spectrometric approach and has the added advantage that the sample is not destroyed during the analytical procedure.

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Chapter 5

The determination of selenium in blood plasma
and serum by flow injection hydride generation
atomic absorption spectrometry

5.1 <u>INTRODUCTION</u>

The process of determination of volatile hydride-forming elements by atomic absorption spectrometry was initially introduced in the late 1960's by Holak [1] to overcome the problems associated with flame atomic absorption spectrometric, AAS, determination of arsenic. In subsequent years, the method has been applied to virtually all the hydride-forming elements, namely arsenic, antimony, bismuth, germanium, lead, selenium, tellurium and tin, and, especially in the case of arsenic and selenium, is a widely recognised method of analysis.

The popularity of hydride generation AAS arises for several reasons, with the major one being concerned with the principle of the method. This involves the separation and preconcentration of the analyte from the sample matrix, resulting in the enhanced selectivity and sensitivity of the technique. One important consequence of the analyte separation from the matrix is the suppression of interferences during the atomisation step. However, the method is not immune from the effects of interferents, particularly during hydride evolution from the sample. Another reason underlying the widespread use of the technique is its relative simplicity and low cost of apparatus.

Hydride generation atomic absorption spectrometry involves two completely independent steps: hydride release and hydride atomisation.

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5.1.1 Hydride release

Several agents have been used for the conversion of analyte to hydride, but the most convenient, and currently most widely used, is sodium borohydride. This is used in the form of pellets or, more commonly, in the form of a solution stabilised with either sodium or potassium hydroxide.

Hydrochloric acid is most commonly employed for acidification purposes, but recently it has been shown that selenium hydride can be generated from nitric and sulphuric acid solutions as efficiently as from hydrochloric acid, provided potassium iodide is added to the borohydride solution [2].

Under optimum conditions, and in the absence of a matrix, it can be assumed that the release of hydride from solution approaches 100% efficiency. This has been convincingly substantiated in the case of selenium by use of a ^{75}Se radiotracer [2]. Most authors have reported that, under optimum conditions, selenium hydride is released with an efficiency of 95% or better. Linear calibrations down to the pg/ml range have been reported for selenium, suggesting that the release efficiency does not deteriorate down to these levels [2].

5.1.2. Methods of hydride generation

After generation of the hydride, atomisation can be carried out in one of several ways. The following atomisers are presently in use:

(1) inert gas-hydrogen diffuse flames;

(2) externally heated quartz tube;

(3) graphite furnaces;

(4) flame in quartz tube.

5.1.2.1. Inert gas-hydrogen diffuse flames

The flames commonly used in atomic absorption spectrometry are not ideally suited to the determination of selenium since they absorb strongly in the far ultraviolet region; a 60% background absorption was observed when using an air/acetylene flame as an atom resevoir. Argon/hydrogen and nitrogen/hydrogen diffusion flames are relatively transparent in this region of the spectrum, as the hydrogen burns with the entrained ambient air only in the outer flame zone. They are, however, subject to severe chemical interferences, owing to their relatively low temperature. Nevertheless, hydrogen diffusion flames have been by far the most widely employed flame sources in hydride generation atomic absorption spectrometry systems.

5.1.2.2. Externally heated quartz tube

Heated quartz tubes are frequently preferred to flames as atom resevoirs for hydride generation, as their use permits lower background absorption than hydrogen diffusion flames, and gives longer residence times in the optical path.

Two methods have been employed for heating quartz tubes:

  (1) flame heating;

  (2) electrical heating.

5.1.2.2.1. Flame heated quartz tube

Flame heating is normally achieved using an air/acetylene flame. However, it is difficult to obtain uniform heating of the tube, and the top of the tube is cooler than the directly heated underside.

5.1.2.2.2. Electrically heated quartz tube

Electrical heating of the quartz tube is carried out either by means of a resistance wire wound around the tube, or by a tailored furnace, as shown in Figure 5.1.

The sensitivity obtained in externally heated quartz tubes is considerably higher than in diffuse flames. This superior sensitivity arises from the large dimensions of the quartz tube, and to the low dilution of the hydride either by flame gases or
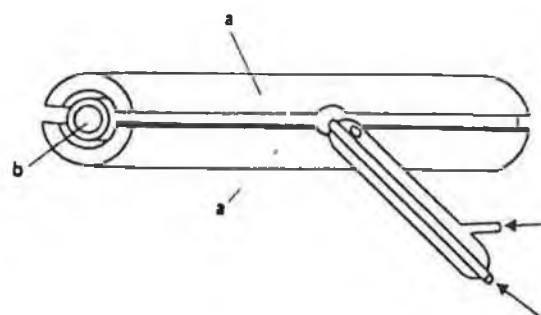
Figure 5.1. Schematic representation of an electrically

heated quartz tube. (a) heated furnace

(b) quartz tube

(reproduced from ref.2)

as a result of thermal expansion. Both of these factors increase the residence time, and consequently the sensitivity.

The length of the quartz tube is limited by the dimensions of the burner compartment of the instrument employed. The atomiser tube is most often around 150 mm long with a diameter typically around 10 mm.

5.1.2.3. Graphite furnaces

Graphite furnaces have been employed quite extensively for hydride atomisation, almost since the introduction of the hydride generation technique. There are two main approaches which can be taken involving their use: namely in-situ trapping of the hydrides and on line atomisation.

5.1.2.3.1. In-situ trapping

In this approach, use is made of graphite furnaces both as the trapping medium and as the atomisation cell. The hydride is purged from the generator and trapped in a heated graphite furnace, normally at a temperature of 300-600 $^{\circ}$C, until hydride evolution is completed, after which the trapped analyte is atomised at temperatures over 2000 $^{\circ}$C.

Interfacing of the hydride generator to the graphite furnace atomisation cell is critical for optimum performance. The generated hydrides are introduced either via the internal gas

line of commercial furnaces, or to the sampling port of the graphite tube through an interface made either of graphite or quartz.

5.1.2.3.2. On-line atomisation

In on-line atomisation, the hydrides are transferred directly from the generator to the furnace, which is preheated to a temperature of usually over 2200°C.

The sensitivity for this approach is lower than that with in-situ trapping, and is also generally lower than in quartz tube atomisers.

5.1.2.4. Flame-in-tube atomiser

Flame-in-tube atomisers are most often externally unheated quartz tubes with a flame burning inside. A typical atomiser, as shown in Figure 5.2, consists of two parts: an intake part and a T-tube, both of which are made of quartz and which are connected by a standard joint. The horizontal part of the T-tube is aligned in the optical path. The hydride transported from the generator by a flow of hydrogen enters the left side of the intake part. Oxygen is introduced through a capillary into the right side of the intake part. Its flow is much smaller than the flow of hydrogen. Thus a very small, almost invisible, flame burns at the end of the capillary. The end is usually 2 to 10 mm in front
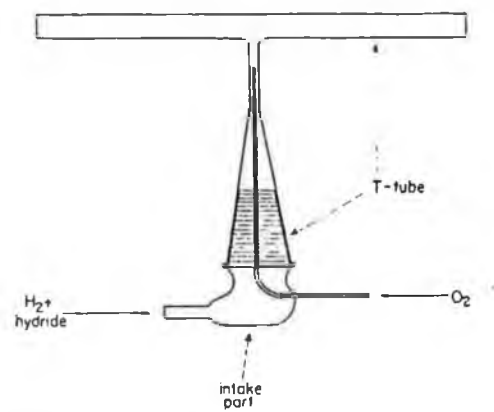
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Figure 5.2. Schematic representation of flame-
in-tube atomiser
(reproduced from ref.2)

of the T-tube junction, so that the flame burns in the inlet arm of the T.

5.1.3. Hydride transport

For selenium hydride, the greatest losses during its transport from sample solution to the atomiser, were reported where unsilanised glass and polypropylene surfaces were used in the apparatus [2]. The magnitude of hydride interaction with surfaces should be inversely proportional to the gas flow and directly proportional to the diameter of the tubing and the generator vessel [2]. The result of this is that hydride losses are less pronounced in apparati with a small free volume and at high gas flow rates. Dedina investigated this by performing hydride generation with samples spiked with ^{75}Se, and found that at flow rates greater than 7 ml/s in an unsilanised glass generator, losses of selenium hydride were below 5% [2].

5.1.4. Methods of hydride transport

There are two basic modes of hydride transport; namely the direct transfer and the collection modes.

5.1.4.1. Direct transfer

In the direct transfer mode, the released hydride is transferred directly to the atomiser, whereas in the collection mode, the hydride is collected in a collection device until evolution is completed, after which the hydride is transferred all at once.

A number of direct transfer modes have been employed. In the continuous flow mode, both the sample and borohydride solutions flow to the generator to establish a steady state analytical signal. In the flow injection mode, both the hydrochloric acid and borohydride solutions flow continuously to the generator, whilst the sample is injected as a small volume into the hydrochloric acid flowing stream. In the batch mode a specific sample volume is reduced all at once.

5.1.4.2. Collection mode

In the collection mode, the hydride may be collected either in a closed vessel under pressure together with the hydrogen resulting from borohydride decomposition, or in a cold trap, which normally consists of a U-tube immersed in liquid nitrogen and through

which hydrogen passes freely and is not collected. These two modes are referred to as "pressure collection" and "cold trap collection" respectively.

5.1.4.2.1. Pressure collection

The most widely used systems in this approach have been those relying on latex balloons for storage of the evolved hydrides. These systems suffer from a number of important limitations, of which the most significant are: (i) the balloons have a limited lifetime due to attack by acid fumes; (ii) the copious amounts of hydrogen simultaneously collected effectively dilute the hydrides; (iii) moisture collected in the balloon can cause some decomposition of the hydrides; and (iv) losses can occur as a result of diffusion of the hydrides through the balloon walls.

5.1.4.2.2. Cold trap collection

In this procedure a liquid nitrogen trap is used to freeze out hydrides as they are evolved, as shown in Figure 5.3. In general, the reaction is allowed to proceed for a fixed period with an inert gas flowing through the system. The trap temperature is then raised and the hydrides are rapidly transferred to the atom resevoir.

There are several advantages which cold trapping procedures offer over pressure collection methods. Firstly, the problem of
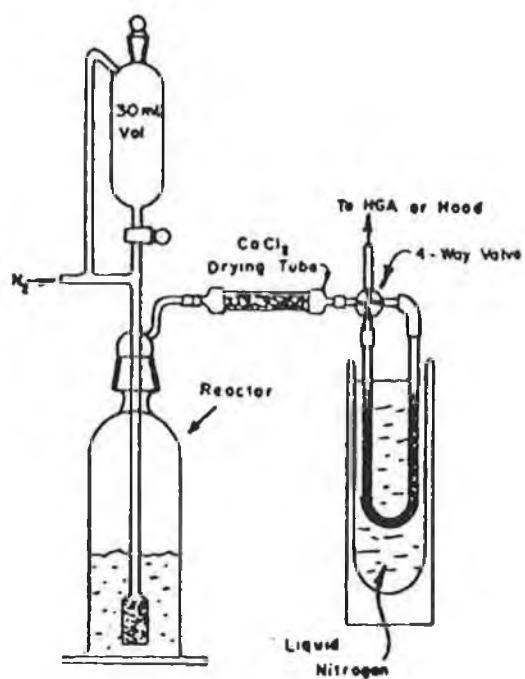
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Figure 5.3. Schematic representation of hydride

generator incorporating a cold trap

collection device

(reproduced from ref.2)

attack of the balloon material by acid fumes no longer exists, and secondly, and more importantly, the hydrides are separated from simultaneously evolved hydrogen, as this passes straight through the trap.

Two distinct procedures have been employed for transferring hydrides to the atom resevoir after freezing out:

(i) the trap is closed off by means of valves or clamps on the inlet and outlet sides, removed from the coolant, and allowed to reach room temperature. The valves are then opened and the hydrides are swept into the atom resevoir by the transport gas;

(ii) after removal from the coolant, the trap temperature is raised by external heating with the transport gas flowing through the system. The hydrides are thus passed to the atom resevoir immediately on vaporisation.

Currently, cold trap collection is the favoured collection mode as the hydride can be collected from a virtually unlimited volume of sample and, as hydrogen is not concomitantly accumulated, when the hydride is subsequently purged into the atomiser, dilution effects are minimised. The trapped hydride is most often purged into an atomiser in an open system simultaneously with evaporation in a heating bath, but it can also be evaporated into a closed volume and subsequently purged into the atomiser after evaporation has been completed. These are referred to as cold trap collection with "open system" and "closed system" heating respectively.

Presently, the greater majority of recent reports on hydride

generation have made use of direct transfer methods.

5.1.5. Choice of reducing agent

Zinc metal was employed as a reductant in most early hydride generation atomic absorption spectrometric methods. Initial methods employing granular zinc were rather slow, with arsine generation taking up to thirty minutes to proceed to completion. The use of 20 - 30 mesh granular zinc was found to result in more rapid reduction with complete reaction occurring in four to five minutes. Methods employing zinc dust or powder have provided reduction times of one minute or less.

In recent years, sodium tetrahydroborate has become the reducing agent of choice. This is due to several factors among which the most important is the high efficiency of hydride generation that is possible. Yields for zinc reduction have been reported as being of the order of 20 % or less, whereas those involving sodium borohydride reduction have been in the range of 90 - 100 %.

If it is used in the solution form, it can be more easily introduced to the sample at a reproducible and constant rate and is particularly suited to use in automated systems.

However, one drawback in the use of sodium tetrahydroborate solutions is their inherent instability. When sodium tetrahydroborate and water are mixed, hydrogen is initially evolved at a moderately rapid rate according to;

$$BH_4^- + 2\ H_2O \longrightarrow BO_2^- + 4\ H_2 \quad [5.1]$$

The formation of the strongly basic metaborate ion increases the pH of the solution and suppresses further hydrogen production. Therefore, by dissolving the sodium tetrahydroborate in a slightly basic solution, the initial generation of hydrogen may be largely prevented. For this reason, it has been common practice to make up sodium tetrahydroborate solutions in dilute sodium hydroxide.

The problem of the instability of the sodium tetrahydroborate can be overcome if the reagent is used in the pellet form. However, although pellets can be more conveniently handled than solutions, solutions do have the advantage that they can be added at a controlled rate over a fixed period of time.

5.1.6. Choice of acid

The majority of workers have found hydrochloric acid to be the acid of choice for hydride generation using both the zinc and sodium borohydride reduction systems. Vijan et al. [3] have investigated the effect of a range of acids and acid mixtures on the determination of selenium with the sodium borohydride reduction system, the results of which are shown in Table 5.1.

From the results obtained, the optimum response was observed using either HCl or an H_2SO_4/HCl (1:4) mixture.

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Table 5.1.   Effect of various acid matrices on

selenium absorbance.

(reproduced from ref.3)

Acid (20% v/v)	Absorbance for 10 ng/ml Se(IV) solution
H_2SO_4	0.042
HCl	0.051
HNO_3	0.0078
Fuming HNO_3	0.0055
$HClO_4$	0.023
H_2SO_4 – HCl	0.054
$HClO_4$	0.0035

5.1.7. Interference effects

When hydride generation techniques were first introduced, it was expected, that, as the generation process effectively separated the determinands from the sample matrix constituents, interference effects would be minimised. However, in practice, this has not proved to be the case with a number of suppressive interferences, mainly resulting from changes in the rates and efficiencies of hydride generation.

The most significant interferences observed can be separated into three main categories: (i) interferences from metal ions; (ii) interferences from other volatile hydride forming elements; (iii) interferences from oxyanions and acids.

5.1.7.1. Interference from metal ions

The suppression of hydrogen selenide generation has been reported [4] in the presence of Ag, Au, Cd, Co, Cu, Fe, Hg, Ni, Pb, Pt and V. It has been suggested that these effects can be explained by the preferential reduction of the metal ion interferent to a different oxidation state or to the free metal, causing precipitation. This precipitate can either co-precipitate the analyte, absorb the hydride formed, catalytically decompose it, or slow down or completely stop its evolution from solution. In addition, there is evidence to suggest that certain interferences may be due to the fact that, after reduction, selenium can form

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very stable selenides with some metals.

5.1.7.2. Interferences from other hydride forming elements

The determination of selenium has been found to be subject to interference from arsenic, antimony, tin, bismuth, tellurium and germanium. Such mutual interferences from the volatile hydride forming elements has been suggested to arise from compound formation in the atom resevoir. While this can account for the interferences observed in the relatively cool hydrogen diffusion flame, it cannot account for those observed in the determination of arsenic by a hydride generation technique coupled with detection by inductively coupled plasma optical emmission spectroscopy, ICP-OES. In this case, 10 mg/l of Se and Te suppressed the signal from 0.1 mg/l As by 66% and 46% respectively; 20 mg/l of Se and Te suppressed the signal from 5 mg/l As by 37% and 50% respectively. It was suggested that these effects were caused by the formation and precipitation of some form of compound between the determinand and other substances during the reaction. Some evidence to support this hypothesis was provided by the results of a semi-quantitative analysis of precipitates remaining in the generation vessel after reaction in the presence of interferents. A similar explanation has been proposed by Verlinden and Deelstra [5] to account for the interference effects of hydride forming elements on the determination of selenium by hydride generation AAS.

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In systems in which quartz tube atomisers are employed, formation of deposits on the tube walls and end windows can give rise to memory effects and also contribute to observed interference effects. Verlinden and Deelstra [5] have reported that in the determination of selenium in the presence of an excess of Sb, Ge, and As using an electrically heated quartz tube system, a greyish film of Sb_2Se_3, a yellowish film of $GeSe_2$, and a layer of a goldish modification of As, respectively, were formed on the tube walls and end windows.

5.1.7.3. Interference from acids

As nitric, sulphuric and perchloric acids may be employed in digestion procedures for the determination of selenium in biological materials, the possibility of interferences resulting from their use is of importance. Whilst interferences arising from nitric acid have been widely studied, it has been shown that all three acids inhibit the generation of the volatile hydride to some extent.

5.1.8. Procedures for overcoming interferences

The two main approaches taken to overcome interferences encountered during hydride generation are masking and separation procedures.

5.1.8.1. Masking procedures

The aim of these procedures is to attempt to remove interferences by the addition of a variety of complexing agents. One of the first applications of this approach involved the use of EDTA. Whilst this was shown to suppress interference from Co, Ni, Zn, Fe, Bi, Cd and Ag in the determination of As in 0.1M hydrochloric acid, it did not prove to be as successful in the case of selenium. However, tellurium(IV) has been shown to be extremely useful in minimising interference effects in the determination of selenium. In the presence of sodium tetrahydroborate, Te(IV) is reduced to telluride which can form very stable compounds with many metal ions and hence suppress their effect on the generation of hydrogen selenide. Interferents whose effects have been shown to be reduced in the presence of Te(IV) include Ag, Cu, Ni, Au, As, Sb, Pt, and Pd [4]. The interferences observed in the absence of Te(IV) were considered to be as a result of the formation of stable interfering metal selenides, whereas the reduced interference observed in the presence of Te(IV) was considered to result from the formation of tellurides of the interferents, which are of higher stability than the corresponding selenides. The masking efficiency observed for Cu was poorer than that for Ni, Pt and Pd. In this case the difference in the stability between the telluride and selenide is less than in the case of the other metals. In spite of this, the interference of a 50-fold excess of Cu (15 mg/l) on the determination of 300 ug/l Se was

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removed.

5.1.8.2. Separation procedures

The two separation procedures which have been used for the separation of selenium from interfering elements are ion exchange chromatography and co-precipitation.

5.1.8.2.1. Ion exchange chromatography

Use was made of a Dowex 50W resin in an attempt to eliminate interfering metals in a hydride generation method for the determination of selenium in vegetation [2]. The use of the resin did not meet with great success because of the high acidity of the sample digest.

5.1.8.2.2. Co-precipitation

For the determination of selenium, a co-precipitation procedure involving ferric hydroxide was reported to allow concentration factors of up to 100-fold and the suppression of diverse metal ion interferences. This involved the formation of a precipitate which was subsequently floated with the aid of a surfactant and small air bubbles, then separated and dissolved in HCl prior to analysis. Other hydride-forming elements were also co-precipitated by the iron hydroxide, and consequently the

suppressive effects of these elements were not alleviated. However, in the cases of Cu, Ni and Hg, the threshold levels of these elements were considerably increased. Lanthanum hydroxide has also been used in several studies to eliminate interferences from a diverse range of interfering ions, and allow concentration of the determinand to be achieved.

5.2. EXPERIMENTAL

5.2.1. Reagents

All reagents used were of analytical grade. All solutions were prepared in doubly distilled water, obtained by passing distilled water through a Millipore Milli-Q water purification system.

The sodium tetrahydroborate, $NaBH_4$, solution was prepared by dissolving sodium tetrahydroborate in a 1% sodium hydroxide solution to form a 1% sodium tetrahydroborate solution. Before use, it is necessary to filter this solution through a 5 um filter. A fresh 1% solution of sodium tetrahydroborate solution was prepared each day.

For the hydride generation studies, a 100 ng/ml stock solution of selenium(IV) was prepared daily by diluting a standard solution (BDH, Poole, Dorset, UK) containing 1 mg/ml of selenium (IV). Aliquots were diluted with 1 M hydrochloric acid to obtain appropriate working reference solutions for calibration purposes.

For the graphite furnace method, working standards were prepared by dilution of the standard solution with 0.2% nitric acid. To prepare the calibration graphs, 50-ul of pooled serum or plasma were "spiked" with an equal volume of either 0.2% nitric acid (blank), or a standard. A 100-ul portion of diluent (0.2% nitric acid + 0.2% Triton X-100) was then added to each serum and

plasma standard. Subsequently, three volumes of diluent (150 ul) were added to the 50 ul serum and plasma test samples. The different concentrations of Triton X-100 added to the serum/plasma standards and to the samples were found to have no effect on the results. The matrix modifier was prepared by dissolving 1.86 g of $Cu(NO_3)_2$ and 1.73 g of $Mg(NO_3)_2$. $6H_2O$ (Aldrich, Gillingham, Dorset, UK) in one litre of doubly distilled water.

Pooled blood serum samples were obtained from the Department of Medicine, The Queen's University of Belfast, and a plasma sample from one unit of whole blood was obtained from the Blood Transfusion Service, Dublin.

All glassware was washed for four days in 4 M nitric acid, rinsed and soaked in doubly distilled water until use.

5.2.2. Apparatus

For the hydride generation studies, an Instrumentation Laboratory Model 357 atomic absorption spectrometer was used in conjunction with a selenium hollow cathode lamp (S&J Juniper and Co., Harlow, Essex, UK). The signal from the spectrometer was displayed on a chart recorder (Philips PM 8251A).

During the optimisation of the "T" shape atomisation cell temperature, the air and acetylene flow rates were varied and the corresponding cell temperature was measured using a mineral insulated thermocouple and a digital thermometer (Eirelec,

Dundalk, Ireland).

In the flow injection manifold, a peristaltic pump (Watson Marlow 501U), a four way rotary valve (Tecator 5001), a Kel-F mixing "T" (Plasma Therm, London, UK) and a gas/liquid separator (Plasma Therm) were used.

Teflon tubing of 1 mm internal diameter was used for the sample loop, whilst silicone tubing was used for delivery of the reagents.

For the graphite furnace atomic absorption spectrometric studies, a dedicated Perkin-Elmer Zeeman 3030 atomic absorption spectrometer with a stabilised temperature platform furnace was used in conjunction with an AS60 autosampler.

Sample digestions were carried out in standard Erlenmeyer flasks, which were heated on a hot-plate.

5.2.3. Digestion procedure

The blood plasma and serum samples must undergo digestion prior to any analysis procedure by hydride generation atomic absorption spectroscopy.

In the digestion procedure, a 2 ml sample of serum or plasma was measured accurately into a 50-ml Erlenmeyer flask and 5 ml of 16 M nitric acid were added. Two precleaned glass beads were then placed in the flask, and a precleaned glass funnel was placed in the mouth of the flask.

The flask was then placed on a hot-plate, and the temperature

was raised over twenty minutes to 140^OC and maintained at this temperature for twenty minutes. The flask was then cooled, 2.5 ml of 18 M sulphuric acid and 1 ml of 11.6 M perchloric acid were added, and the temperature was slowly raised again to 140^OC and maitained for fifteen minutes. The temperature was then raised over fifteen minutes to 205^OC and maintained at this temperature until white fumes of perchloric acid were evident.

The flask was then cooled, 5 ml of 5 M hydrochloric acid were added and the mixture was heated at 95^OC for thirty minutes. After cooling, the contents were diluted to 25 ml, and 330 ul aliquots were taken for selenium measurements.

5.2.4. Development of a simple hydride generator

The first approach taken in the developement of a hydride generation system involved the construction of a simple apparatus from readily available laboratory materials. The first design is shown in Figure 5.4.

This system consisted of a 23 mm i.d, 150 mm long boiling tube, into the mouth of which was fitted a rubber bung containing three openings. Through the first of these passed a length of tubing whereby argon gas entered the system. A glass tube which was connected to the nebuliser inlet tubing and a length of tubing, connected to a syringe on the exterior, were fitted in the second and third openings respectively.

A 5 ml aliquot of standard was pippetted into the tube and the rubber bung placed firmly in the mouth. This created a sealed chamber from which the only exit was through the glass tube and via the nebuliser inlet tubing into the atomic absorption spectrometer. The system was allowed to remain in this state for a period of time to allow the argon to purge any air from the system.

The atom cell used in the atomic absorption spectrometer was an argon/hydrogen entrained flame.

The sodium tetrahydroborate solution was injected via the plastic tubing from the syringe into the standard solution. The force of this injection brought about the mixing of the sodium tetrahydroborate solution with the standard solution.
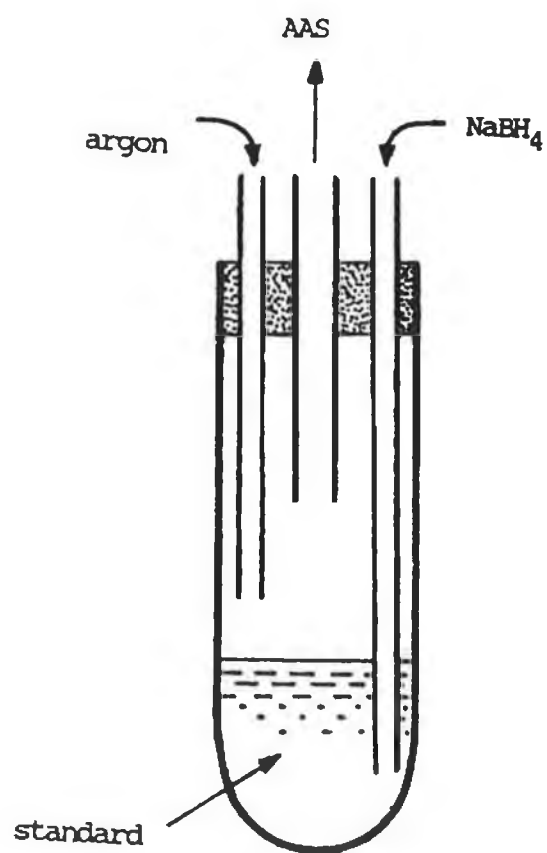
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Figure 5.4.   Batch type hydride generator

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The generated hydrides and hydrogen were taken up via the nebuliser and into the atomic absorption spectrometer.

Blank measurements were carried out by placing 10 ml of 1M HCL in the tube and proceeding as described beforehand.

It was found that, when the sodium tetrahydroborate (III) solution was injected, the force of the resulting reaction with the acidified standard drove some of the reaction mixture back up the injection tubing. It was felt that this could lead to the introduction of errors into the system, and therefore, a slightly improved version was made which differed from the first in two aspects:

(a) the tubing through which the sodium tetrahydroborate solution was injected was shortened. This meant that the sodium tetrahydroborate was injected slightly above, and not as before directly into, the standard solution;

(b) mixing was achieved by use of a magnetic stirrer.

Whilst it was possible to determine selenium by this system, it was found that a lot of operator time and manipulation was required, i.e between each blank/standard determination the system has to be dismantled, cleaned and then reassembled.

Therefore, it was decided that a new approach, in which this requirement for operator attention could be minimised, would have to be taken.

5.2.5. Use of a gas/liquid separator

The next approach taken in the development of a hydride generation system was the introduction of a gas/liquid separator The gas/liquid separator consists of two glass sections, which are fitted together at a ground glass joint to give the overall structure shown in Figure 5.5.

Argon gas is entrained into the system via Tube 1, whilst the products of the hydride generation reaction enter the system via Tube 2. The hydride generation reaction occurs at a Kel-F mixing "T" from which the products are transported via a length of Teflon tubing to Tube 2 and, ultimately, to the atomic absorption spectrometer.

The first design incorporating this mixing "T" and gas/liquid separator is shown in Figure 5.5.

The sodium tetrahydroborate solution and the standard solutions are delivered to the mixing "T" by the use of a peristaltic pump. In this approach, blank measurements were made by delivery of the hydrochloric acid and sodium tetrahydroborate along their respective carrier lines. Standard measurements were made by changing the acid carrier line from the acid container to that of the standard. To repeat the blank measurement, the reverse procedure was performed.

In this system, reduction of the selenium(IV) occurs at the
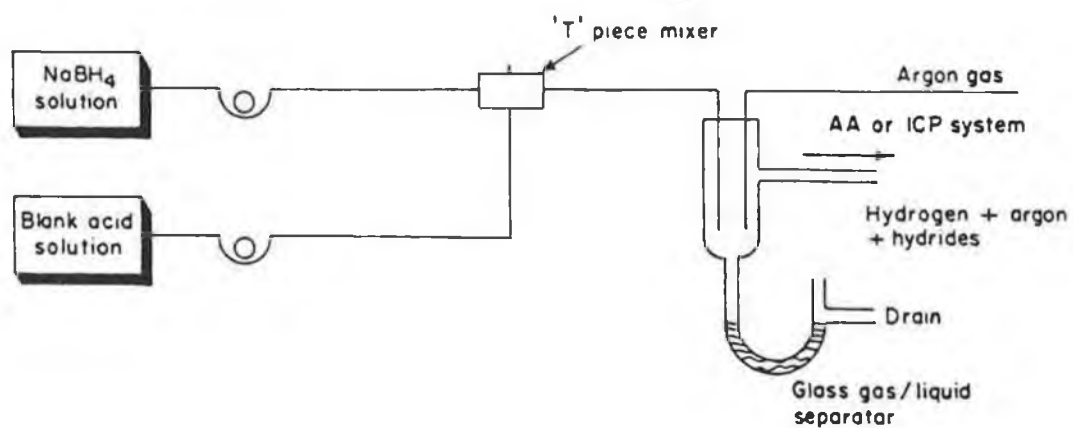
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Figure 5.5. Continuous flow hydride generation system incorporating a gas/liquid separator

"T" piece and the reaction is completed by the time the flow reaches the gas/liquid separator. At this point, the liquid products are separated from the gaseous products, which are either hydrogen or a mixture of hydrogen and hydrides. The liquid waste products flow via a "U" tube to a free running drain while the gaseous products are taken via the nebuliser inlet tubing into the argon/hydrogen entrained flame.

Blank measurements were made by delivering 1M HCL and sodium tetrahydroborate solution to the mixing "T". Standard measurements were then made by delivering the standard and sodium tetrahydroborate to the "T" piece. After this measurement had been completed, the blank measurement was repeated.

In this system, much of the previous burden upon the operator for reagent/standard delivery had now been reduced to a large extent.

5.2.6. Use of flow injection technique of sample introduction

The next step in the development of the eventual hydride generation system was the introduction of a rapid and reliable means of sample introduction. Therefore, a four way rotary valve was introduced into the system. A schematic diagram of the flow injection manifold is shown in Figure 5.6.

A change in the type of atom cell was also introduced into the system in order to obtain improved detection limits and sensitivity. Suitable burner modifications were made to allow a
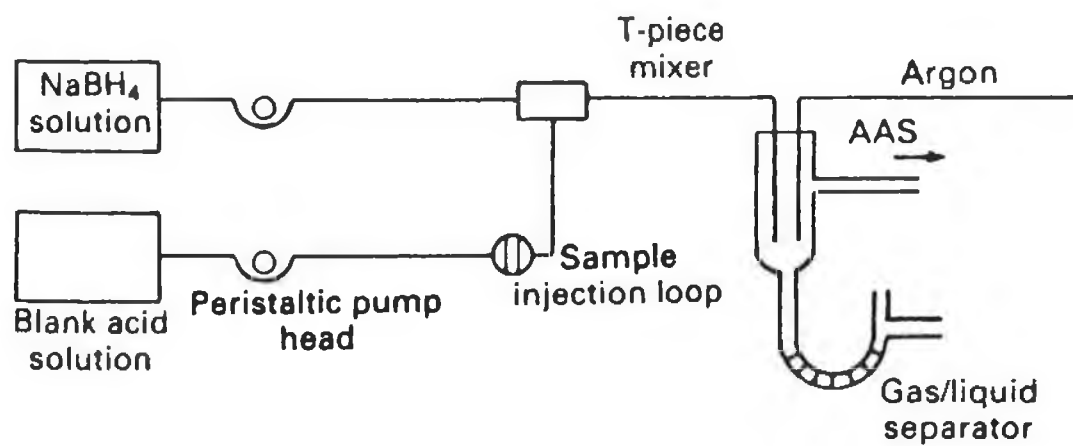
Figure 5.6.   Schematic representation of flow
              injection hydride generation system

quartz atomisation cell to be supported in an air/acetylene flame about 5 mm above the slot of a 5 cm single slot burner. The atomisation cell, made in our laboratories, consisted of a 120 x 8 mm i.d quartz tube fused at the centre with a 150 x 2 mm i.d. quartz tube to form a "T" shape.

Before analysis, the atomic absorption spectrometer was allowed to warm up until the atomisation cell attained thermal equilibrium (840-860°C). The signal from the spectrometer was displayed on a chart recorder. During the optimisation of the atomisation cel temperature, the air and acetylene flow rates were varied, and the corresponding cell temperature measured using a mineral insulated thermocouple. The optimum cell temperature was achieved by using air and acetylene flow rates of 8.5 and 1.9 L/min respectively.

The flow injection manifold shown in Figure 5.6., consisted of a peristaltic pump, a four way rotary valve with external loop for sample injection, a Kel-F mixing "T" and a gas/liquid separator. Sample loops of various volumes were prepared using 1-mm i.d. Teflon tubing; appropriate lengths of which were cut to provide sample loop volumes ranging from 120 to 460 ul. This 1-mm i.d. Teflon tubing was used throughout the flow injection system. The tube lengths from the rotary valve to the "T", and from this to the gas/liquid separator were 10 cm and 6.5 cm respectively. The sodium tetrahydroborate solution was pumped at 3.9 ml/min and the 1M hydrochloric acid at 5.4 ml/min; the two

flow rates being achieved using 1.5 mm i.d and 2.0 mm i.d. silicone tubing respectively.

In the operation of this system, two sampling cycles were used. In the first, the acid and sodium tetrahydroborate streams were allowed to mix at the T-piece, and the peak height signal was measured over a 20 second integration time period and recorded. During this period, the hydrogen generated, enables the blank signal to be monitored.

The second period occurs immediately after the first and involves the injection of the sample via the four-way rotary into the acid carrier stream; once again the peak height signal was measured over 20 seconds and recorded. At the end of this period, the rotary valve was switched back to the injection position and the cycle was started again. This sequence of events did not include a specific time period for washing the system, as experimentation had shown that, in the period immediately after the analysis period, the signal had returned to the baseline. This ensured that the blank level was achieved between each cycle and that, within each cycle, the analyte was monitored above the blank level.

5.2.7. Graphite furnace method

In the graphite furnace method, the volume of the blank, standards and samples injected on to the pyrolytical graphite coated L'vov platform was 20 ul and to this were added 10 ul of matrix modifier. Argon was used as the purge gas.

The optimum conditions used for the drying stage varied between 30 and 70 seconds depending on the age of the graphite tube and platform.

At the end of the drying stage, the temperature was raised to $900^{\circ}C$ (ramp, 5 seconds) and held for 60-70 seconds. With maximum power heating, the temperature was then raised to $2100^{\circ}C$, the gas flow was stopped and readings were taken over the next 3 seconds. The background-corrected peak area was measured for the last 2.5 seconds of atomisation and recorded on the printer.

5.3. RESULTS AND DISCUSSION

5.3.1. Initial considerations

The determination of selenium(IV) by hydride generation atomic absorption spectrometry can be defined as occurring in three distinct stages:

    (1) the conversion of Se(IV) to hydrogen selenide;

    (2) the separation of the volatile hydride from the liquid phase;

    (3) the transport of the hydride to the atom cell.

There are a number of practical approaches which can be adopted in order to carry out these steps: namely, batch, continuous flow or flow injection methodologies.

5.3.2. The use of a batch hydride generation system

In the batch generation system described, 1% sodium tetrahydro-borate was injected forcefully into the standard solution by means of a syringe. The combination of the force of the injection and the stirring of the solution helped to ensure that good mixing of the reagents occurred.

This is of considerable importance, as Agterdenbos and Bax [6] have reported that formation of hydrogen selenide takes place at time values of less than one millisecond after reagent mixing. It was found, in this investigation, that if mixing of the

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reagents was dependent upon the force of the injection alone, the $NaBH_4$ reacted with the standard solution only in the immediate area of the injection.

The principle parameters which affect the ultimate sensitivity of the batch hydride generation process are the hydrochloric acid and $NaBH_4$ concentrations, the volume of $NaBH_4$ injected and the nature of the argon- hydrogen flame.

After generation in the reaction vessel, the hydrides are taken via the nebuliser, into the argon-hydrogen flame. A nebuliser flow rate of 3 ml/min was used throughout all the parameter investigations in this evaluation of the batch system.

5.3.2.1. Effect of hydrochloric acid concentration

The effect of the hydrochloric acid concentration was investigated using 2 ml of a 1% $NaBH_4$ solution, and 5 ml of a 100 ng/ml Se(IV) solution, made up in varying concentrations of hydrochloric acid, ranging from 0.5 M to 5 M. The resulting relationship between the hydrochloric acid concentration and the absorbance at 196 nm is shown in Figure 5.7. As a 1 M concentration allowed the best sensitivity, it was selected for further use.

5.3.2.2. Influence of $NaBH_4$ concentration and volume

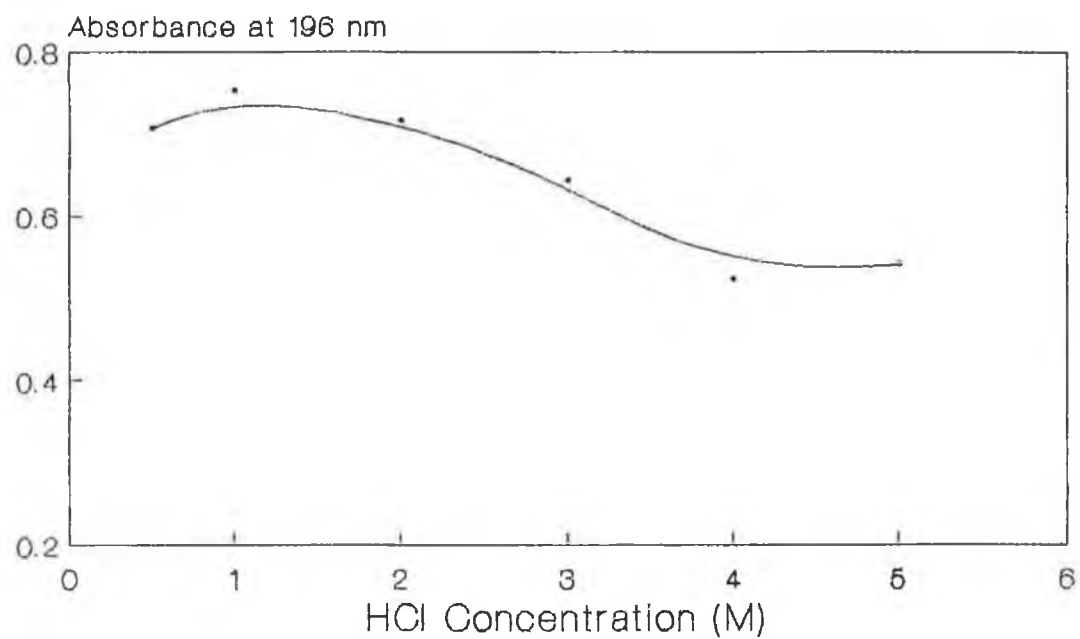The relationship between the sodium borohydride concentration

Figure 5.7. Influence of hydrochloric acid

concentration on absorbance response

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and the sensitivity of the absorbance response at 196 nm was investigated using a 100 ng/ml solution of Se(IV) in 1 M hydrochloric acid and 2 ml of $NaBH_4$ solutions of varying concentration from 0.2 % to 1%. As shown in Figure 5.8., a 0.6% solution of $NaBH_4$ allowed the most sensitive response.

The effect of the volume of $NaBH_4$ injected was evaluated using a 100 ng/ml solution of Se(IV) in 1 M hydrochloric acid, a 0.6% solution of $NaBH_4$ and by varying the volume injected between 1 ml and 4 ml. It was found that an injection volume of 3 ml allowed for the most sensitive response.

5.3.2.3. Influence of fuel/oxidant ratio

The fuel/oxidant ratio plays a crucial role in determining the ultimate sensitivity of the technique, as it controls the temperature of the atom cell, in this case the flame, and consequently the degree of atomisation which occurs.

The effect of the fuel/oxidant ratio upon the absorbance response was determined using 2 ml of a 100 ng/ml solution of Se(IV) in 1 M hydrochloric acid and 3 ml of 0.6 % $NaBH_4$. As shown in Figure 5.9., a fuel/oxidant ratio of 4/16 standard cubic feet per hour (SCFH) provided a flame temperature which allowed the most sensitive response.
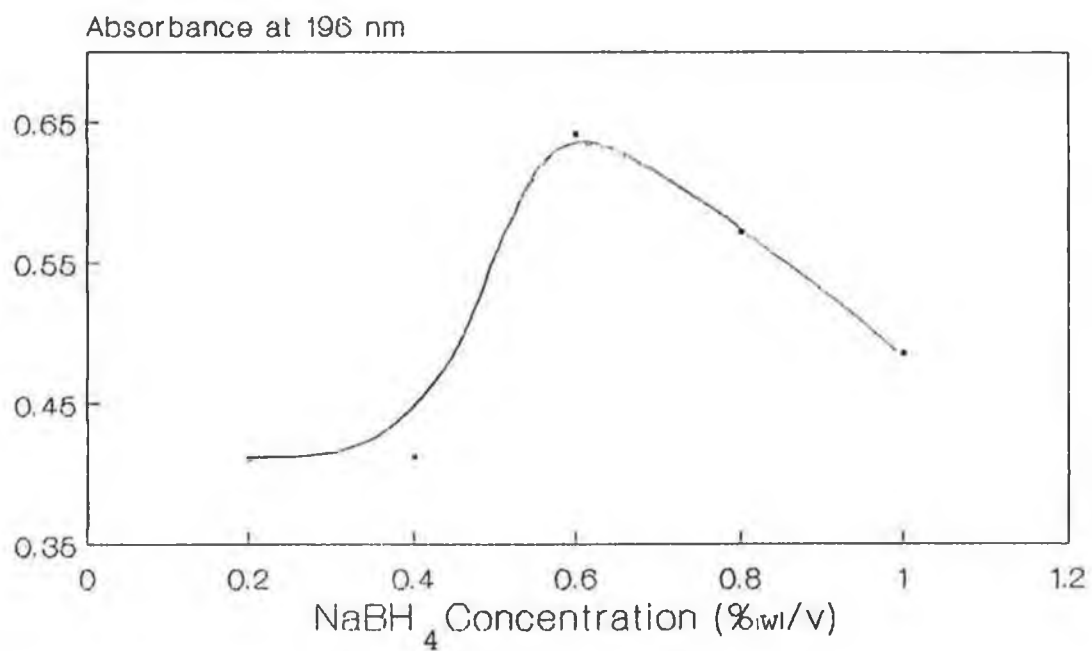
196

Figure 5.8. Effect of NaBH$_4$ concentration on the
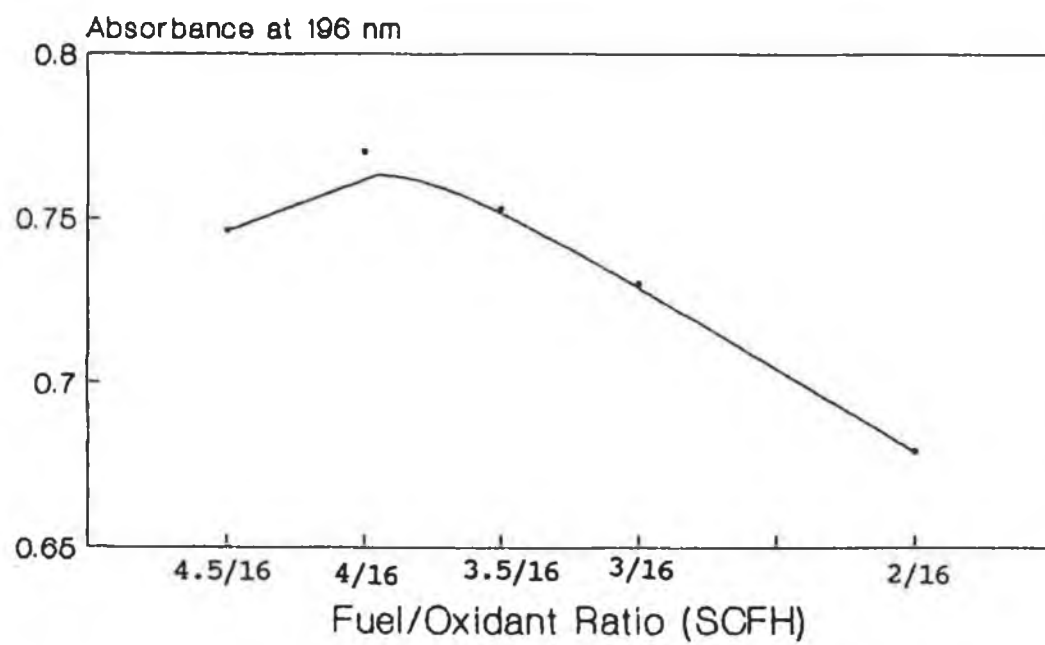
sensitivity of the absorbance response

Figure 5.9. Influence of fuel/oxidant ratio on the sensitivity of the response

5.3.3. Evaluation of the batch hydride generation procedure

The batch hydride generation apparatus used in this study is based upon the design first described by Holak [1]. It can be readily assembled from materials normally found in the laboratory and requires a minimum of operator expertise for its operation. Using this system. a linear calibration range up to 70 ng/ml was obtained. The reproducibility, expressed as the relative standard deviation (RSD), was typically in the range of 4-10% at the 50 ng/ml Se(IV) level (n=10).

The reproducibility of the technique was, to a very large extent, dependent upon the reproducibilty with which the $NaBH_4$ was injected into the hydride generation apparatus. In addition, the necessity to dismantle the apparatus, remove the reactants from the reaction vessel, and subsequently clean, refill and reassemble the apparatus between measurements meant that the procedure, as a whole, was extremely time consuming.

In an effort to circumvent these problems, a peristaltic pump was introduced for reagent and standard delivery. In conjunction with this, it was necessary to include a gas/liquid separator to remove the hydrides from the waste solution.

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5.3.4. Continuous flow hydride generation system

In the continuous flow hydride generation system, the reaction vessel in the previous batch system was now replaced with a T - shape, into which the $NaBH_4$ and the hydrochloric acid/sample streams were delivered into the respective arms, and from which the generated hydrides and waste products were delivered to the gas/liquid separator, as shown in Figure 5.5 . The hydrides were taken from the gas/liquid separator, via the nebuliser, into the flame atom cell. As in the case of the batch system, a nebuliser flow rate of 3 ml/min was used in this investigation.

The concentration of the $NaBH_4$ and the hydrochloric acid are of importance, as was the case in the batch system.

5.3.4.1. Effect of hydrochloric acid and $NaBH_4$ concentrations

The relationship between the hydrochloric acid concentration and the absorbance at 196 nm was investigated using a 0.6% $NaBH_4$ solution and a 100 ng/ml Se(IV) standard solution, made up in varying concentrations of hydrochloric acid. The optimum absorbance response was achieved when a 1 M concentration was used.

The effect of the $NaBH_4$ concentration upon the sensitivity of the response was determined using a 100 ng/ml Se(IV) standard in 1 M hydrochloric acid and varying concentrations of sodium tetrahydroborate. As was the case in the batch system, a 0.6%

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solution of NaBH$_4$ permitted the most sensitive response.

The major advantage of the use of a peristaltic pump for the delivery of the respective reagents, is the reduced requirements for manual manipulation placed on the operator. Moreover, the incorporation of a gas/liquid separator into the system means that the time consuming steps of dismantling, cleaning and reassembling of the apparatus, as encountered in the previous batch system, can now be eliminated, thus simplifying the analytical methodology considerably.

Use of this system, termed a continuous flow hydride generation system, gives a linear range up to 60 ng/ml. The reproducibility for a series of ten measurements for a 50 ng/ml Se(IV) solution, expressed as the RSD, was typically in the region of 2 - 3%.

The reproducibility of the continuous flow hydride generation system shows a marked improvement over that of the batch system. This is due to the more reproducible reagent introduction and sample handling characteristics afforded by a flow system. In addition, the overall procedure is considerably faster than that for the batch system.

Continuous flow systems do, however, consume considerable amounts of reagents and sample. Therefore, in an attempt to minimise this, the incorporation of a discrete sample injection method into the flow system, i.e. a flow injection system, was investigated.

5.3.5. Flow injection hydride generation system

In the flow injection system, two major changes were made from the previously described continuous flow system. These were:

(i) the introduction of a rotary valve with an external sample loop, to allow the injection of a discrete sample volume;

(ii) the use of a flame heated quartz atom cell.

In this system, the hydrides were carried into the atom cell by a carrier gas, and not, as was the case in the previous systems, by the nebuliser. As argon was used in both the batch and continuous flow systems to flush any air out of the hydride generation/atomisation system, it was therefore the natural choice as a carrier gas.

5.3.5.1. Influence of $NaBH_4$ concentration

The influence of the $NaBH_4$ concentration upon the sensitivity of the absorbance response at 196 nm, was investigated using a 10 ng/ml solution of Se(IV) in 1 M hydrochloric acid, an argon carrier gas flow rate of 0.5 l/min, a sample loop injection volume of 100 ul, a cell temperature of approximately 830°C, and by varying the $NaBH_4$ concentration from 0.5% to 3%. From the resulting relationship, shown in Figure 5.10., it can be seen that a $NaBH_4$ concentration of 1% allows the optimum sensitivity, and was used throughout this investigation.
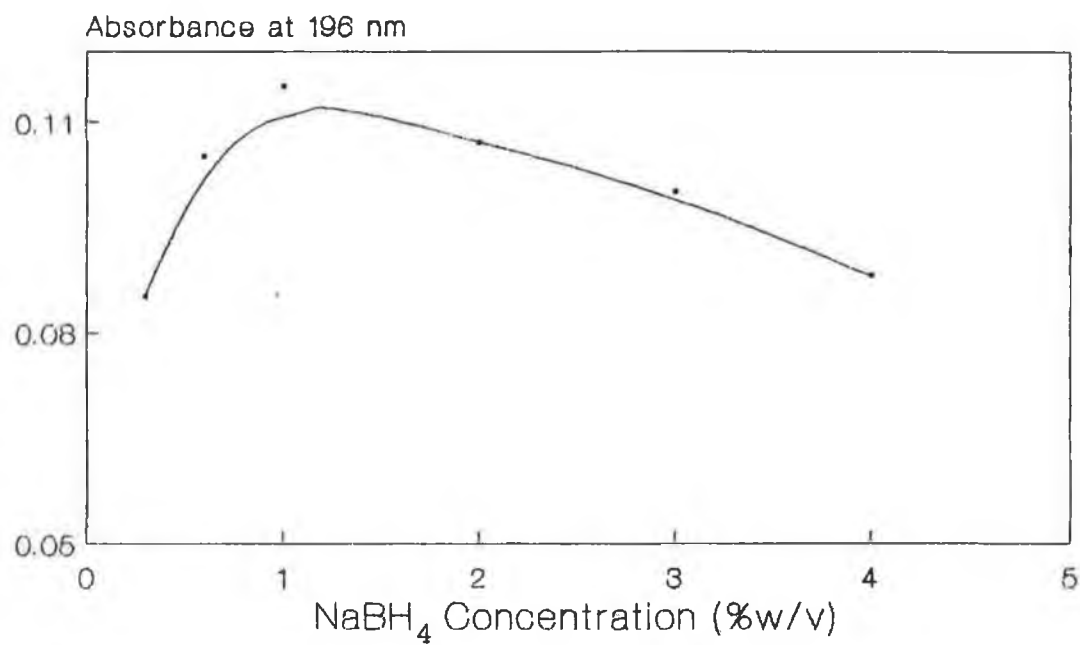
Figure 5.10.   Influence of NaBH$_4$ concentration on

the absorbance response at 196 nm in the

flow injection system

5.3.5.2. Influence of hydrochloric acid concentration

The relationship between the hydrochloric acid concentration and the absorbance signal at 196 nm was investigated using a 1% solution of $NaBH_4$, an argon carrier gas flow rate of 0.5 l/min, a cell temperature of approximately $830^{o}C$, a sample loop volume of 100 ul and 10 ng/ml solutions of Se(IV) in varying concentrations of hydrochloric acid, ranging from 0.5 – 5 M.

As shown in Figure 5.11., high acid concentrations enhanced the sensitivity over that provided by lower acid concentrations.

However, a 1 M hydrochloric acid concentration was selected for further use, as the corrosive properties of the highly concentrated acid make it unsuitable for use with the apparatus employed in this study.

5.3.5.3. Effect of carrier gas flow rate

In addition to transporting the hydrogen selenide to the atomisation cell, the carrier gas also expels any air present in the system, thus eliminating the possibility of any background absorption and allowing precise measurements to be made in the far ultraviolet region.

The argon gas flow rate influence on the absorbance signal was determined using a 1% solution of $NaBH_4$, a 10 ng/ml solution of Se(IV) in 1 M hydochloric acid, a sample loop injection
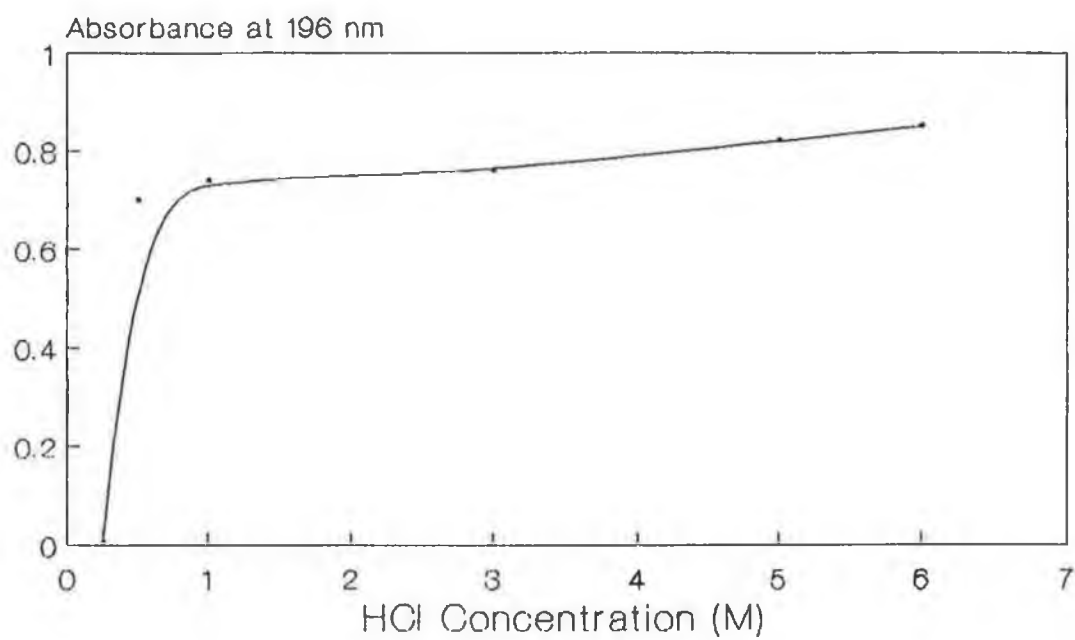
Figure 5.11. Dependence of the absorbance response
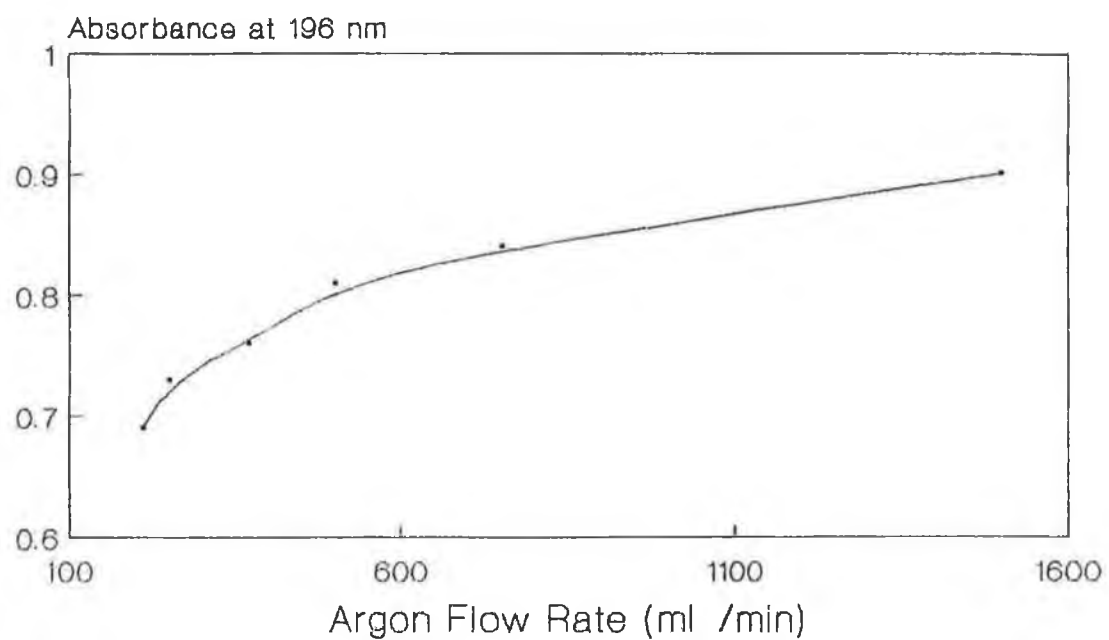on the hydrochloric acid concentration

Figure 5.12. The effect of the carrier gas flow rate

on the response

volume of 100 ul, a cell temperature of approximately 830°C, and by varying the argon flow rate over the range 0.25-1.5 l/min. The resulting relationship, shown in Figure 5.12., shows an increase in the sensitivity of the response with increasing carrier gas flow rate, and a carrier gas flow rate of 1.5 l/min was used in all further studies.

5.3.5.4. Influence of atomisation cell temperature

The influence of the atomisation cell temperature on the sensitivity of the absorbance response was determined using a 1% solution of NaBH$_4$, a 10 ng/ml solution of Se(IV) in 1 M HCl, a sample loop injection volume of 100 ul, an argon flow rate of 1.5 l/min and by varying the fuel/oxidant ratio.

From the resulting relationship, shown in Figure 5.13., an atomisation temperature in the range 830-860°C allows the optimum sensitivity, and is in agreement with the results of Verlinden and Deelstra [7]. This temperature was obtained by using air and acetylene flow rates of 8.5 and 1.9 l/min respectively.

The use of a flame heated atomisation cell is not the ideal approach, as the temperature of the atomisation cell is higher on the lower surface than on the upper one. This situation can be effectively overcome by the use of an electrically heated system, as described in section 5.1.2.2.

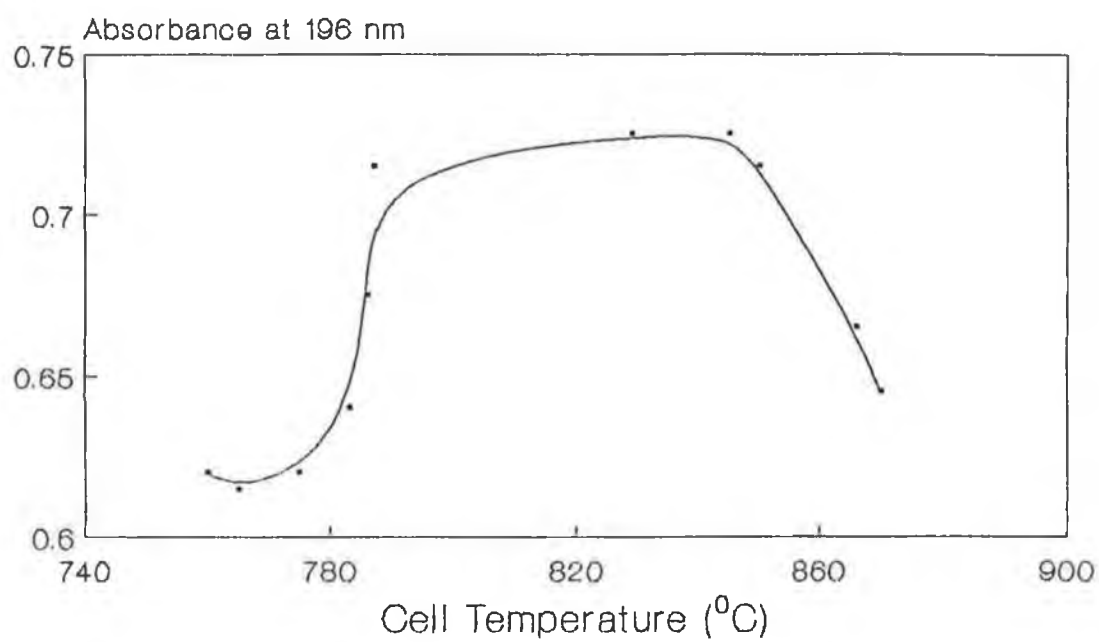The atomisation cell has a limited lifetime, as the quartz glass

Figure 5.13. Relationship between atomisation cell temperature and the absorbance response at 196 nm

becomes progressively clouded and weakened with use, until it
eventually fractures. This situation has been previously
described, but as yet, there has been no experimental
investigation into the exact causes of this phenomenon and how
the structure of the quartz is affected.

Normally, a quartz atomisation cell can be used for 50-70 hours
before fracturing of the glass necessitates a change.

5.3.5.5. <u>Effect of integration time</u>

The effect of the integration time was investigated using a 1%
solution of $NaBH_4$, a 10 ng/ml solution of Se(IV) in 1 M HCl, a
sample loop injection volume of 100 ul, an argon flow rate of
1.5 l/min and a cell temperature of approximately 830 ^{O}C.

From the resulting relationship, shown in Figure 5.14., there is
an increase in the sensitivity up to approximately 15 seconds
after which the rsponse is steady. The integration time in this
system, is measured from the moment of sample injection and
therefore includes the time of transport from the point of
injection to the atomisation cell.

An integration time of 20 seconds was used throughout this
investigation, as this ensured that all the selenium injected
had been transported to and atomised in the cell when the
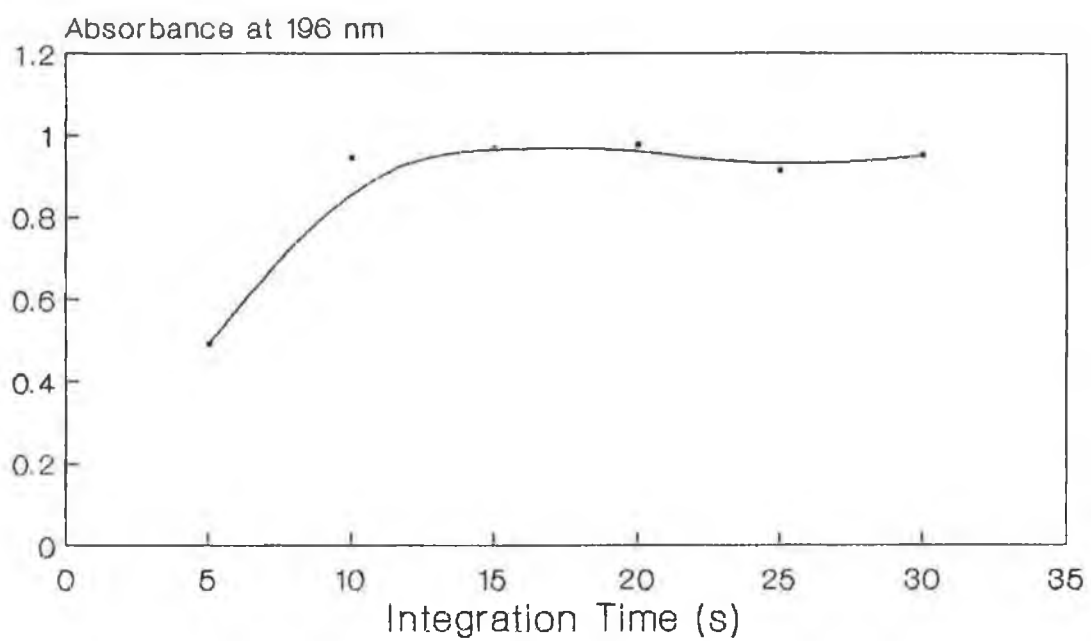absorbance measurement was recorded.

Figure 5.14.   Effect of integration time on the

absorbance response

5.3.5.6. Influence of sample volume

The relationship between the volume of sample/standard injected into the flowing stream, via the sample loop, and the absorbance response was investigated using a 1% solution of $NaBH_4$, a 10 ng/ml solution of Se(IV) in 1 M hydrochloric acid, an argon flow rate of 1.5 l/min and a quartz tube temperature of 850°C, reagent flow rates of 3 ml/min and by varying the volume between 100 ul and 370 ul.

As shown in Figure 5.15., there was a steady increase in the response with increasing volume up to approximately 300 ul, after which the rate of increase was less.

A sample/standard volume of 330 ul was chosen for throughout the remainder of this investigation.

5.3.5.7. Effect of reagent flow rates

The effect of the hydrochloric acid and sodium tetrahydroborate (III) flow rates upon the sensitivity of the absorbance response was determined using a 1% solution of $NaBH_4$, a 10 ng/ml soluion of Se(IV) in 1 M hydrochloric acid, an injection volume of 330 ul, an argon flow rate of 1.5 l/min and a quartz tube temperature of 840°C.

The resulting relationship is shown in Figure 5.16 . As can be seen, the sensitivity of the absorbance response increases as the ratio of the hydrohloric acid to the $NaBH_4$ flow rates

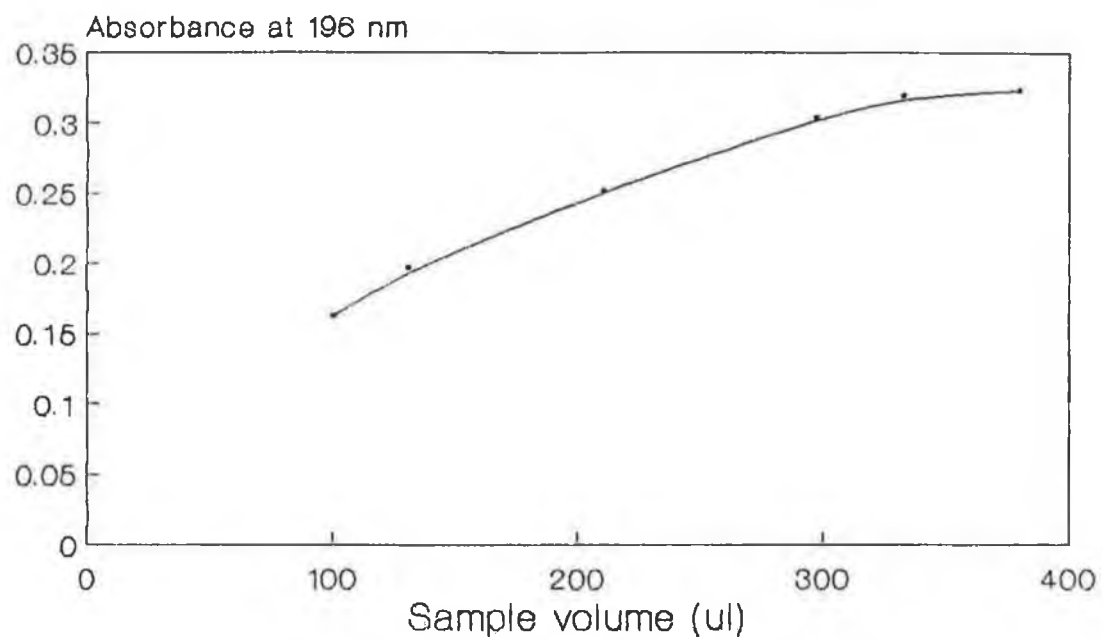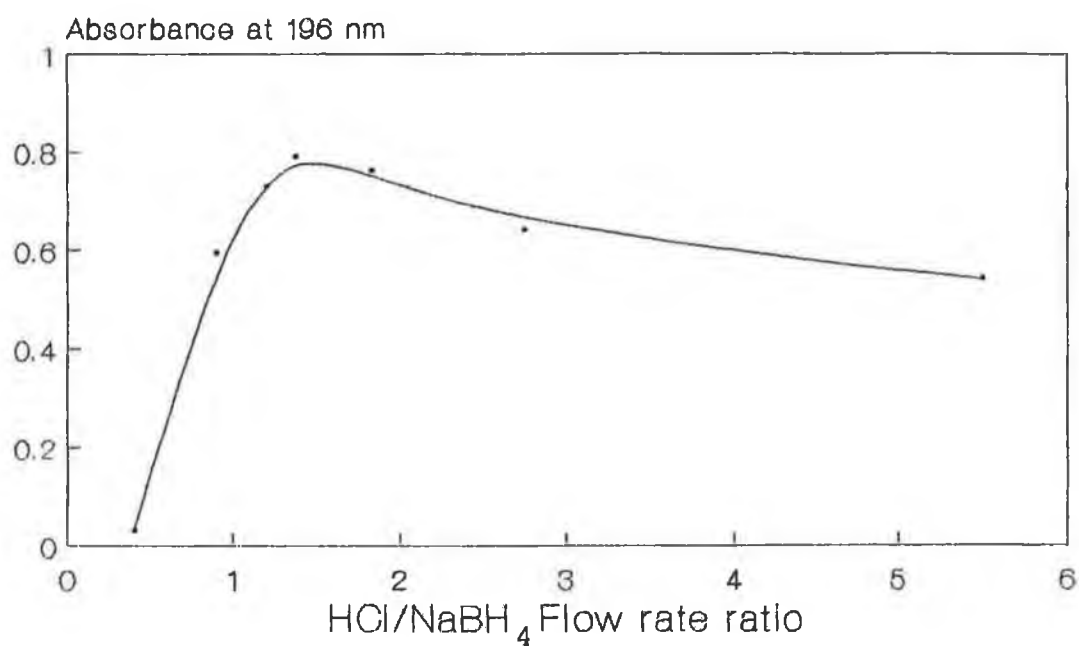Figure 5.15. Dependence of the absorbance response
on the sample injection volume

Absorbance at 196 nm

HCl/NaBH$_4$ Flow rate ratio

Figure 5.16. Influence of hydrochloric acid and NaBH$_4$

flow rates on the absorbance response

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becomes progressively larger. It was found that a ratio of 1.4 to 1.8 of acid to sodium tetrahydroborate flow rates gave the most sensitive results. This finding is in accordance with the findings of Ward and Stockwell [8], who reported a similar relationship for a continuous flow hydride generation system. This finding is also in accordance with those in the earlier batch system, in which the ratio of acid to $NaBH_4$ was of approximately the same order. In the continuous flow hydride generation described by Ward and Stockwell [8], a $HCl/NaBH_4$ flow rate ratio of two was found to give the optimum absorbance response.

The sensitivity of the absorbance response increased as the flow rates of both reagents were increased. However, there is a practical limit as to how far this can be carried out; too high a flow rate would cause a build up of internal pressure and increase the likelihood of rupturing the tubing.

In this study, flow rates of 5.6 and 3.9 ml/min were used for the hydrochloric acid and sodium tetrahydroborate solutions respectively.

5.3.6. Blood serum and plasma analysis

The analysis of the blood serum and plasma samples can be considered as consisting of three phases namely;

    (1) the collection, transport and storage of samples.

    (2) the digestion and analysis of the samples.

    (3) the comparision and validation of the results.

5.3.6.1. Collection, transport and storage of samples

One pooled blood plasma sample was obtained from the Blood Transfusion Service, Dublin. This had been routinely screened for hepatitis and HIV and been declared free from both. The sample was frozen immediately after being collected, and was kept in this state during transport and storage, until it was required for analysis.

Nine blood serum samples were obtained from the Department of Medicine, The Queen's University of Belfast. These samples had been collected from patients in the Royal Victoria Hospital, Belfast for routine clinical analysis, and also for evaluating the selenium status of the patient.

The samples had not been screened for the presence of hepatitis or HIV and, therefore, the status of the samples with regard to both of these was unknown.

After collection, the samples were placed in heparinised tubes and frozen. Each sample was labelled with the initials of the

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patient from whom it had been collected. The volume of serum sample was approximately 10 ml in each case.

The serum samples were transported in the frozen state and were kept in this form until ready for analysis by hydride generation atomic absorption spectroscopy.

For each of the ten samples, approximately 2 ml was supplied to the Department of Medicine for analysis by Zeeman corrected graphite furnace atomic absorption spectroscopy.

During the analysis procedure, care was taken during the handling of the samples, and gloves were worn at all times. All glassware which had come in contact with the samples were scruplously cleaned afterwards.

5.3.6.2. Digestion and analysis of samples

For the determination of selenium in samples by hydride generation AAS, it is essential to ensure that all the selenium present is free, i.e. it is not in the form of an organo-selenium compounds, and, that it is present in the +4 state. Therefore, it is necessary to carry out a decompsition procedure on the sample.

As it has been reported that some organoselenium compounds, and in particular the trimethylselenonium cation, are resistant to acid digestion unless perchloric acid is included in the mixture, the majority of reported digestion methods include perchloric acid. Consequently, a great deal of caution needs to

be exercised in such procedures because of the explosive potential of perchloric acid if heated to dryness in the presence of organic material.

As a result of this, several alternative digestion procedures have been reported, e.g. nitric acid/phosphoric acid/hydrogen peroxide. Bunker and Delves [9] have recently reported that a nitric acid/sulphuric acid digestion mixture gave the same digestion efficiency as a nitric acid/sulphuric acid/perchloric acid mixture, thus suggesting that the digestion of most biological materials for the determination of selenium does not require the use of perchloric acid.

Despite these alternatives, digestion mixtures employing perchloric acid are widely used for the determination of selenium in biological matrices, and therefore, in this investigation a similar digestion process was adopted.

The digestion of the blood serum and plasma samples was carried out in standard Erlenmeyer flasks on a hotplate. Using this system it was only possible to carry out the digestion of one sample at a time. The normal procedure involved placing four flasks on the hotplate. A thermometer was inserted into one of these flasks, and this served as a temperature control throughout the digestion. At all times during the digestion, the contents of this flask contained all the reagents as were used in the others, with the exception of sample.

Of the remaining three flasks, one was used for the blank whilst the other two were used for the digestion of the relevant sample

in duplicate. During the digestion a glass filter funnel was placed in the mouth of each flask to improve the refluxing conditions during the digestion.

As there is normally a difference in the temperature across the surface of the hotplate, the surface had been calibrated to determine those positions where the temperature was similar. These positions were then used for the positioning of the flasks.

The use of sulphuric acid in the digestion mixture prevents the flask from drying out, thus circumventing any risk of an explosion occurring because of the perchloric acid. However, care must be taken as its use also increases the risk of charring, which may result in losses of selenium through volatilisation.

Before the determination of selenium by hydride generation atomic absorption spectrometry can occur, it is necessary to ensure that all of the selenium present in the sample is in the +4 state. This is normally achieved by heating the sample with 4-6 M HCl for a fixed period of time. This step is of critical importance to the overall accuracy and precision of the technique, as it has been reported that the reduction process is so dependent on the heating time that deviation by more than a minute can result in 20% differences in recovery, whereas excessive heating results in the reduction proceeding in part to elemental selenium.

5.3.6.3. Validation of results

The validity of the flow injection hydride generation atomic absorption spectrometric method for the determination of selenium in the blood plasma and serum samples was assessed by calculating several parameters; the recovery, detection limit, sensitivity and with-in run and between-run precisions.

The recovery of the method was determined by, firstly, carrying a series of standards through the digestion procedure and comparing the response with similar standards made up in HCl.

Typically, the recovery was of the order of 95 to 104 %. Initially some problems were encountered with regard to the recovery of the selenium standards. This was eventually overcome by adhering strictly to the time periods for each step in the digestion procedure.

The recovery from the sample matrix was investigated by digesting a series of four plasma samples; one of these was the plasma sample alone, whilst the remaining three were the plasma sample with "spikes" of Se(IV) standard. This was performed in duplicate.

The recovery ranged from 95 to 109%, indicating that there was no appreciable matrix effects.

The detection limit of the method was determined from between-batch measurements of the blank [10]. The blank reading expressed as the mean +/- standard deviation was 0.31 +/- 0.41 ng/ml. The detection limit, defined as three times the standard

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deviation of the blank measurements, was found to be 1.2 ng/ml. The sensitivity was 2.1 ng/ml and the linear range extended up to 50 ng/ml, as shown in Figure 5.17.

The within-run precision was 5.8% at 20 ng/ml (n=6), 4.2% at 40 ng/ml (n=6), 5.4% at 69 ng/ml (n=6) and 4.5% at 80 ng/ml.

The between-run precision was determined by carrying out measuerements on two of the sample digests on three successive days. The between-run precision was 4.8% at 69 ng/ml (n=18) and 3.4% at 80 ng/ml (n=20).

For the graphite furnace atomic absorption spectrometric studies, a sensitivty of approximately 37 pg per 0.0044 A s was achieved, with a within-run precision of 4.4% at 66 ng/ml (n=11) and a between-run precision of 5.7% at 88 ng/ml (n=28).

5.3.6.4. Interlaboratory comparison study

Further assessment of the validity of the hydride generation method was obtained by participation in an inter-laboratory comparison study with the Department of Medicine, The Queen's University of Belfast.

The determinations in each laboratory were carried out independently of each other, and, when the complete analysis had been performed, the results were delivered to the other laboratory.

The results obtained for both methods for a group of ten blood plasma and serum samples is shown in Figure 5.18. The regression
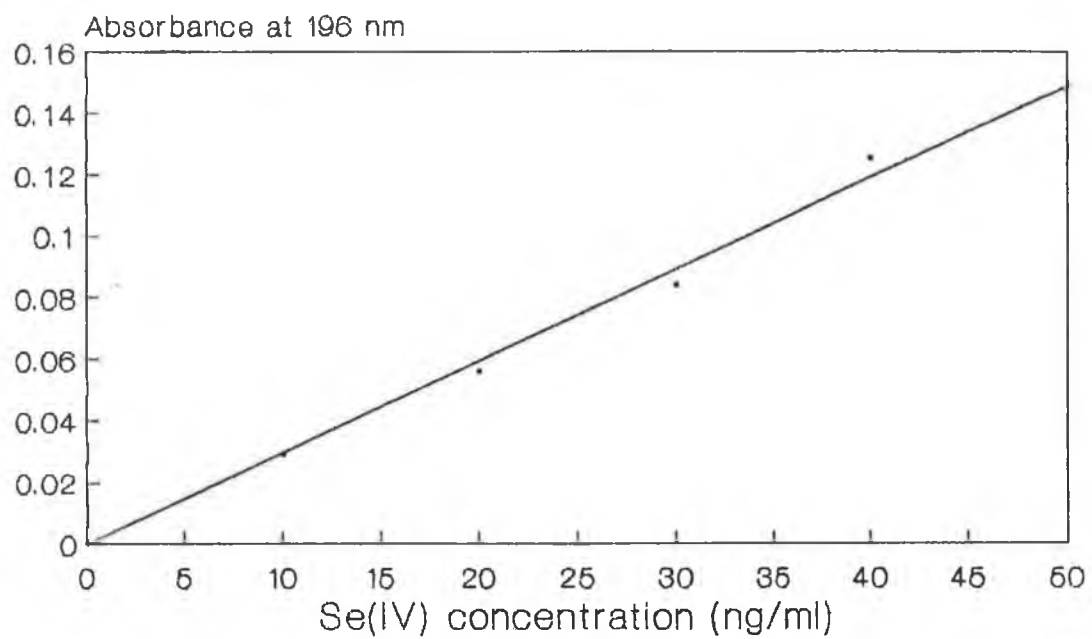
Figure 5.17. Plot of Se(IV) concentration vs
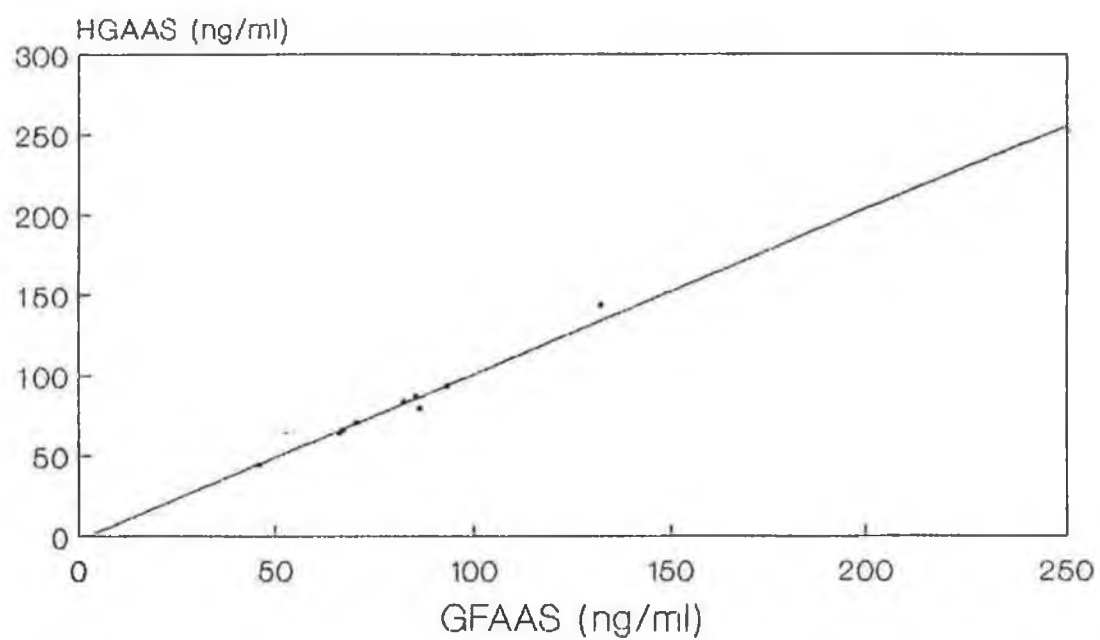
absorbance response

Figure 5.18. Relationship between blood selenium
concentrations as determined by GFAAS
and by HGAAS

equation was found to be y = 1.03x + 1.91, where x corresponds to the GFAAS method and y corresponds to the flow injection hydride generation method.

The corelation coefficient was 0.997, showing that there was excellent agreement between the two methods.

5.4. CONCLUSIONS

Since it's initial description by Holak [1], hydride generation
atomic absorption spectrometry has become a widely accepted
method for the analysis of hydride forming elements, and in
particular, arsenic and selenium.

The batch-type generation apparatus described in section 5.2.4.,
represents the simplest and, until recently, the most common
approach for the generation of hydrides for subsequent analysis
by atomic absorption spectrometry. When used in conjunction with
an externally heated quartz atomisation cell, this approach
gives detection limits of the order of 1-2 ng/ml.

However, as explained in section 5.3.2., a considerable degree
of operator handling is required for the operation of this
system and, as such, it is not entirely suited to the analysis
of a large number of samples in a given period of time.

In recent years, automated hydride generation systems have
become more widely available. These systems have been based on a
continuous flow approach which is similar in principle to the
system described in section 5.2.5. The commercially available
Plasma-Therm hydride generator, which was described by Ward and
Stockwell [8], represents state-of-the-art hydride generation
apparatus and allows the determination of selenium at very low
levels with excellent reproducibility. As pointed out in section
5.3.4, this reproducibility is a direct consequence of the
excellent reagent and sample handling characteristics offered by

the use of flowing streams. However, continuous flow systems are, by the nature of their operation, notoriously profligate with sample solution, and in the case where the sample volume is limited, this can prove to be a disadvantage.

The use of flow injection offers a combination of the excellent handling conditions of flowing streams allied with the ability to use small sample injection volumes.

The flow injection hydride generation system described here utilises the same mixing chamber and gas/liquid separator and a similar type of atomisation cell as in the continuous flow system described by Ward and Stockwell [8].

The detection limit in the continuous flow system was 0.07 ng/ml compared with the 1.2 ng/ml level obtained for the flow injection system. This is in agreement with the results of Pyen and Browner [11], who reported that the signal magnitude obtained in a flow injection system was lower than that in a continuous flow system. Consequently, the detection limits in the flow injection system are higher. In terms of the injection volume used in each system, this corresponds to absolute detection limits of 0.5 and 0.4 ng/ml for the continuous flow and flow injection systems, respectively.

The detection limit of 1.2 ng/ml for the flow injection system is approximately one-fortieth of the selenium concentration found in the least concentrated serum sample, and compares favourably with other systems used for the determination of selenium in biological matrices.

Based upon the time required for a single analysis, a sampling rate of 90 injections per hour can be achieved in the flow injection system compared with a rate of 42 injections per hour for the continuous flow system. This would represent a significant improvement for a direct method of analysis, but in this instance, where the majority of the time for a complete analysis of a serum/plasma sample would be taken up by the digestion process (3 hours), the saving in time is relatively small, and further work to scale down the digestion is necessary.

The digestion process is of critical importance to the overall accuracy and precision of the overall procedure, and a considerable degree of attention must be paid to maintaining the correct time and temperature conditions throughout the whole digestion of the sample.

The use of flow injection methodology in combination with the hydride generation approach results in a system which allows a rapid and economical analysis to be carried out. It is also easily assembled and requires the minimum of operator manipulation and expertise.

5.5. REFERENCES

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Chapter 6

Conclusion

6.1. CONCLUSIONS

The recognition, in the earlier part of the century, of the toxic properties of selenium and the increased awareness in recent years of its biological importance in animals, has meant that analytical methods of analysis, which allow the accurate determination of this trace element, have always been very important.

Selenium is widely regarded as being a difficult element to measure in biological matrices, and, consequently, a wide range of techniques have been developed and applied to its analysis. At the present time, a group of four techniques are most commonly used for this purpose; namely neutron activation analysis, fluorimetry, atomic absorption spectrometry and stripping voltammetry.

Neutron activation analysis [1] is regarded as being the method of choice for the accurate analysis of many metals including selenium. However, its widespread use is not possible because of the sophistication of the technique and apparatus allied with its cost and requirement for skilled personnel. Despite this, it plays an important central role in the analysis of selenium as it is the method of choice for certifying the selenium content of biological reference materials which are ultimately used for validating the other methods of analysis.

Fluorimetric methods of analysis have been widely used for the determination of selenium [2]. This has been due to the

excellent inherent sensitivity of the technique coupled with its low cost and relative ease of operation. In addition, many potential interferences can be circumvented during the extraction step required to remove the fluorimetric complex, normally a piazselenol, from the matrix. This step, however, represents the major drawback of the method as the overall procedure can be somewhat time consuming and tedious and not entirely suited to the analysis of a large number of samples.

In the last 10-15 years, there has been a great increase in the use of atomic absorption spectrometric methods for the analysis of metals. In the particular case of selenium, this development has been along two distinct lines; namely graphite furnace and hydride generation atomic absorption spectrometry.

There has been a considerable degree of research into the theory of graphite furnace AAS with the result that the equipment available today represents state of the art technology. This development has been of particular importance for the successful analysis of selenium, as the use of Zeeman background correction has been recommended for use in matrices containing iron [3], and especially in the case of whole blood.

In the interlaboratory comparison study, described in section 5.3.6.4., the graphite furnace formed part of a system dedicated to the analysis of selenium in blood samples as part of the MONICA project, set up to study the factors involved in cardio-vascular disease in Northern Ireland [4]. The choice of a Zeeman background corrected graphite furnace was based upon the

requirements to analyse a large number of small volume blood samples reproducibly with the minimum of sample handling and operator attention [4].

However, the cost in setting up this system is quite considerable, and may not be justified in those situations where the analysis of selenium is not performed on a regular basis and where a Zeeman background correction system may be superfluous to the overall requirements of the atomic absorption spectrometer in use.

The use of hydride generation AAS has increased considerably since its first description in 1969 by Holak [5]. Whilst there has been a certain degree of development of commercially available hydride generation systems, the apparatus required for a sensitive, reproducible analysis can be easily manufactured from readily available laboratory materials; in distinct contrast to the previously described graphite furnace system.

The majority of hydride generation methods described in the literature are based upon a batch system approach with the use of continuous flow systems coming to the fore, particularly in commercially available equipment, in the last five years. In order to develop a hydride generation system capable of handling small sample volumes reproducibly, a flow injection system was designed and evaluated.

In comparison with an analogous continuous flow system, the consumption of sample was lower and the sample throughput was higher in the flow injection system. In addition, the hydride

generation approach was shown to be extremely accurate in the interlaboratory comparison study with the graphite furnace method.

However, in contrast to the graphite furnace method, the blood serum and plasma samples had to be digested in a $HNO_3/H_2SO_4/HClO_4$ acid mixture before analysis. This not only required a considerable degree of operator attention, but also introduced a major potential source of error in the technique as a whole. It has been previously reported that the digestion procedure is of critcial importance to the accuracy of the technique [6], a fact which was borne out in this investigation.

That such care must be exercised in the control of the temperature parameter throughout the three hour digestion procedure, represents a major disadvantage of the hydride generation method, and other methods requiring such sample pretreatment, e.g. fluorimetry, cathodic and anodic stripping voltammetry.

The use of commercially available digestion apparatus can overcome this to a certain extent. In the final analysis, in deciding upon which atomic absorption spectrometric method to use, serious consideration should be given to the sample pretreatment requirements as well as to the analytical capabilities of the techniques.

Since the development of high quality hanging mercury drop electrodes, the use of stripping voltammetric techniques has

gained in popularity. In this investigation, the use of mercury coated carbon fibre electrodes for the differential pulse cathodic stripping voltammetry of selenium was described for the first time. The detection limit obtainable at such electrodes was of the same order as at conventional macroelectrodes, and lower than that of the hydride generation technique.

With the use of mercury coated carbon fibre electrodes, the nature of the mercury film and the conditions under which it is formed and maintained on the carbon fibre are of critical importance to the success of the eventual stripping voltammetry. These conditions were rigorously investigated in this study and, in the stripping voltammetric procedure, the need for a specific deposition time and convective transport conditions, normally a prerequisite at macroelectrodes, was eliminated. Therefore, the stripping voltammetric procedure could be speeded up and simplified considerably with these electrodes. Their true potential, however, lies in the development of microlitre volume electrochemical cells, which would allow the analysis of selenium to be performed in very small sample volumes, whilst still offering excellent sensitivity and reproducibility.

The anodic stripping voltammetric approach has not been as widely used for the analysis of selenium as the related cathodic stripping method. This is due to the better detection limits attainable at the latter and to the difficulties associated with the use of gold electrodes. The response obtained at a gold electrode can be irreproducible due to the formation of oxides

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on the gold surface, thus necessitating the inclusion of a cleaning and replating procedure in the analytical methodology. In the conventional electrochemical system, this entails a lot of manual changing of solutions and is very tedious. This can be overcome in the flow system studied in this investigation, allowing the complete procedure to be greatly speeded up. Moreover, the use of the fibre electrode in conjunction with the flow cell, illustrates how the use of such electrodes can simplify the development of electrochemical detection systems radically. The electrode manufacture procedure can be easily reproduced in any laboratory situation quickly and cost effectively. The electrode material can be simply changed to suit the analysis required and the flow cell can be altered to adapt to specific apparatus.

In conclusion, the analysis of selenium can be performed by a number of methods, the choice of which is governed by a variety of factors. Atomic absorption spectrometric methods have, in the last decade, become extremely popular because of the excellent sensitivity they provide. Stripping voltammetric methods, and in particular cathodic stripping voltammetry, have similarly gained in popularity, and the use of fibre electrodes shows considerable potential in the future development of small electrochemical detection systems, which should be particularly suited to the analysis of very small sample volumes.

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